UNIVERSITY OF COPENHAGEN
FACULTY OR DEPARTMENT

PhD Thesis

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Nickel allergy: effect of repeated exposures and skin barrier integrity

This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen, Denmark 31 August 2018.

NATIONAL ALLERGY RESEARCH CENTRE

REGION Herlev og Gentofte Hospital

Nickel allergy:

effect of repeated exposures and skin barrier integrity

PhD Thesis

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Academic Dissertation
Friday, 7 December 2018
at Auditory 2, Gentofte Hospital, Hellerup

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PREFACE

This thesis is based on scientific work carried out at the National Allergy Research Centre and the Department of Dermatology and Allergy, Herlev and Gentofte Hospital from 2015 to 2018. The project received funding from the Ministry of Environment and the Aage Bang foundation, which is gratefully acknowledged.

First, I would like to express my gratitude to my inspiring supervisors, all whom I admire. To my principal supervisor, Jeanne Duus Johansen, I was thrilled when you gave me the chance to be a part of your outstanding team, and I still cannot imagine a better environment to start as a researcher. Thank you for guiding me through all the studies, your professionalism, which reaches through all aspects of research, from the smallest (but important) details to seeing the big picture. Jeanne, you have provided a solid foundation for my development as a researcher, and for that I am forever grateful. To Jacob Pontoppidan Thyssen, thank you for believing in me from the start, for your endless enthusiasm and knowledge about nickel, your positive spirit and humour and your genuine interest in our studies. To Torkil Menné, thank you for generously sharing your invaluable wisdom, for being there when I needed help, for giving me a wider and historical perspective and for being a great support all way.

To my collaborators from Karolinska Institutet, Klara Midander, Anneli Julander and Carola Lidén, you introduced me to a different, creative and inspiring work environment. Thank you for guiding me through and completing the extensive laboratory work and for all our educative discussions. I have enjoyed working with you and have learned so much from you.

Thank you, Claus Rikard Johnsen for generously helping me in the chamber during Study II. A special thanks to Anne Marie Topp, you have been working hard by my side all along. These studies would not have been possible without your practical as well as psychological support, and all the laughter along the way. Thank you, Lene Toft-Jensen for your positive spirit and for making things possible. Also thank you, Josefine Bandier for your great introduction to the centre, Anna Bünning Olsson for graphical support and Nina Glasser Ulrich and Niels Højsager Bennike for being there. A huge thank you to all my colleagues at the National Allergy Research Centre for daily discussions, practical support and well-needed coffee breaks. I am very grateful to all participants in both studies: without you, there would have been no studies.

Finally, I would like to thank my parents and my parents-in-law for your support and help with the children in critical times, and my grandmother Kerstin, and to my fantastic friends for listening and being supportive.
My biggest thanks go to my loving husband Magnus, who has been the best support one could ask for, both intellectually and practically. Thank you for listening and understanding, for your unyielding encouragement and efforts at reading my work and assisting me with statistics. Our daughters, Alma and Laura, thank you for putting things into perspective every day, you are everything.

Malin Glindvad Ahlström, August 2018

LIST OF SCIENTIFIC PAPERS

I. Ahlström MG, Menné T, Thyssen JP, Johansen JD. Nickel allergy in a Danish population 25 years after the first nickel regulation. Contact Dermatitis. 2017:76(6): 325-332.

II. Ahlström MG, Thyssen JP, Menné T, Midander K, Julander A, Lidén C, Johnsen CR, Johansen JD. Short contact with nickel causes allergic contact dermatitis: an experimental study. Accepted to British Journal of Dermatology 29 June 2018 doi: 10.1111/bjd.16935

III. Ahlström MG, Midander K, Menné T, Lidén C, Johansen JD, Julander A, Thyssen JP. Nickel deposition and penetration into the SC after short metallic nickel contact: an experimental study. Submitted for publication in Contact Dermatitis 30 August 2018

ADDITIONAL PUBLICATIONS

Publications during doctoral studies not included in this thesis.

Ahlström MG, Thyssen JP, Menné T, Jellesen MS, Westermann PJS, Johansen JD. Nickel and cobalt release from fidget spinners on the Danish market. Contact Dermatitis. 2018 May;78(5):357-359

Ahlström MG, Thyssen JP, Menné T, Johansen JD. Consumer Behaviour Among Nickel-allergic Patients. Acta Derm Venereol. 2017 Nov 15;97(10):1247-1248

Ahlström MG, Jacob P. Thyssen, Torkil Menné, Jeanne D. Johansen. Prevalence of nickel allergy in Europe following the EU nickel regulation – a review. Contact Dermatitis 2017 Oct;77(4):193-200

Ahlström MG, Torkil Menné, Jacob P. Thyssen, Jeanne D. Johansen. The European Nickel Regulation and Changes since Introduction. Contact Dermatitis 2017 Jun;76(6):382-384

Schmidt JD, **Ahlström MG**, Johansen JD, Dyring-Andersen B, Agerbeck C, Nielsen MM, Poulsen SS, Woetmann A, Ødum N, Thomsen AR, Geisler C, Bonefeld CM. Rapid allergen-induced interleukin-17 and interferon-γ secretion by skin-resident memory $CD8_+$ T cells. Contact Dermatitis. 2017 Apr;76(4):218-227.

LIST OF ABBREVATIONS

AD	Atopic dermatitis
ECHA	European Chemicals Agency
EU	European Union
FLG	Filaggrin gene
ICP-MS	Inductively coupled plasma mass spectrometry
In	Indium
LDF	Laser Doppler Flowmetry
LDM	Laser Doppler Perfusion Monitoring
LDPI	Laser Doppler Perfusion Imaging
MOAHLFA	**M**ale, **O**ccupational dermatitis, **A**topic dermatitis, **H**and dermatitis, **L**eg dermatitis, **F**ace dermatitis, **A**ge >40 years
NMF	Natural Moisturizing Factors
PCA	2-pyrrolidone-5-carboxylic acid
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
Rh	Rhodium
ROAT	Repeated Open Application Test
SC	Stratum Corneum
SD	Standard Deviation
SLS	Sodium Lauryl Sulphate
TPU	Tissue Perfusion Units

LIST OF DEFINITIONS

Alloy:	A metallic material, homogenous on a macroscopic scale, consisting of two or more elements so combined that they cannot be readily separated by mechanical means
Dermal Absorption:	The diffusion of a chemical from the outer surface of the skin through the skin and eventually into the systemic circulation
Dermal Penetration:	The diffusion of a chemical from the outer surface of the skin into the stratum corneum (SC)
Dermal Permeation	The diffusion of a chemical through one layer into another, which is both functionally and structurally different from the first layer
Primary prevention:	Aims to avoid the onset of specific diseases via risk reduction by altering behaviours or exposures that can lead to disease or enhancing resistance
REACH:	Regulation of the European Union, adopted to improve the protection of human health and the environment from the risks potentially posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. It also promotes alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals
Representativeness:	The level of how well a sample drawn for questionnaire research reflects the population of interest
Secondary prevention:	Aims to reduce the impact of a disease that has already occurred by detecting and treating disease as soon as possible to halt or slow its progress
Systemic immunomodulatory treatment:	Drugs taken orally or by injection that modify the response of the immune system by increasing (immunostimulators) or decreasing (immunosuppressives) the production of serum antibodies

SUMMARY

Nickel allergy is frequent in the European population, affecting both adults (8–18%) and children and adolescents (8–10%). This is despite nickel being restricted in Europe. Up-to-date knowledge of items causing allergic skin reactions is warranted to evaluate whether amendments of the restriction is needed. For items to be covered by the current restriction, the duration of intended skin contact is '*30 minutes on one occasion or 10 minutes on at least three occasions within a period of two weeks*'. This is based on theoretical modelling, but no real-life evaluation has been made. This thesis consists of two studies: a questionnaire study and a clinical experimental study. The overall aim was to evaluate whether the current EU restriction on nickel is sufficiently protective regarding short repeated nickel contact and to identify current sources of nickel allergy in Denmark.

The questionnaire study comprised 342 patients with nickel allergy from the Department of Dermatology and Allergy at Gentofte Hospital. Of the patients, 51% (173/342) had experienced dermatitis from metallic items during 2010–2015. Overall, the most commonly reported items causing dermatitis after implementation of the restriction were earrings, other jewellery, buttons, belt buckles and wrist watches (in order of frequency). Ten-minute durations of skin contact with metallic items caused dermatitis in 21.4% of patients, and 30-minute durations caused dermatitis in 30.7%. In the experimental study, 16 individuals with nickel allergy and 10 control individuals were exposed to metallic nickel discs for three 10-minute periods with a 10-minute interval between each period. These exposures resulted in nickel amounts that elicited allergic nickel dermatitis in pre-irritated skin (63%) and in normal skin with previous dermatitis (19%) in individuals with nickel allergy. Nickel penetration into the stratum corneum (SC) was rapid, within an hour, and nickel was recovered up to 72 hours after exposure. None of the control individuals reacted to nickel exposure, and significantly lower amounts of nickel skin deposition and SC penetration were found.

In conclusion, relatively short nickel skin contact (three 10-minute periods) may lead to considerable nickel skin deposition and SC penetration capable of eliciting allergic nickel dermatitis in individuals with nickel allergy. Short repeated skin contact with nickel releasing items may contribute to the continuing nickel allergy problem. The results imply that the risk of developing nickel allergy may differ between individuals due to large variations in skin deposition after contact. Nevertheless, currently, the primary sources of nickel allergy and dermatitis in Denmark are the same consumer items as those that led to the implementation of the restriction.

SUMMARY IN DANISH

På trods af nikkellovgivning, er forekomsten af nikkelallergi i Europa høj for voksne (8-18%) og for børn/unge (8-10%). For at kunne vurdere om der er behov for ændringer i lovgivningen, er der brug for opdateret viden om de genstande der forårsager allergiske hudreaktioner på grund af nikkel. I den nuværende lovgivning, er genstande som er i kontakt med huden *'i 30 minutter én gang eller 10 minutter 3 gange indenfor en periode på 2 uger´* omfattet. Tidsintervallerne blev defineret 2014 og er baseret på teoretiske beregninger, mens den kliniske relevans ikke er undersøgt.

Denne afhandling består af to studier: en spørgeskemaundersøgelse og et klinisk eksperimentelt studie. Det overordnede formål var at vurdere, om den nuværende EU nikkelregulering er tilstrækkelig beskyttende med hensyn til kort gentagen nikkelkontakt samt at kortlægge nuværende eksponeringskilder til nikkelallergi i Danmark.

Spørgeskemaundersøgelsen inkluderede 342 nikkelallergiske patienter fra Hud- og Allergiafdelingen på Gentofte Hospital. I perioden 2010-2015 havde 51% (173/342) af patienterne oplevet eksem fra metalgenstande. Patienterne oplyste, at de hyppigste genstande som havde ført til eksem efter implementering af reguleringen, var øreringe, andre smykker, knapper, bæltespænder og armbåndsure (prioriteret rækkefølge). Hudkontakt med varighed op til 30 minutter havde ført til eksem hos 30,7% og varighed op til 10 minutter hos 21,4% af nikkelallergikere.

I det eksperimentelle studie blev 16 nikkelallergikere og 10 kontrolpersoner uden nikkelallergi eksponeret for nikkelskiver i tre 10-minutters perioder med 10 minutters intervaller. I alt fik 63% af nikkelallergikere et allergisk nikkeleksem på irriteret hud og 19% på normal hud hvor der tidligere havde været eksem. Nikkelpenetration i stratum corneum (SC) foregik hurtigt - inden for en time - og kunne måles op til 72 timer efter eksponeringen. I kontrolgruppen udviklede ingen allergisk nikkeleksem, og der blev fundet signifikant lavere nikkel mængder både på huden og i SC hos disse personer.

Vi konkluderer, at relativt kort hudkontakt (tre 10-minutters perioder) kan føre til tilstrækkelig deponering af nikkel på huden og penetration i SC til, at fremkalde allergisk nikkeleksem hos nikkelallergiker. Kort og gentagen berøring med nikkelfrigivende metalgenstande kan medvirke til fortsat sensibilisering og elicitation af allergisk nikkeleksem. Endvidere indikerer resultaterne, at risikoen for at udvikle nikkelallergi kan være individuelt forskellig grundet stor variation i nikkeldeponering efter hudkontakt. De primære årsager til nikkelallergi i Danmark i dag er fortsat de forbrugergenstande, der førte til implementering af en nikkelregulering.

TABLE OF CONTENTS

1. INTRODUCTION

Nickel is a transition metal with the atomic number 28. It is widely used in products and materials that come into contact with the skin of workers and consumers. Nickel has many advantages, for example, its good ductility, resistance to oxidation and corrosion, and low cost. Its primary uses are in stainless steel and other alloys; it is also used in its pure form in plating and in nickel compounds (Nickel Institute https://www.nickelinstitute.org/).

1.1 Contact allergy to nickel

During skin contact with items containing nickel, nickel ions can be released by sweat and transferred to the skin. This can lead to contact allergy to nickel. Contact allergy is a type IV immunological reaction that includes two phases: a sensitization phase and an elicitation phase. In the sensitization phase, immunological memory of a specific allergen is generated upon exposure and the person becomes sensitized. Following sensitization, a subsequent exposure triggers specific T-cells, which may result in elicitation of allergic contact dermatitis to the specific allergen, with symptoms such as itching, erythema, swelling and vesicles (1). A lower amount of the allergen is needed for the elicitation of dermatitis compared with the sensitization phase—for nickel, estimated in the range of 100–1000 times lower (2). The risk of developing nickel allergy (sensitization) or allergic nickel dermatitis (elicitation) depends on the rate of nickel ion release from a material (3). No relationship exists between the content of nickel in an alloy and its ability to cause an allergic reaction (4). Allergic nickel dermatitis is localized to the site of skin contact or to the hands, or it may be more widespread (5). Mild cases clear with avoidance of nickel exposure and topical treatment, but some cases can become more persistent, especially in patients with hand dermatitis or atopic dermatitis (6,7).

Nickel is the most frequently occurring contact allergen worldwide. In Europe, it is estimated that 8–18% of adults and 8–10% of children and adolescents of both sexes are allergic to nickel (8). Nickel allergy is much more common in women than in men (four to ten times) and in girls than in boys (9). Individuals with nickel allergy are more prone than those without to report hand dermatitis (30–40% vs. 15–20%), usually of the recurrent and vesicular type (10–12). Further, nickel allergy prevalence is increased in patients with atopic dermatitis (13,14).

1.2 Prevention of nickel allergy in Europe

Owing to the high prevalence of nickel allergy in women, the European Community decided in 1994 on a restriction aimed at primary and secondary prevention of the allergy. It was inspired by the Danish nickel regulation, which came into force in 1990. In 2000, the EU nickel restriction came into force, and in 2009 it was subsumed into REACH (the EU Chemicals Regulation). The restriction was not an attempt to eradicate nickel allergy, rather to protect most of the population (4). An overview of nickel restrictions, and changes over time, can be found in Table 1.

The EU nickel restriction covers two categories of items: a) articles intended for direct and prolonged contact with the skin such as earrings, necklaces, bracelets and chains, anklets, finger rings, wristwatch cases, watch straps and tighteners, rivet buttons, tighteners, rivets, zippers and metal marks in garments, spectacle frames and mobile phones; b) any post assemblies inserted into pierced parts of the body (Entry 27 of Annex XVII to REACH: Nickel and its compounds). These items are not allowed to be marketed if the nickel release exceeds the limits of the restriction. Initially, no definition existed of the actual duration of '*prolonged contact'* in relation to the EU restriction. However, in 2014, due to the persisting high prevalence of nickel allergy in EU countries despite the restriction, the European Chemical Agency (ECHA) defined '*prolonged contact*' as *'30 minutes on one occasion or 10 minutes on at least three occasions within a period of two weeks*' (15). The definition was based on information on nickel release, reactivity in nickel-sensitized individuals, nickel skin uptake, and penetration (15). Additionally, the ECHA was requested to compile a list of items covered by the definition; the list is in preparation.

There are convincing data to demonstrate a decrease in nickel allergy prevalence in some European countries. The decrease, monitored both in patch test populations and in the general population, albeit less often, is primarily found in women from northern European countries (8). Nonetheless, nickel allergy remains frequent. There is evidence of continuing sensitization in the youngest part of the population in Denmark and Sweden, who experienced antecedent nickel regulations (16,17). A recent large population study found that 7.5% of Swedish 16-year-olds of both sexes were sensitized to nickel (girls: 9.8%, boys: 4.9%) (17). Another novel, multicentre study of the general population found a prevalence corresponding to 8–18% of adults of both sexes (18). This is worrying; the causes are debated and understanding remains incomplete. Large differences in the prevalence appear between countries, sexes, age groups and over time, with predominance in the southern- compared with the northern parts of Europe and in women

compared with men (Fig 1). The differences are presumably a result of variations in exposure due to differing regulations between countries and over time coupled with compliance with these regulations. The actual accepted nickel release from items covered by the restriction has changed markedly over time as a result of differences in the interpretation of the analytical method to test for nickel release (Standard reference test, EN1811) (19). Further, several market surveys of items covered by the restriction have demonstrated continuing nickel release exceeding the limit in the restriction (20–23). Another cause—one that has yet to received notable attention—is the possible contribution from items intended only for short-duration skin contact and therefore not covered by the restriction (24,25).

Country	Regulation	Limit	Product category	In force	Analytical method
Denmark	Bekendtgørelse nr. 472 af 27 June 1989 amended 16 December 1991	Release ≤0.5 μg/cm^2/week	Jewellery, watches, spectacles, metal in clothing	10 July 1989	Dimethyl glyoxime test
Sweden	General advice regarding ear piercing National Board of Health and Welfare, Sweden SOSFS 1989: 40	Content ≤0.05% nickel or nickel coating thinner than 0.01 μm	Ear piercing with nickel-containing piercers or rings	1990	Atomic absorption
Germany			Nickel containing consumer items labelled 'contains nickel and may cause an allergic reaction'	1991	
EU	EU Communities Directive 94/27/EC	(A) Release ≤0.5 μg/cm^2/week (B) Content ≤0.05% (C) Release ≤0.5 μg/cm^2/week	(A) Consumer products in prolonged contact with skin (B) Piercing posts (C) Coated products such as in (A), for a period of 2 years of normal use	20 July 2000	EN1811 (1999)* EN1810 (atomic absorption) EN12472†
EU	Commission Directive 2004/96/EC In 2006 REACH 1907/2006 Entry 27	Changed (B) to release ≤0.2 μg/cm^2/week	Piercing posts	1 September 2005	EN1811 (1999)*

*Objects to be tested are stored in artificial sweat at 30°C for 1 week. Nickel release into the solution is analysed. The interpretation of the outcome of EN1811 has been amended several times; see Tables 2–4.
†Objects are subjected to corrosion and wear by abrasive chips, simulating 2 years of normal use. Nickel release is then tested with EN1811. Amended 2005 and 2009.

Table 1. Overview of nickel restrictions from (19).

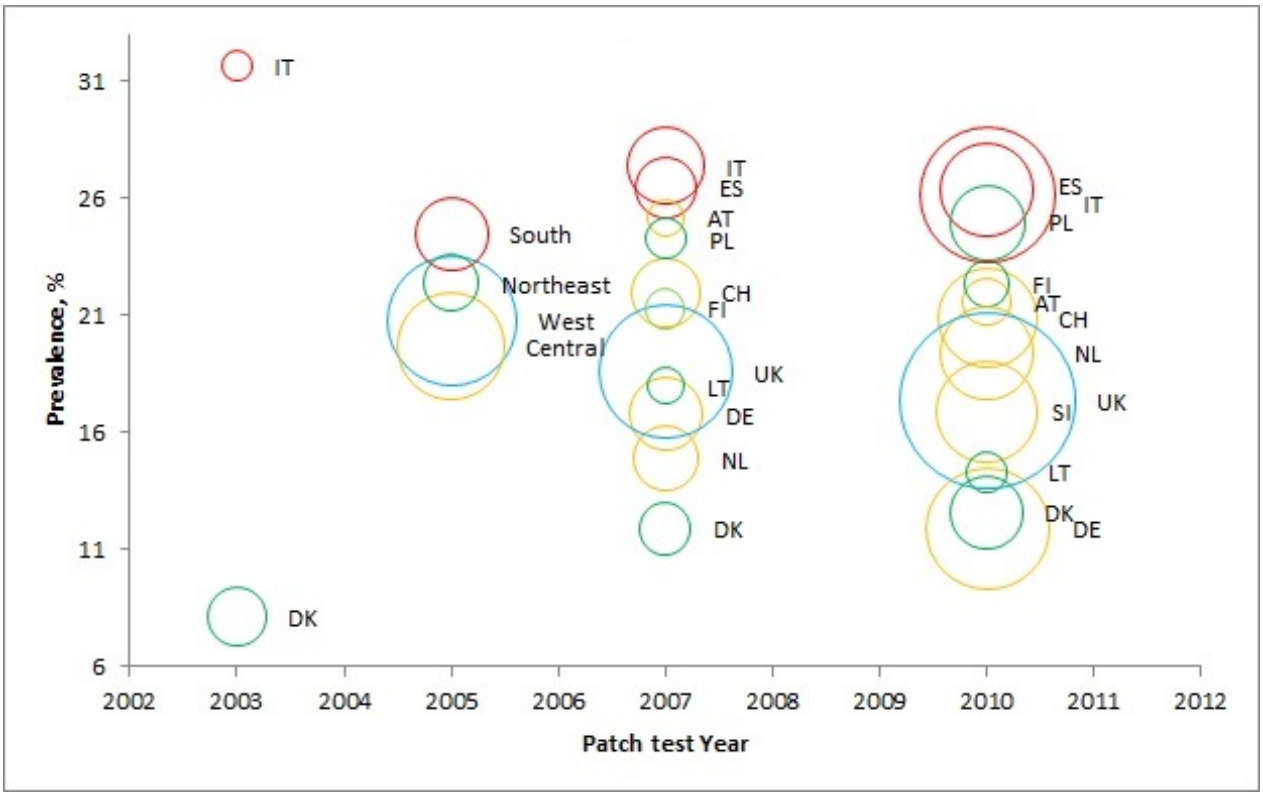

Figure 1. Prevalence of nickel allergy in patients with dermatitis, of both sexes, by country or region from the European Surveillance System on Contact Allergies network from 2002 to 2012 (26–29). Major differences in prevalence between northern and southern European countries are apparent. Results in 2002/2003 are presented from 2 departments: Padova in Italy (IT) and Gentofte in Denmark (DK). Countries were clustered into regions in 2005/2006; South (red): Italy (IT) and Spain (ES). Northeast (green): Finland (FI), Lithuania (LT) and Poland (PL). West (blue): United Kingdom (UK). Central (yellow): The Netherlands (NL), Switzerland (CH), Austria (AT) and Germany (DE). The prevalence was specified by country at the other two test periods. Denmark (DK), Slovenia (SI). The size of the circles indicates the number of patch tested patients by contributing clinics by country. Figure was modified from (8).

1.3 Sources of nickel exposure

Historically, the sources of nickel allergy have varied over time. Initially, nickel allergy was a male occupational disease in nickel platers (30); from the 1930s, it shifted towards being more common in women and stemming from consumer items (5). Over the years, trends in fashion have determined the leading causes of nickel allergy. The main causes in the 1950s and 1960s were stocking suspenders; metal in blue jeans (buttons, zippers) in the 1970s; and in the 1980s the prevalence rose extensively in young women due to inexpensive earrings and jewellery (31–33). Following implementation of the EU nickel restriction, market surveys have been undertaken aimed at monitoring nickel release from selected items. Three consecutive and systematic Swedish market surveys showed that the implementation in Sweden had been effective (22,34,35). Nevertheless, critical nickel release from items both covered and not covered by the restriction has been identified in some EU countries. Such items include earrings,

hair clasps and jewellery (36,37); children's toys (38); tools (39,40); and accessories, utensils, and electronic devices (25). Dermatitis resulting from items such as coins and mobile phones has also been reported (41,42). A wide variety of items have caused nickel allergy and dermatitis in occupations in the industrial setting, construction work and the service and healthcare sectors (9,43). In a questionnaire study in 2006–2007 among nickel allergic dermatitis patients from private Danish dermatology clinics, the most commonly reported causes were watches, earrings, buttons, jewellery and belt buckles (9). In one study, patients with nickel allergic dermatitis from a tertiary referral centre identified items suspected of causing their disease and the items were subsequently tested. Critical nickel release was found from diverse items, but most commonly from mobile phones, spectacle frames, hair clasps, watches and keys (44). Continued identification of the current sources of nickel allergy, including identification of new risk items, is crucial for the surveillance of the protection of European citizens.

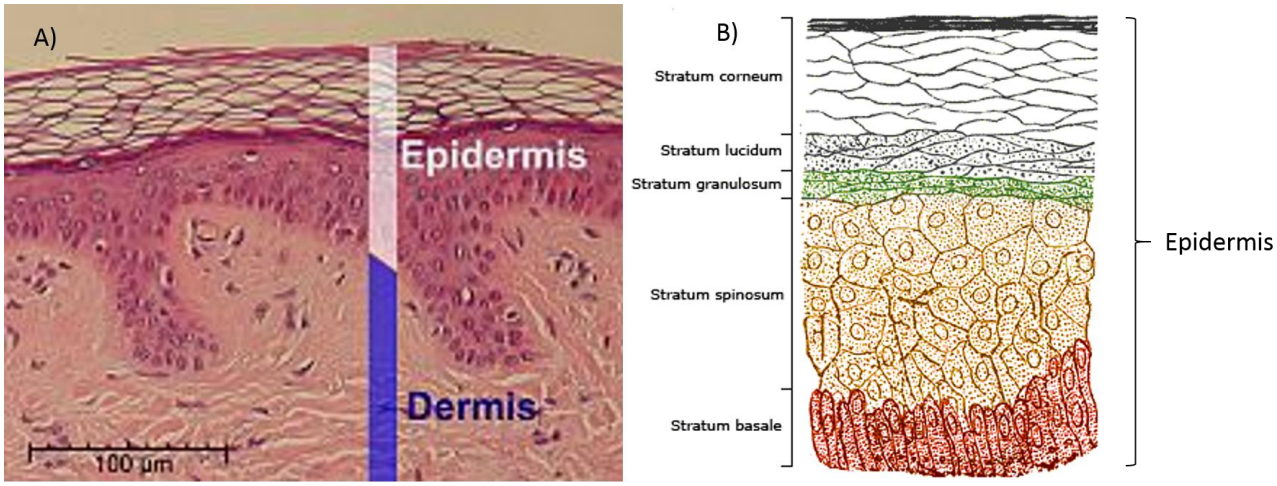

Figure 2. Epidermis. A) Microscopic image of the epidermis (white bar) with underlying dermis (blue bar) stained with haematoxylin and eosin (45) B) Illustration of the epidermis layers, modified from (46).

1.4 The skin barrier and nickel penetration

The skin can be divided into three layers: 1) the epidermis, which is the outer compartment; 2) the underlying dermis, containing blood vessels and lymphatic ducts; and 3) the subcutis, built up of subcutaneous fat. All layers are penetrated by skin appendages, hair follicles and sweat ducts. The stratum corneum (SC) is in the outermost layer of the epidermis, characterized by multiple lipid layers surrounding flattened corneocytes (Figure 2) (47,48). Underneath is the viable epidermis, which contains basal cells that replace the SC cells when they disappear due to desquamation and dendritic cells involved in immune defence. The turnover time of the SC and the epidermis is approximately two and four weeks, respectively (49). Nickel deposited onto the

skin surface may permeate the SC and reach the viable epidermis where it can be detected by immune cells.

The SC is the major barrier to nickel absorption in the skin and the penetration of nickel is slow through this layer (50,51). The lag time of nickel through the SC is thought to be due to chelation of nickel by histidine-rich filaggrin proteins (52). FLG is expressed just below the SC (53,54) and mutation in this gene may consequently facilitate percutaneous nickel penetration (55).
FLG is the main precursor protein of the amino acid-derived components of natural moisturizing factors (NMF). Loss-of-function mutations in the FLG gene lead to reduced levels of NMF in the SC and consequent dryness of the skin (56). A biomarker for the FLG genotype can be found by measuring the degradation components of natural moisturizing factors (NMF), for example, histidine, 2-pyrrolidone-5-carboxylic acid (PCA) or urocanic acid (54,57). SC penetration, depending on allergen size, polarity and lipophilicity, occurs via the slow intercellular (lipophile large molecules), transcellular (small ionic substances) and fast appendageal (large molecules and particles) route (58). The understanding of the interrelated mechanisms that regulate the SC nickel penetration is incomplete (59). SC penetration largely depends on the integrity of the SC, and impaired/irritated skin can augment metal skin absorption 10–100 times (60). The skin barrier can be constitutionally impaired (such as FLG null mutations) or acquired (due to skin irritant exposure, e.g. SLS). Other essential factors that influence nickel penetration and thereby the risk of sensitization or elicitation are, for example, the allergen dose and size of the exposure area, the presence of penetration-enhancing factors, the anatomical site, occlusion, temperature and pH (59).
Overall, estimation of nickel SC penetration is critical because this process is the rate-determining step required for sensitization or elicitation of allergic nickel dermatitis.

1.5 The importance of short repeated nickel skin contact

In everyday life, the skin comes into contact with metal many times a day. Short repeated skin contacts with metallic items, such as keys, tools, handles, scissors and coins, may lead to nickel building up in the skin over time. The contribution of these contacts to the persistent high prevalence of nickel allergy is unknown. In contrast to continuous exposure to a single nickel-releasing item, the causality regarding these exposures may be more difficult to elucidate. The relevance may be even more difficult to assess in cases where exposures occur in combination with irritants (61,62). To evaluate the risk of such exposures, studies of the actual skin deposition, penetration and absorption in combination with the elicitation potential are central.

The rate of nickel release from a metallic item is crucial for the amount of nickel skin deposition, which in turn determines the risk of sensitization and elicitation (4). Determining factors are duration and frequency of contact, temperature, amount and pH of sweat, type of material (pure nickel/nickel alloy) and its surface (63). The nickel release from nickel alloys and pure nickel in artificial sweat is initially very high and thereafter decreases rapidly (64,65). Similarly, nickel skin deposition is rapid after contact (66,67). Thus, the same pattern of high initial release/deposition in artificial sweat and touch seems to be applicable (24,68,69).

The elicitation potential of repeated low-dose nickel exposure in an open setting (ROAT) was compared with nickel patch test reactions in one study. The accumulated nickel dose in the ROAT that led to nickel dermatitis corresponded to one single nickel dose in the patch test, which emphasises the relevance of repeated exposures (70).

Taken together, the role of short repeated metallic exposures with respect to sensitization and elicitation of nickel allergy in the European population is unknown. The high initial nickel release from nickel materials and the importance of accumulated exposures may be important.

2. AIMS

The overall aim was to evaluate whether the current EU nickel restriction is sufficiently protective regarding short repeated nickel contact and to determine up-to-date risk exposures.

Specific aims:

1. To identify current sources of nickel allergy and allergic nickel dermatitis in individuals with nickel allergy (Paper I)

2. To evaluate self-reported time-exposure relationships with nickel-containing items in individuals with nickel allergy (Paper I)

3. To determine whether relatively short repeated skin contact with nickel (3 x 10 minutes) on normal and irritated skin in individuals with nickel allergy can
    i. lead to cutaneous blood flow increase (Paper II)
    ii. elicit allergic nickel dermatitis (Paper II)

4. To quantify nickel surface skin doses and nickel penetration into the SC over time following relatively short repeated skin contact (3 x 10 minutes) with nickel in normal and irritated skin in individuals with and without nickel allergy (Paper III)

3. METHODS

3.1 OVERVIEW

The studies presented were conducted at the Department of Dermatology and Allergy, Herlev and Gentofte Hospital, Hellerup, Denmark between January 2015 and July 2018. The thesis consists of a questionnaire study and a clinical experimental study.

Paper I: Questionnaire study of individuals with nickel

Paper II and III: Clinical experimental study in individuals with and without nickel allergy

3.1.1 The National Database of Contact Allergy

The National Allergy Research Centre was founded by the Ministry of Environment in 2001. In relation to this, the surveillance database of contact allergy, the 'National Database of Contact Allergy', was established and included patch test results from dermatology departments of university hospitals and from dermatologists in private practice in Denmark. The aim was to continuously monitor frequencies of contact allergy and clinical data from patients with dermatitis. Data from the database may be extracted for research purposes, either from selected or single centres.

3.1.2 Patch Testing

Patch testing is the gold standard method to diagnose contact allergy. To test for nickel allergy, nickel sulphate 5% (2.0 mg/cm^2) in petrolatum (pet.) is applied in an 8 mm Finn chamber affixed with Scanpore tape to the upper back for two days; the test site is then evaluated on the day of removal (Day 2), Day 3–4, and Day 7. The patch test reading is based on visual scoring and palpation of the skin reaction and is classified as `+1´, `+2´, `+3´, `+?´, `irritant reaction´ or `negative´ according to current guidelines (71). A positive patch test is defined as a score of minimum `+1´, which is generally defined as a sign of nickel allergy, i.e. sensitization has occurred.

3.1.3 Information from the database used in the studies

In this thesis, individuals with a previous positive nickel patch test at the Department of Dermatology and Allergy, Copenhagen University Hospital, Herlev-Gentofte, Denmark were recruited from the National Database of Contact Allergy. Personal data such as date of birth, sex

and maximal nickel patch test reactivity were registered. Further, clinical diagnoses of atopic dermatitis and hand dermatitis, for example, had been evaluated by the attending dermatologist and registered in the patient's file using the internationally recognized MOAHLFA index (**M**ale, **O**ccupational dermatitis, **A**topic dermatitis, **H**and dermatitis, **L**eg dermatitis, **F**ace dermatitis, **A**ge >40 years). The patch testing procedure was standardized according to the guidelines from the European Society of Contact Dermatitis (71).

3.1.4 Ethics statement

The questionnaire study (Paper I) was reported to the Regional Ethics Committee of Copenhagen (H-15010935) and approved by the Data Protection Agency (GEH-2015-075, I-Suite no. 03723). For the experimental study (Papers II–III), all participants signed a written informed consent before inclusion. The study was approved by the Regional Ethics Committee of Copenhagen (H-16050296) and the Data Protection Agency (HGH-2017-027, I-Suite no: 05519). Further, it was registered at ClinicalTrials.gov (NCT03309215).

3.2 QUESTIONNAIRE STUDY (Paper I)

3.2.1 Study population

All nickel patch test positive patients from 2010–2014 were invited to participate in this study. To create the subgroups of patients, the return date of the questionnaire was combined with the date of birth to generate age at questionnaire completion.

3.2.2 The questionnaire

A new questionnaire was constructed for patients with nickel allergy aimed at revealing current metallic exposures and minimum duration of contact possibly leading to dermatitis. Personal data and responses to questions were explored in this study. Examples of selected questions and response categories can be found in Supporting Table S1, Paper I.

3.2.3 Validation of the questionnaire

The complete questionnaire was pre-tested by 10 colleagues from the Department of Dermatology and Allergy with no known nickel allergy. Cognitive interviewing regarding their interpretation of the questions, difficulties with the wording or selection of response categories was conducted in each case. After revision, a pilot study was conducted including six patients with nickel allergy from the Department of Dermatology and Allergy at Gentofte Hospital. Subsequently, the procedure with interviews was repeated and the questionnaire was finally revised. Prior to analysis of questionnaire responses, 10% randomly chosen questionnaires (No.:

35) were checked for data entry errors. The questionnaire contained 197 questions; 10 questionnaires contained errors; one error was found in five questionnaires, two errors in four questionnaires, and three errors in one questionnaire. In total, 16 errors were found in these 35 questionnaires, resulting in a data entry error frequency of 0.23% of the questions.

3.3 EXPERIMENTAL STUDY (Papers II–III)

3.3.1 Study population

Nickel patch test positive patients during 2015-2017, aged 18–75 years, were invited to participate in the study. Further, control participants with no known metal dermatitis were recruited by advertising on the Internet (www.forsoegsperson.dk). Interested potential participants in both groups were interviewed by telephone regarding eligibility criteria. For control participants, it was confirmed that they had not experienced dermatitis after contact with metallic items. On the first study day, all participants were examined for exclusion criteria and a patch test with nickel sulphate 5% in pet. was applied to confirm their nickel allergy status. In addition, the importance of following the restrictions applicable during the study period (regarding showers, emollients, physical exercise etc.) was stressed to all participants, and eventual individual approaches to comply with the restrictions were discussed. All participants completed all study days.

3.3.2 Sodium lauryl sulphate (SLS)

Sodium lauryl sulphate (SLS) (formula: $C_{12}H_{25}NaO_4S$) is a anionic detergent often found in cosmetic products, for example (72). In dermatological research, SLS is widely used to induce irritant contact dermatitis. Exposure to SLS both disrupts the skin barrier and induces skin inflammation (73). Recently, SLS has been shown to decrease the quantity of epidermal filaggrin protein and NMF levels (74–76).

3.3.3 Metallic discs

Metallic discs releasing nickel (>99% wt. nickel) or control discs (>99% wt. aluminium) were produced specifically for this study. Before each use, the surface on one side of the discs was ground with wet sandpaper in a standardized way (see Paper II, supporting information). The method of disc preparation was developed for this study. A variation of the procedure described in (24) was used to allow for preparation of many discs (96 discs per exposure day). Instead of grinding by hand, a sander was used on up to 23 discs at a time which were placed in a disc holder produced for the purpose (Fig. 3). Different disc holders were used for grinding the different materials (nickel and aluminium). To quantify nickel release from the prepared discs

used in the study, discs were immersed in artificial sweat for different periods and a wipe test was performed to mimic the exposure procedure of the study (see Paper III, Table II).

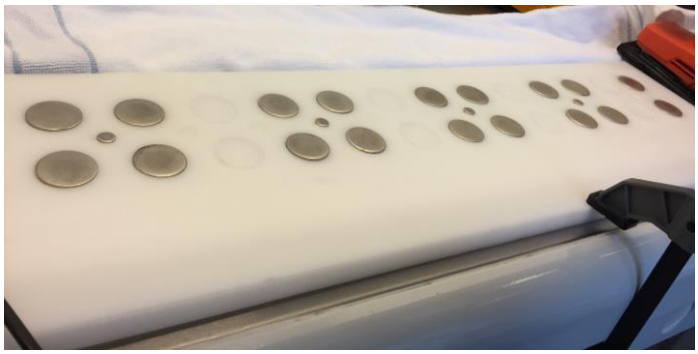

Figure 3. Nickel discs (large and small) to be ground in a specially produced disc holder.

3.3.4 Study design

The study design is described in detail in Paper II and III. In short, on day 0, the forearms were randomized for SLS pre-treatment on either the left or right forearm by the randomization function in excel; the other forearm was left untreated. Participants with nickel allergy (both from the pilot and main study) and control participants were randomized in two separate groups. Nickel earlobe exposures followed the side randomized to SLS pre-treatment.

All participants were exposed to eight nickel-releasing discs: three large discs on each forearm (ø: 3 cm), one on the upper back (ø: 3 cm) and one small disc on one earlobe (ø: 1 cm). In addition, four aluminium-releasing discs were applied: one large disc on each forearm and one on the upper back (ø: 3 cm) and one small disc on the other earlobe (ø: 1 cm) (Fig. 4). Readings and laser doppler measurements of the same forearm exposure areas were performed over time (area 3 and 4) (Paper II), whereas all areas (1–4) on the forearms were used for tape stripping (Paper III) (Figure 5). Metallic disc exposures on the upper back were fastened with tape for 48 hours, directly beneath the nickel patch test. The metallic disc exposures on the back were done to study differences in reactivity compared with the nickel patch test and were evaluated according to current guidelines (71). Scoring of forearm reactions, however, was done using a modified scale to identify weak reactions and for a more detailed discrimination of subgroups (77,78). The subgroups were (+): weak erythema; +: erythema, infiltration; +(+): erythema, infiltration and a few papules; ++: erythema, infiltration, papules; ++(+): erythema, infiltration, papules and a few vesicles; and +++: intensive erythema, infiltration and coalescing vesicles. Earlobe reactions were assessed as present/not present. A detailed study design can be found in Table 2.

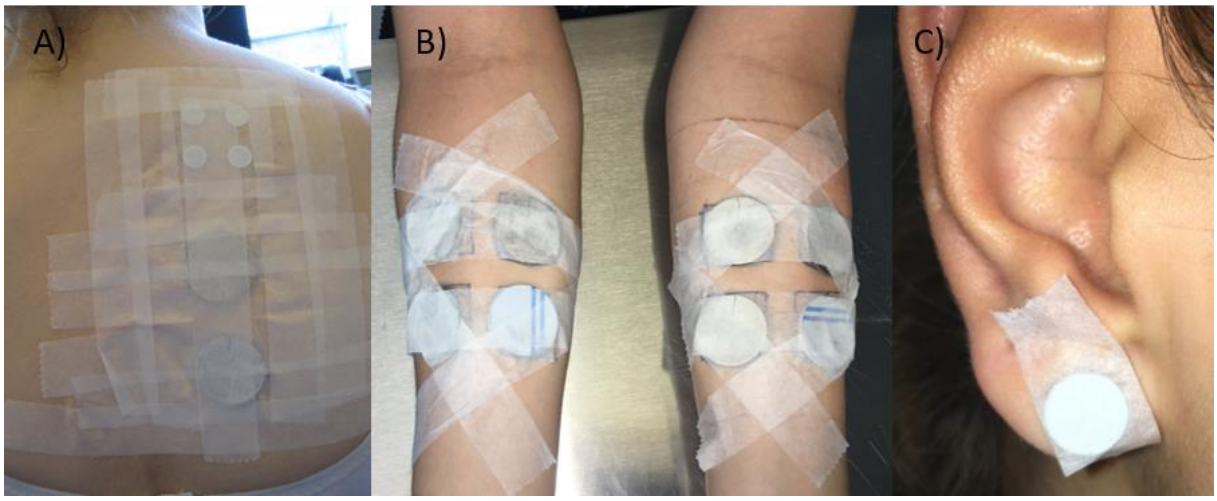

Figure 4. A) Study exposures on upper back with nickel sulphate 5% in pet. in one Finn Chamber (8 mm, upper), one nickel disc (30 mm, middle) and one aluminium disc (30 mm, lower) on day 0 B) metallic nickel- or aluminium-containing discs on both volar forearms on day 1 and C) nickel disc exposure on one earlobe on day 1.

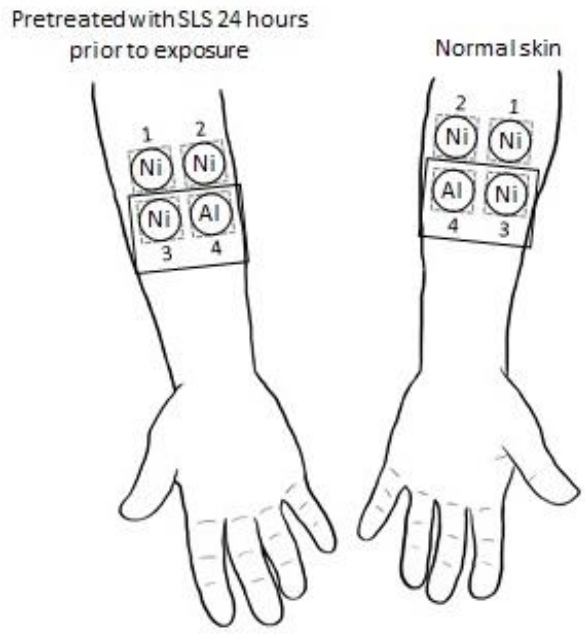

Figure 5. Illustration of the four exposure areas on each mid-volar forearm. The rectangles mark the two areas (3 and 4) used for all evaluations (clinical and blood flow) during the study. Tape stripping was done on both forearms as follows: day 1: area 1, day 2: area 2, day 4: area 3 and 4.

	Day			
Action	**0**	**1**	**2**	**4**
Metallic disc preparation	X	X		
SLS-patch on/off	X	X		
Pregnancy test	X			
Randomization	X			
Questionnaire	X			
Filaggrin genotype	X			
Nickel-patch on/off	X		X	
Metal discs back on/off	X		X	
Photography		X	X	X
Metallic discs on forearms		X		
Optical density of tape strips	X		X	X
Clinical evaluation				
-exposure site discs		X	X	X
-nickel patch site			X	X
Tape Stripping				
-upper arm	X			
-nickel disc area		X	X	X
-aluminium disc area				X
Blank tapes		X	X	X
Blood flow analysis		X	X	X

Table 2. Trial design.

3.3.5 Laser doppler flowmetry

Laser Doppler Flowmetry (LDF) is an established method for measuring cutaneous microcirculation using either an optic probe (laser Doppler perfusion monitoring, LDPM) or an imaging system (laser Doppler perfusion imaging, LDPI). The technique is based on the Doppler frequency shift that occurs when laser light is scattered by a moving object (red blood cells); the higher the speed, the higher the frequency shift and the higher the flux (79,80). The output, the flux of blood cells, reflects the concentration of moving red blood cells times their average velocity. The technique is used in the field of contact allergy to detect cutaneous inflammation (81–83). Although it cannot discriminate between allergic and irritative dermatitis, it has been shown to correlate with visual scoring of the skin (81). The LDPM used in this study measured blood flow at a wavelength of 785nm +/-10 nm and a flux range of 0–1000 PU. Three consecutive measurements of approximately 20–30 seconds were made (Figure 6). Within each measurement a steady state region of interest of approximately 15 seconds was selected, and a mean value was found. In the analysis of blood flow values, the mean value of the three measurements was used.

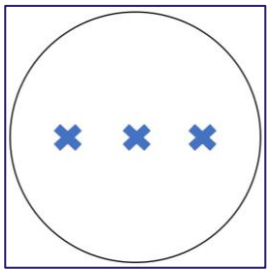

Figure 6. For all exposure areas on forearms, a mean of three consecutive measurements corresponding to the centre of the test area and on either side midway on an imaginary horizontal line between the centre and the peripheral disc exposure area.

3.3.6 Tape stripping

Tape stripping is a minimally invasive technique widely used in skin barrier research for removal or collection of the SC (84,85). Consecutive tapes are applied to a defined skin area, collecting a fine layer of skin cells from the SC on each tape. In general, approximately 15–45 consecutive tape strips are needed to remove all the SC (84,86–89), although substantial individual differences exist (90). In the present study, tape stripping of exposure areas was done to quantify the nickel skin dose (µg/cm^2) by following chemical analysis using inductively coupled plasma-mass spectrometry (ICP-MS). Tape stripping to study metal penetration in the SC has previously been done for silver from silver garments and for nickel salts in aqueous solutions (91,92). In the present study, tape strips (D-Squame®; Monaderm, Monaco, France) were used for analysis of both nickel (no. 15) from exposure areas on forearms and 2-pyrrolidone-5-carboxylic acid (PCA) (no. 8) from both inner upper overarms. To determine the amount of protein removed by each tape, optical density was used for each tape before storing (93). Optical absorption was measured with the Squamescan instrument at 850 nm in an area of 1.8 cm^2, corresponding to approximately 50% of the entire tape (93).

3.3.7 Analysis of FLG and PCA

We measured the three most common mutations in the filaggrin gene (FLG) by multiplex analysis of buccal swaps. In addition, PCA was measured in tape strips from the upper inner arms using ultra-performance liquid chromatography.

3.3.8 The Standard Reference Test EN1811

EN1811 is the standard reference test method for nickel restriction. Examined items are stored in artificial sweat at 30℃ for 1 week, and nickel release into the solution is analysed (94). Nickel release from the metallic discs used in the present study was tested with EN1811 and also for shorter periods matching the duration of exposure.

3.3.9 Inductively coupled plasma mass spectrometry

Chemical metal analysis of tape strips, wipe extracts and artificial sweat was performed with inductively coupled plasma-mass spectrometry (ICP-MS) at Karolinska Institutet, Solna, Sweden (Fig. 7).

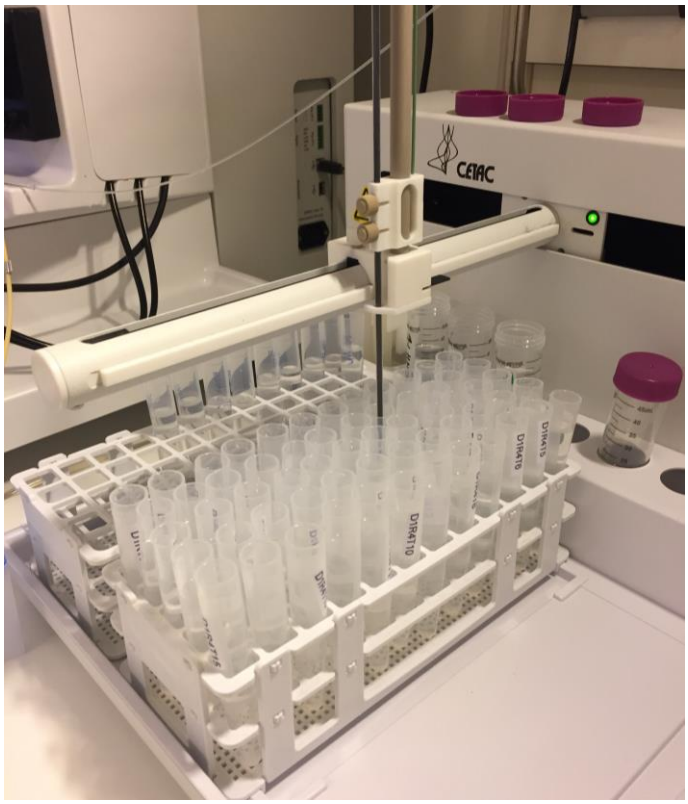

Figure 7. Inductively coupled plasma mass spectrometry (ICP-MS) of study samples.

4. RESULTS AND PAPERS

This section summarizes the key findings of the papers included. The original papers are included after each summary.

Paper I: Nickel allergy in a Danish population 25 years after the first nickel regulation

- Exposure sources reported to cause first-time dermatitis in patients with nickel allergy differed between women and men. In order of frequency,
    - women reported earrings (67.8%), buttons on clothing (55.4%), wrist watches (51.4%), other jewellery (50%), zips (23.6%) and belt buckles (19.2%).
    - men reported wrist watches (55.6%), belt buckles (50%), spectacle frames (22.2%), other jewellery (22.2%), earrings (16.7%), buttons on clothing (16.7%) and keys (16.7%).
- The most recent dermatitis was caused by the same items as the initial dermatitis, although the order of frequency was partly changed. In addition, zips and tools were reported as frequently as earrings for men.
- Young nickel allergic patients (≤25 years) of both sexes reported the same main problematic items as all women in the study, with the addition of hair clips.
- Subgroups of patients who either reported first-time dermatitis after the EU nickel restriction was enforced in Denmark or their most recent dermatitis within 5 years of the study reported the same main dermatitis-causing items as all women. First time dermatitis was also commonly caused by coins and spectacles.
- Over 50% (173/342) of patients with nickel allergy reported having experienced nickel dermatitis during 2010–2015.
- In addition to keys, items intended for short-duration contact, such as coins, scissors and tools, were reported to some extent in all patients and in the subgroups.
- Skin contact of up to 10 minutes with a metallic item had caused dermatitis in 21.4% of patients, and 30.7% had experienced dermatitis following 30 minutes of contact.
- There was a non-significant trend for faster reactions (≤ 30 minutes of contact) with increasing patch test reactions.

Nickel allergy in a Danish population 25 years after the first nickel regulation

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doi:10.1111/cod.12782

Summary

Background. Nickel in metallic items has been regulated in Denmark since 1990; however, 10% of young Danish women are still sensitized to nickel. There is a need for continuous surveillance of the effect of regulation.

Objectives. To identify current self-reported metallic exposures leading to dermatitis in nickel-allergic patients, and the minimum contact time needed for dermatitis to occur.

Methods. A questionnaire was sent to all patients who reacted positively to nickel sulfate 5% pet. within the last 5 years at the Department of Dermatology and Allergy, Gentofte Hospital.

Results. The response rate was 63.2%. Earrings were the foremost cause of dermatitis after the EU Nickel Directive had been implemented, followed by other jewellery, buttons on clothing, belt buckles, and wrist watches. Dermatitis reactions within 10 min of contact were reported by 21.4% of patients, and dermatitis reactions within 30 min of contact were reported by 30.7% of patients.

Conclusions. Nickel exposures that led to the implementation of a nickel regulation seem to persist. The durations of contact with metallic items to fall under the current REACH regulation of nickel correspond well with the results of this study.

Key words: allergic nickel dermatitis; EU directive; metallic items; nickel; prolonged direct contact.

Nickel is the most common cause of contact allergy in the general population (1, 2) and among patients with dermatitis (3, 4). The initial outbreak of consumer nickel allergy and dermatitis was caused by stocking suspenders (in the 1950s and 1960s) (5), followed by buttons, rivets and zippers in blue jeans (1970s) (6), and finally by ear-piercings and jewellery (1980s) (7).

In 1990, a nickel regulation was introduced in Denmark in response to the high frequency of nickel allergy and dermatitis, especially among young women (8). In Sweden, a regulation reducing the content of nickel in ear-piercing posts and earrings was adopted in 1991 (9), and, in the same year, nickel-containing consumer items were labelled 'contains nickel and may cause an allergic reaction' in Germany. In 1994, the EU Nickel Directive was introduced, coming into full force only in 2001 (10). The last amendment was made in 2005, and concerned piercing posts (11). During 2009, the EU Nickel Directive was included in Annex XVII in REACH, the EU chemicals regulation, and the EU Nickel Directive from that time onwards will be referred to as 'nickel regulation'.

The nickel regulation has led to a significant decrease in the prevalence of allergic nickel contact dermatitis in young women (1, 12), in women ear-pierced after its implementation as compared with those ear-pierced before its implementation (12, 13), and in young women

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Conflicts of interest: The authors declare no conflicts of interests pertinent to this study.

Accepted for publication 18 January 2017

with dermatitis (4, 14, 15). However, a significant proportion of young individuals still become sensitized to nickel and report dermatitis (16, 17), and a high prevalence of allergy, exceeding 10%, is seen both among young women in the general population and among young female patients with dermatitis in Europe (1–3). There may be several explanations for the persistence of nickel allergy, in particular related to the nickel regulation and its enforcement (4). Notably, the limits of nickel release have been unchanged since 2005, but the analytical methods used to measure nickel release from metallic items (EN1811) and their interpretation have been changed several times (11, 18–23).

For a metallic item to be covered by the nickel regulation, it must fulfil the criterion of being intended for 'prolonged direct contact with the skin'. The definition of prolonged contact was agreed in the EU in 2014 as being >10 min on three or more occasions or >30 min on one or more occasion within a 2-week period. This was based on data from studies examining time-related release of nickel from alloys, combined with studies on skin uptake and penetration of nickel, and finally reactions to different doses of nickel in nickel-sensitized patients (24).

There is a need for continuous surveillance of the effect of the nickel regulation. The importance of this is highlighted by the fact that, owing to changes in the interpretation of EN1811, the actual permitted nickel release has been changing over time (10). The main objective of this study was to identify current self-reported metallic exposures leading to dermatitis in nickel-allergic patients from a hospital clinic and the minimum contact time needed for dermatitis to occur.

Methods

Patients were included in the study if they had shown a positive patch test reaction to nickel sulfate 5% pet. (Trolab, Smartpractice-Almirall Hermal, Reinbek, Germany) within the past 5 years (1 January 2010 to 31 December 2014) at the Department of Dermatology and Allergy, Gentofte Hospital. If multiple patch tests had been conducted in this period, the result of the most recent test was used. The European baseline series had been used for patch testing. Patches had been applied on the upper back and left in place for 2 days, and readings were performed on day (D) 2, D3 or D4 and D7, according to ESCD criteria (25). A positive reaction was defined as at least homogeneous erythema and palpable infiltration in the test area. Reactions not fulfilling these criteria were classified as negative. A total of 541 nickel-sensitized patients were identified. Of these individuals, 6 had died, 9 could not be contacted, and 2 had emigrated, leaving 524 potential participants. The study was reported to the Regional Ethics Committee of Copenhagen (H-15010935), and approved by the Data Protection Agency.

A questionnaire was sent to the 524 potential participants in 2015. Non-respondents were sent the questionnaire up to three times, with at least 3 weeks between each reminder. The questionnaire items addressed dermatitis after exposure to consumer goods with a metallic surface and the shortest time duration needed for a reaction to occur. Moreover, patients were asked about the first time they had experienced dermatitis caused by a shiny metallic item, their age at onset, and what item(s) they reacted to (multiple choices were allowed). They were given a list of 15 groups of items to select from, and could also add items not already listed. The initial dermatitis reaction was interpreted as the sensitization event; accordingly, the items causing the first dermatitis were considered to be of particular importance. An overview of the main questions and answer options is shown in Table S1.

All included patients were registered with the date of their patch test reaction, date of birth, sex, the maximum patch test reaction to nickel, and basic characteristics regarding their nickel allergy. All data from the questionnaires were entered into Epidata software (The Epidata Association, Odense, Denmark) by two investigators, and analysed with IBM SPSS statistics, version 22 (IBM, Armok, NY, USA). Before the analyses, 10% randomly chosen questionnaires (35 questionnaires) were checked for data entry errors. Among these, incorrect input was found in 0.23% of the questions.

Results

A total of 342 patients (318 women and 24 men) responded to the questionnaire, corresponding to a participation rate of 63.2%. Non-respondents were younger than respondents (60.9% versus 39.1% aged <40 years, $p < 0.01$), whereas no significant difference in the sex distribution was found (male non-responders 10.6% versus male responders 7.0%, $p = 0.15$). Further study population characteristics are shown in Table 1. The only difference between male and female patients concerned the prevalence of ear-piercing, whereby 95.8% of women with nickel contact allergy had pierced ears as compared with 30.4% of men ($p < 0.001$).

Metallic items causing dermatitis

Women reported first-time skin dermatitis caused by shiny metallic items at a median age of 16 years and men at a median age of 18 years. Data on first-time dermatitis are shown in Table 2; the patients often listed more

Table 1. Characteristics of patients

	Women n = 318	Men n = 24	Total n = 342	*p*-Value*
Age at test (years): Median; 25/75	47 (35–55)	56 (45–55)	47 (36–56)	–
Atopic dermatitis, n (%)	63 (19.8)	5 (20.8)	68 (19.9)	NS
Hand dermatitis at time of test, n (%)	114 (35.8)	8 (33.3)	122 (35.7)	NS
Facial dermatitis at time of test, n (%)	96 (30.2)	6 (25.0)	102 (29.8)	NS
Leg dermatitis at time of test, n (%)	3 (0.9)	1 (4.2)	4 (1.2)	NS
Pierced ears, n (%)	299/312 (95.8)	7/23 (30.4)	306/335 (91.3)	<0.001
Other piercings, n (%)	60/312 (19.2)	2/23 (8.6)	62/335 (18.5)	NS

NS, not significant.
*Chi^2 test, except if expected n < 6, when Fisher's test was used.

Table 2. First-time and most recent dermatitis caused by shiny metallic item(s)

	First-time dermatitis				Most recent dermatitis			
	Women n = 276	Men n = 18	Total n = 294	*p*-Value	Women n = 276	Men n = 18	Total n = 294	*p*-Value
Age at dermatitis (years): Median;	16	18	16	–	40	47	40	–
25/75	12–25	25–50	13–25	–	30–51	33–63	30–51	–
Items causing dermatitis, n (%)								
Earrings	187 (67.8)	3 (16.7)	190 (64.6)	< 0.001	133 (48.2)	1/18 (5.6)	134 (45.6)	< 0.001
Buttons on clothing	153 (55.4)	3 (16.7)	156 (53.1)	0.001	85 (30.8)	3/18 (16.7)	88 (29.9)	NS
Wrist watches	142 (51.4)	10 (55.6)	152 (51.7)	NS	62/275 (22.5)	5/18 (27.8)	67/293 (22.9)	NS
Other jewellery	138 (50)	4 (22.2)	142 (48.3)	0.02	96 (34.8)	2/18 (11.1)	98 (33.3)	0.04
Zips	65 (23.6)	1 (5.6)	66 (22.4)	NS	33 (12.0)	1/18 (5.6)	34 (11.6)	NS
Belt buckles	53 (19.2)	9 (50.0)	62 (21.1)	0.002	26 (9.4)	9/18 (50)	35 (11.9)	< 0.001
Spectacles	20 (7.2)	4 (22.2)	24 (8.2)	0.048	21 (7.8)	4/18 (22.2)	25 (8.5)	0.05
Hair clips	21 (7.6)	0	21 (7.1)	NS	17 (6.2)	0	17 (5.8)	NS
Keys	16 (5.8)	3 (16.7)	19 (6.5)	NS	27/275 (9.8)	2/18 (11.1)	29/293 (9.9)	NS
Coins	16 (5.8)	1 (5.6)	17 (5.8)	NS	23 (8.3)	0	23 (7.8)	NS
Scissors	6 (2.2)	0	6 (2.0)	NS	4 (1.4)	0	4 (1.4)	NS
Tools	3 (1.1)	1 (5.6)	4 (1.4)	NS	3 (1.4)	1/18 (5.6)	4 (1.4)	NS
Computers	3 (1.1)	0	3 (1.0)	NS	4 (1.4)	0	2 (1.4)	NS
Mobile phones	1 (0.4)	0	1 (0.3)	NS	3 (1.1)	0	3 (1.0)	NS
Lighters	1 (0.4)	0	1 (0.3)	NS	2 (0.7)	0	2 (0.7)	NS

NS, not significant.
Items that were added to the list by patients were: hooks on clothing (brassieres) and coat hangers (7); shoes (2); water taps (2); cutlery (1); door handles (1), pins/knives (1); MP3 ear plugs (1); workplace identity card holders; (1) and handles of office stamps (1).

than one item. In women, the most important cause of first-time dermatitis was earrings (67.8%), followed by buttons on clothing (55.4%), wrist watches (51.4%), other jewellery (50%), zips (23.6%), and belt buckles (19.2%). Few women reported work tools, computers, mobile phones or lighters as causes of first-time dermatitis. In men, wrist watches and belt buckles were the items most commonly cited as causing the initial episode of dermatitis (55.6% and 50%, respectively), followed by spectacle frames (22.2%), jewellery other than earrings (22.2%), earrings (16.7%), buttons on clothing (16.7%), and keys (16.7%). None of the men reported computers, mobile phones or scissors as the cause of first-time dermatitis.

More than one item could be listed as the cause of first-time dermatitis. In women, 88.7% reported jewellery (earrings, other jewellery, and/or wrist watches) and 62.0% reported metallic items on clothing (i.e. buttons, zips, and belt buckles) as the cause of first-time dermatitis. An overlap between the groups was seen, as some patients reported metallic items from both groups as the cause of first-time dermatitis. Among men, 66.7% reported jewellery as the cause of first-time dermatitis, and 55.6% reported metallic items on clothing as the cause of first-time dermatitis (Fig. 1a,b). In total, 73 (33%) patients listed only one item as having caused first-time dermatitis: of these, 31 (42%) reported earrings, followed by wrist watches (n = 13), and buttons (n = 11).

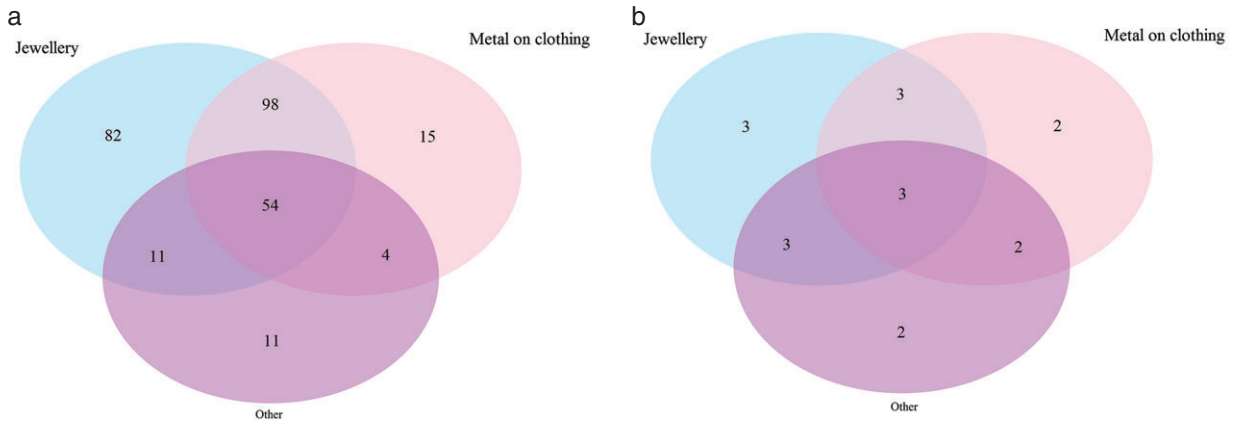

Fig. 1. Venn diagram of metallic items as the cause of first-time dermatitis clustered in groups for (a) women and (b) men. Jewellery: earrings, other jewellery, and wrist watches. Metal on clothing: buttons on clothing, zips, and belt buckles. Other: keys, coins, spectacles, hair clips, scissors, tools, computers, lighters, and mobile phones. Self-added metallic items were not included in this analysis.

Table 3. Dermatitis caused by shiny metallic items in subgroups of patients

	Aged ≤25 years in 2015	First dermatitis within the past 10 years	Most recent dermatitis within the past 5 years
Number	20	30	173
Sex, male/female	1:19	5:25	12:161
Age (years), median	21	38	49
25/75 percentiles	19–24	25–58	35–58
Items causing dermatitis, n (%)			
Earrings	11 (55)	10 (33.3)	73 (42.2)
Other jewellery	11 (55)	9 (30.0)	58 (33.5)
Buttons on clothing	4 (20)	8 (26.7)	49 (28.3)
Wrist watches	2 (10)	4 (13.3)	28 (16.2)
Zips	2 (10)	3 (10)	26 (15.0)
Keys	0	4 (13.3)	22 (12.7)
Belt buckles	4 (20)	9 (30.0)	23 (13.3)
Coins	0	3 (10.0)	16 (9.2)
Spectacles	0	4 (13.3)	17 (9.8)
Hair clips	2 (10)	1 (3.3)	11 (6.4)
Scissors	0	0	4 (2.3)
Tools	0	1 (3.3)	4 (2.3)
Computers	1 (5)	2 (6.7)	4 (2.3)
Lighters	0	1 (3.3)	2 (1.2)
Mobile phones	0	0	2 (1.2)

The same patient may occur in more than one column of this table.

A statistically significant overlap was found between earrings and wrist watches as causes of first-time dermatitis ($p < 0.001$), earrings and buttons ($p < 0.001$), earrings and other jewellery ($p < 0.001$), and earrings and zips ($p < 0.01$).

Table 2 also contains data on items causing the most recent episode of dermatitis. Women had a median age of 40 years and men had a median age of 47 years when they experienced their most recent episode of dermatitis caused by a shiny metallic item. Women reported the same five most frequent causes as for the first episode of dermatitis; however, the order was partly changed, earrings (48.2%), buttons on clothing, other jewellery, wrist watches and zips. Men most commonly reported belt buckles (50%) as causing their most recent episode of dermatitis, followed by wrist watches, spectacles, buttons on clothing, and jewellery. Earrings were reported by only 1 man, and none of the men mentioned computers, mobile phones, or scissors.

Patients were also analysed in subgroups to further evaluate the effect of the nickel regulation, as shown in Table 3. The first subgroup comprised the young patients with nickel allergy. They were aged ≤25 years at the time of answering the questionnaire, and had thus lived their entire lives under the protection of a nickel regulation; the first regulation came into force in Denmark in 1990. Another subgroup comprised patients who reported first-time dermatitis in the previous 10 years, that is, during the time in which the EU regulation on nickel has been in force in Denmark. The last subgroup of interest comprised patients who reported their most recent dermatitis in the past 5 years, indicating continued exposures of clinical significance.

Together, the above findings show that earrings remained the foremost cause of reactions to nickel-releasing items in all patients and in all subgroups. In patients who reported reactions after a regulation on nickel had been implemented, the causes, in order of priority, were: other jewellery, buttons on clothing, belt buckles, and wrist watches.

Length of contact needed to elicit dermatitis

All patients who reported dermatitis caused by metallic items were asked how short a contact with a shiny metallic item was sufficient for dermatitis to develop; 290 answered (99.3%) (Fig. 2). Of these, 6.6% (5.6% of men and 6.6% of women) reported a skin reaction after 2 min of contact, 21.4% (16.7% of men and 21.7% of women) reported a skin reaction after ≤10 min of contact, and 30.7% (22.2% of men and 31.3% of women) reported a skin reaction after ≤30 min of contact. To investigate whether patients with the strongest patch test reactions reported dermatitis after a shorter time of direct contact than patients with weaker reactions, the length of direct contact was analysed with respect to the strength of the patch test reactivity (Fig. 3). Patients were stratified into groups according to the reported length of time before dermatitis developed: within 30 min (2, 5, 10 or 30 min) or more than 30 min (1, 2–5 h, or a longer time). There was a non-significant trend for shorter contact time (≤30 min) for increasing patch test reactions (Cochran–Armitage test for trend, $p = 0.10$). There was no trend for faster reactors in the group who had been diagnosed with atopic dermatitis (Cochran–Armitage test for trend, $p = 0.81$).

Discussion

In this questionnaire study of 541 nickel-allergic patients from a hospital clinic, earrings were the metallic items that most commonly caused self-reported dermatitis, for both first and last episodes of dermatitis. Other culprit metallic items, in order of frequency, were other jewellery, buttons on clothing, belt buckles, and wrist watches. Furthermore, dermatitis was reported after ≤10 min of skin contact with a shiny metallic item by 21.4% of all patients, and after ≤30 min by 30.7% of all patients.

The response rate was 63.2%, which was satisfactory. The distribution of sex (male/female ratio of 1:13) and age in the present study was in line with previous studies (26, 27). The high number of women with nickel allergy as compared with men is most likely attributable to differences in exposure between the sexes. The key exposures leading to nickel contact allergy seem to occur early in life. Thus, in this investigation, the initial dermatitis caused by metallic items was experienced at a median age of 16 years for women and 18 years for men.

We assume that the initial dermatitis mostly represents the sensitization event, as the items causing it are usually worn every day for prolonged periods (28, 29). We found earrings to be the major cause of sensitization in all patients and also in the subgroups expected to have been protected by a regulation; that is, patients aged ≤25 years and those who had experienced their first-time dermatitis within the past 10 years. Furthermore, earrings were reported as the major cause of elicitation after the implementation of the EU Nickel Directive.

It is well known that earrings for pierced ears constitute a special risk of inducing nickel sensitization (30), as the skin is broken and the metal therefore bypasses the normal skin barrier. The pivotal limit of nickel release used in the nickel regulation has been standardized only for metallic items placed on intact skin (31). The safety of piercing in relation to the use of different metallic alloys has been scarcely investigated. Even the use of high-quality stainless steel ear-piercing post assemblies may not exclude allergic reactions in those who are allergic to nickel (32). Ingber et al. (32) studied the clinical reaction after ear-piercing with post assemblies containing AISI 316 L stainless steel in 25 individuals with proven nickel allergy. Within 48 h of the piercing, 2 of those with nickel sensitivity developed redness and itching related to the piercing area, but the authors nevertheless concluded that the symptoms did not represent allergic nickel dermatitis. However, as redness and itching are the initial symptoms of allergic contact dermatitis, we conclude that ASNI 316 L stainless steel may not be safe for ear-piercing in nickel-allergic individuals, even if the studs comply with the recommended nickel release test.

Our finding that earrings remain the major cause of sensitization (as indicated by first-time dermatitis), together with the high prevalence of nickel contact allergy among young women (1), which does not seem to be declining any further (14), raises the question of whether the nickel regulation is sufficiently protective regarding piercing post assemblies. It has been shown that the EU Nickel Directive has been particularly effective in the group of women who have never had their ears pierced (13). However, clinical experimental trials examining the clinical reaction after piercing are needed to further explore this matter.

Overall, 'other jewellery' was the second most commonly reported item causing dermatitis after the implementation of a nickel regulation. High nickel release over the limits of the regulation has been found in jewellery, including earrings, purchased in several EU countries (33–36). Other frequently reported items in this study were buttons, belt buckles, and wrist watches, which have also been found to exceed the limits of permitted nickel release in a Danish study (33). The most commonly reported items in our study are in line with a previous questionnaire study of dermatitis patients from private dermatology clinics, with the addition of zips, belt buckles, and keys (37). Items such as mobile phones, computers, tools and scissors were relatively rare causes of self-reported dermatitis. This may be attributable to less

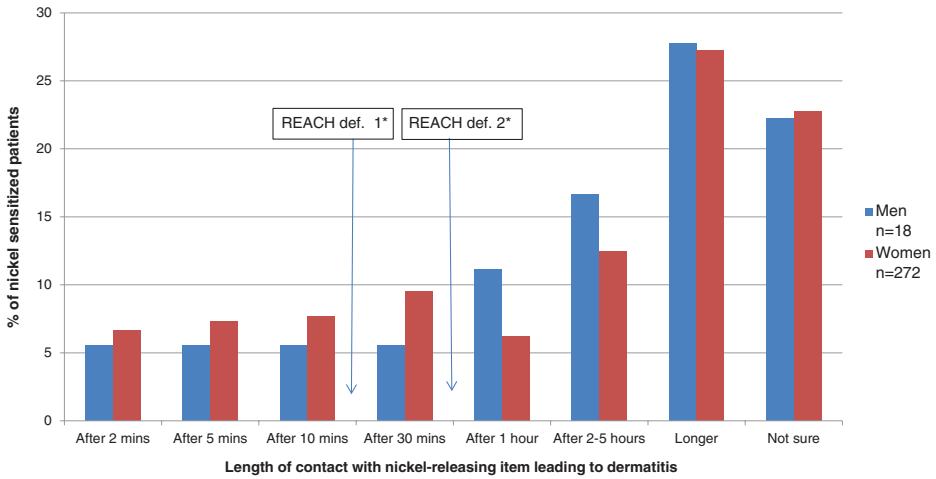

Fig. 2. Length of contact capable of eliciting dermatitis in 290 nickel patch test-positive patients seen at the Department of Dermatology and Allergy, Gentofte Hospital, during 2010–2014. (*1) Prolonged contact with skin, REACH definition 1: Prolonged contact defined as contact for >10 min on three or more occasions within a 2-week period. (*2) Prolonged contact with skin, REACH definition 2: Prolonged contact defined as contact for >30 min on one or more occasions within a 2-week period. [Definitions are given in REACH Annex XVII Entry 27, Q&A no. (935)].

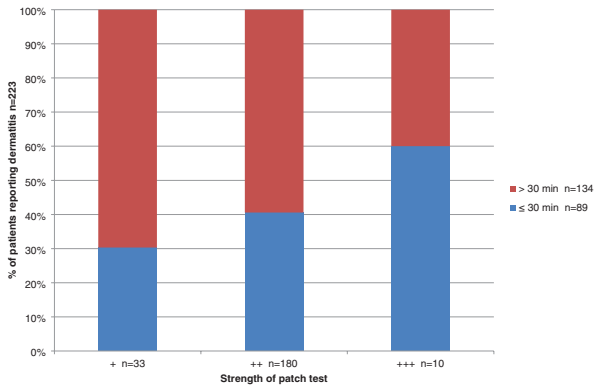

Fig. 3. Length of time from contact to dermatitis versus patch test reactivity to 5% nickel sulfate among 223 nickel patch test-positive patients seen at the Department of Dermatology and Allergy, Gentofte Hospital, during 2010–2014.

intense and intermittent contact with the skin and/or less use of nickel in alloys and coatings. The low number of male patients in this study sample make the interpretation of the result for men uncertain.

In this investigation, patients could select the culprit object from a list of 15 different types of shiny metallic item, which may mean that exposures were overlooked, although it was possible to add items not listed. The most frequently added item was hooks on underwear. This is an exposure known to lead to problems with nickel allergy, owing to the close and prolonged contact with the skin.

The definitions of time duration for items to fall under the EU Nickel Directive are based on existing data on release kinetics from materials in artificial sweat, studies on skin uptake and penetration of nickel, and elicitation thresholds in sensitized patients. The question has been raised of whether such durations of exposure can actually cause symptoms in a significant proportion of those with nickel allergy. In this investigation, 21.4% reported onset of dermatitis caused by a metallic item with exposures of ≤10 min, and 30.7% reported dermatitis with exposures of ≤30 min (Fig. 1). Therefore, the time limits in the definition seem to correspond well with the results of this study. We even found that 6.6% reported dermatitis after 2 min of contact.

Allergic nickel dermatitis after short skin contact with metallic items has not been studied in individuals with nickel allergy. However, skin deposition of nickel may be high following short and repeated skin contact of seconds to minutes with metallic surfaces, as has been shown in experimental studies, where nickel release was highest initially (38, 39). For another contact allergen used in black hair dyes, *p*-phenylenediamine, it is known that even 2 min of exposure can produce a positive patch test reaction when it is read after 48 h (40). In a recent publication, it was speculated whether some patients may react with contact urticaria rather than type IV reactions after contact with items releasing nickel. Saluja et al. (41) described positive prick test results for nickel 5% pet. in 11 patients with a history of nickel allergy, but with negative patch test results. All patients included in our study had

positive patch test reactions to nickel, showing contact allergy. However, we cannot exclude the possibility that the immediate self-reported reactions were attributable to an urticarial reaction. Another explanation for patients reporting eczema after short direct skin contact may concern local memory T cells in the skin. In a recent study, we showed the existence of distinct local memory in 10 patients with nickel contact allergy (42). The patients were patch tested with nickel or pet. on two occasions at the exact same skin areas, with a 21-day interval. Visible reactions were only found in skin areas that had been exposed to nickel on both occasions, and the clinical scores correlated with both accumulation of $CD8^+$ T cells and the presence of interleukin (IL)-1β, IL-1α and IL-17A in the skin.

We found an interesting, but non-significant, trend towards faster reactions in patients with strong patch test reactions. There were only 10 patients in the group with strong patch test reactions (+++), meaning that the statistical power of the study was probably insufficient in this regard. For further investigation, clinical trials testing the reaction time according to patch test reactivity are needed.

A weakness of the present study is that we did not include a control group. Furthermore, the numbers of patients in two of the subgroups were relatively small, being 20 and 30 patients (young patients and patients with first-time dermatitis during the past 10 years, respectively), making these result less robust. All nickel-allergic patients diagnosed within the past 5 years were included (n = 541). An explanation for the small sizes of these subgroups may be that patients with nickel allergy in Denmark are often not seen by a physician, and, if they are, they are seen primarily by dermatologists in private practice. They are only referred to hospitals if other issues arise or if the dermatitis is more severe. This means that there is a delay relative to the initial events in nickel sensitization, and the patient group therefore has a relatively high median age. Drawing conclusions about the prevalence and incidence of nickel allergy among dermatitis patients was not an aim of this study, and these should be drawn from large-scale cohort studies, as cross-sectional questionnaire studies are more suited for descriptive analyses.

Conclusion

The same nickel exposures that motivated a regulation limiting nickel release seem to persist today. This may be explained by differences in the actual permitted nickel release over time (10). Earrings remain the major cause of nickel sensitization and elicitation after implementation of the nickel regulation. Other important items are other jewellery, buttons on clothing, belt buckles, and wrist watches. The recent defined time durations for a metallic item to fall under the nickel regulation correspond well with the self-reported minimum contact time capable of eliciting contact dermatitis in this study.

Acknowledgements

We would like to thank research nurse Anne Marie Topp for participation in the collection of data, and the Danish Environmental Protection Agency for participation in reviewing the report. This study was funded by the Environmental Protection Agency under the Ministry of Environment and Food of Denmark. The data presented in this article were presented in the report 'An investigation of causes of nickel allergy' as a LOUS (The List of Undesirable Substances) follow-up project, environmental Project No. 1869, 2016.

Supporting Information

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

Table S1. Core questions in the questionnaire (translated from Danish).

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Supplementary material: Core questions in the questionnaire (Translated from Danish)

Questions	Answers
" Have you ever had dermatitis* after skin contact with shiny metallic items such as earrings or ear studs, watches, buttons or metallic fastenings?" (multiple answers allowed)	("Yes", "no").
"If yes, how old were you at the first occurrence?"	(Give age in years)
"If yes, which metallic items led to dermatitis at the first occurrence?"	(“earring/ear stud”, “other jewellery”, “watch”, “key”, “button”, “spectacles”, “scissors”, “belt buckle”, “coin”, “tool”, “mobile phone”, “hair clip”, “zip”, “lighter”, “computer”, “other”. Lastly, patients who responded “other” were asked to specify causative items)
"If yes, which metallic items led to dermatitis a the most recent occurrence?" (multiple answers allowed)	(“earring/ear stud”, “other jewellery”, “watch”, “key”, “button”, “spectacles”, “scissors”, “belt buckle”, “coin”, “tool”, “mobile phone”, “hair clip”, “zip”, “lighter”, “computer”, “other”. Lastly, patients who responded “other” were asked to specify causative items)
"How old were you at the most recent occurrence?"	(Give age in years)
“Do you develop dermatitis following short skin contact with metallic items?”	(“yes; after 2 minutes”, “yes; after 5 minutes”, “yes; after 10 minutes”, “yes, after 30 minutes”, “yes, after 1 hour”, “yes, after 2–5 hours”, “no, longer contact is needed”).

* Prior to the questions, dermatitis was described "dermatitisis is characterized by ichyness, red skin, bumps, small blisters and swelling. The skin eventuelly becomes rough. Dermatitis is usually located to the same areas during a long period of time."

Paper II: Short contact with nickel causes allergic contact dermatitis: an experimental study

Relatively short repeated exposures (3 x 10 minutes) to metallic nickel caused

- Allergic nickel dermatitis in 63% (10/16) of participants with nickel allergy on irritated skin and in 19% (3/16) on normal earlobe skin with previous dermatitis.
- No dermatitis on the normal forearm skin of participants with nickel allergy or on any skin sites of participants without nickel allergy.
- Significantly higher blood flow in irritated skin exposed to nickel compared with aluminium on both the first day and three days after exposure. The differences in blood flow increase between the exposure areas were 42.0 and 52.2 tissue perfusion units (TPU) one day and three days after exposure, respectively.
- A small but significant increase in blood flow in nickel- compared with aluminium-exposed normal skin in participants with nickel allergy one day but not three days after exposure (3.8 TPU) ($p=0.016$).
- No blood flow changes in earlobe skin in any participants or on any sites of participants without nickel allergy.

BJD
British Journal of Dermatology

Short contact with nickel causes allergic contact dermatitis: an experimental study

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Summary

Background Knowledge about the required duration of exposure for elicitation of allergic nickel dermatitis in nickel-allergic individuals is limited. However, it often has been proposed that short skin contact is safe.
Objectives To examine whether repeated skin contact with nickel over short time periods (3 × 10 min) can elicit allergic nickel dermatitis.
Methods Sixteen nickel-allergic adults and 10 controls were exposed to, respectively, nickel- and aluminium-containing discs on each volar forearm and on each earlobe for 3 × 10 min. One arm was pretreated for 24 h with sodium lauryl sulfate (SLS) 0·5% under occlusion before exposure. One aluminium and one nickel exposure site were clinically evaluated, and blood flow was measured with laser Doppler flowmetry at day 2 and day 4.
Results Ten of 16 (63%) nickel-allergic participants developed allergic nickel dermatitis on SLS-pretreated arm skin and three of 16 (19%) developed it on normal skin on the earlobe. On the SLS-pretreated arms of nickel-allergic participants, blood flow increased significantly more on the nickel-exposed skin than on the aluminium-exposed skin on days 2 and 4. No change in clinical reactivity or blood flow was found on normal forearm skin in nickel-allergic participants or on any skin in controls.
Conclusions This experimental study showed that relatively short repeated skin contact (3 × 10 min) with metallic nickel elicits allergic nickel dermatitis in irritated skin and at sites with previous dermatitis. The results support the restrictions in current nickel regulation.

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Accepted for publication
27 June 2018

Funding sources
M.G.A. and J.D.J. were financially supported by an unrestricted grant from the Danish Environmental Protection Agency. This study was supported by the Aage Bangs Foundation. The funding sources did not play any role in the study design or the preparation of the manuscript.

Conflicts of interests
None to declare.

DOI 10.1111/bjd.16935

What's already known about this topic?

- Repeated low-dose nickel exposure may be a more potent trigger of allergic nickel dermatitis than less frequent exposure to a higher single dose.
- High nickel deposition onto skin can be measured after only seconds of direct skin contact.
- An impaired skin barrier may increase the risk of allergic nickel dermatitis.

What does this study add?

- Relatively short repeated skin contact (3 × 10 min) with nickel has the potential to provoke allergic nickel dermatitis in irritated skin and at sites with previous dermatitis.

B J D | Journal Code | 16935 | Manuscript No. | WILEY | Dispatch: 31.8.18 | No. of pages: 8 | CE: Vasanth | PE: Revathi K.

Skin contact with nickel-releasing items can lead to nickel allergy and dermatitis if the duration of exposure is sufficient. Due to a high prevalence of nickel allergy in Europe, nickel release from some consumer items became regulated in European Union (EU) member countries in 2000. Since then, the prevalence of nickel allergy has been reduced in some EU countries, but nickel is still the leading cause of contact allergy.[1] The regulation stated that items intended for 'direct and prolonged contact with the skin' were included, although no exact duration of exposure was determined. In 2014, 'prolonged contact with the skin' was defined by the European Chemicals Agency (ECHA) as being 'more than 10 minutes on three or more occasions within two weeks, or 30 minutes on one or more occasions within two weeks'.[2] Although it is well accepted that long-term skin contact can cause nickel allergy and dermatitis, the significance of relatively short and repeated nickel exposure in relation to induction and elicitation of nickel allergy has been less explored.

To evaluate the regulatory protection against nickel allergy and dermatitis in the EU, improved understanding of the association between short repeated skin contact and nickel allergy and dermatitis is needed. We examined whether relatively short skin contact (3 × 10 min) with metallic nickel has the potential to elicit allergic nickel dermatitis on normal and irritated skin in nickel-allergic individuals.

Materials and methods

Study population

We enrolled 16 current nickel-allergic adult participants and 10 control volunteers with no history of contact allergy. The median age of the nickel-allergic participants (13 women and three men) was 55·5 years (interquartile range 44–63·5) and of the controls (nine women and one man) 34·0 years (interquartile range 25–58). Nickel-allergic participants had previously been diagnosed by patch testing with nickel sulfate 5% in petrolatum (Almirall Hermal, Reinbek, Germany) in Finn Chambers in the time period 2015–2017 at the Department of Dermatology and Allergy, Herlev and Gentofte Hospital.

Exclusion criteria for all participants were active dermatitis, scar tissue or tattoos on areas to be exposed, generalized dermatitis, pregnancy, breastfeeding, treatment with topical corticosteroids or other topical immunosuppressants on or near exposure areas, use of systemic immunomodulatory treatment within 4 weeks, extensive exposure to ultraviolet radiation within 3 weeks (solarium, sunbathing) and participation in other clinical studies within 4 weeks. Recruitment followed approval from the local ethics committee (H-16050296) and the Danish Data Protection Agency. All participants gave written informed consent before the start of the study. The study was registered at ClinicalTrials.gov (NCT03309215).

Restrictions

During the study period, showers and use of emollients in the skin areas undergoing exposure were not allowed. Participants were not allowed to eat, drink coffee or tea, smoke or do physical exercise for 2 h before blood flow measurements, or to use analgesics 24 h before blood flow measurements.

Metallic discs and preparation

The metallic discs used in the study were composed of > 99 wt.% nickel and > 99 wt.% aluminium (active and negative control discs, respectively) and were manufactured by the Technical University of Denmark, Kgs Lyngby, Denmark. The metallic discs had a diameter of 3 cm (area 7·02 cm^2) (back and forearm exposure) and 1 cm (area 0·79 cm^2) (earlobe exposure). In total, 90 metallic discs were used in the study, and the discs were reused for the participants. Nickel release under various conditions was quantified and will be reported separately (manuscript in preparation). To enable reproducible exposure conditions, one side of all discs was prepared in the same manner 1 day before use (Appendix S1; see Supporting Information).

Exposure area

Four exposure areas of 3·5 × 3·5 cm^2 on the mid volar forearms were marked (Richard-Allan® Regular Tip Skin Marker; Aspen Surgical, Caledonia, MI, U.S.A.). The exposure areas on the arms were 'mirrored' and the set-up was identical in all participants (Fig. 1). The exposure areas were slightly adjusted if a local naevus, large vein or haematoma was present. On the earlobes, the metallic discs were placed centrally and no marking of the skin was made (Fig. S1; see Supporting Information). Only the most distal aluminium and nickel areas (3 and 4) of each arm were used for the repeated evaluation of clinical reactivity and blood flow on all study days. The other exposure areas were used to measure nickel penetration over time, and the data will be presented elsewhere (manuscript in preparation).

Pretreatment with sodium lauryl sulfate

A solution of high-purity sodium lauryl sulfate (SLS) (99% purity; Sigma-Aldrich, St Louis, MO, U.S.A.) was prepared in distilled water to a concentration of 0·5% (w/v). A 200-μL aliquot of the solution was applied on a filter paper disc and fitted into an extra-large (18 mm Ø) Finn Chamber on Scanpor Tape (SmartPractice, Phoenix, AZ, U.S.A.), as recommended.[3] Four chambers were applied to one randomized forearm within 1 min after preparation,[4] and occlusive pretreatment was performed during 24 h. The SLS solution was stored in a refrigerator at 4 °C between use.[4]

Pilot study

A pilot study with three nickel-allergic participants preceded the study. The main purpose was to test the logistics and suitability of the equipment and procedures, resulting in changes of the protocol: the method for preparation of discs was

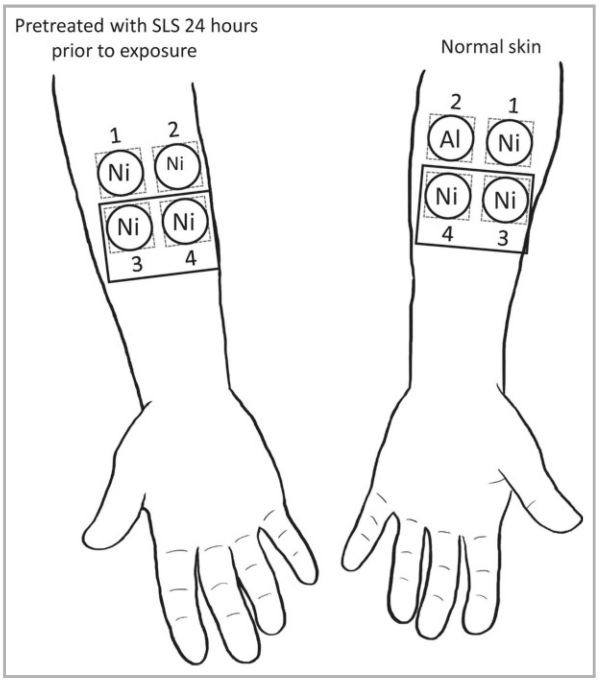

Fig 1. A schematic view of the four exposure areas on each mid volar forearm. The rectangle of each arm marks the two areas (3 and 4) used for all evaluations (clinical and blood flow) during the study. SLS, sodium lauryl sulfate.

changed, points for blood flow measurements were chosen differently, and assessments on day 5 were moved to day 4. More information can be found in Appendix S1 (see Supporting Information).

Experimental study design

The experimental study ran over 12 weeks; the pilot study ran in June 2017 and the main study from September to December 2017. For each participant, the study ran over four study days (Fig. 2) and was conducted at a laboratory and an adjacent exposure chamber located at the Department of Dermatology and Allergy at Herlev and Gentofte Hospital.

On day 0, the four exposure areas on one arm were marked and pretreated with SLS under occlusion (Fig. 1). To verify the nickel allergy status in all participants, patch testing on the back with nickel sulfate 5% in petrolatum in Finn Chambers was conducted. In addition, one prepared disc each of metallic nickel or aluminium was fastened with Scanpor tape on the back. Participants were asked about their previous history of dermatitis on exposure sites and skin diseases in general.

On day 1, the patches with SLS were removed, and the irritant reactions in two areas (3 and 4) were evaluated. The blood flow in the same two areas on the SLS-pretreated arm, on the corresponding two areas on the arm with normal skin and on the skin of both earlobes was measured (Fig. 1) (baseline). On day 1 the participants were acclimatized for 30 min in an exposure chamber with the temperature at 29·2 ± 0·69 °C (range 28·8–29·4) prior to stimulation with the metallic discs, and all metallic disc exposures were performed in the chamber. The exposure chamber (2·1 × 2·2 × 2·3 m), usually used for investigation of airborne allergic reactions, was supplied with fresh air at 0·5 exchange rates per hour and ambient humidity for the season.

Three nickel discs and one aluminium disc were placed centrally in the four premarked exposure areas on each arm. Further, one metallic disc with nickel and one with aluminium were randomly placed centrally on each earlobe. The discs were applied in intervals of 10 min repeated two times with 10-min breaks, thereby being in accordance with the ECHA's definition of prolonged contact.[2] To simulate real-life contact with metallic nickel on both arms and earlobes, friction was created by rotating the metallic discs 90 ° back and forth two times without lifting the discs from the skin. All discs were applied by the same investigator (M.G.A.), fastened with Scanpor tape and left in place for 10 min. Separate newly prepared discs were used for the three applications at each exposure area.

On the following study days 2 and 4 (24 and 72 h after metallic disc exposure, respectively), clinical evaluation and blood flow measurements were made at the same exposure areas (3 and 4) as on day 1 (Fig. 1). The temperature during measurements of blood flow on days 1, 2 and 4 was kept constant at a mean 23·9 ± 0·78 °C (range 23·3–24·2). The metallic discs on the back were removed on day 2 and the clinical evaluations were made on days 2 and 4.

Clinical evaluation

To identify small changes in the exposed forearm skin (irritant and allergic), the exposure areas were evaluated using the modified International Contact Dermatitis Research Group (ICDRG) criteria.[5] Reactions on the earlobes were evaluated as being present or not present. All exposure sites were read by M.G.A. and photos of all exposure sites were subsequently reviewed in a blinded manner by T.M. The clinical evaluation was made and photos were taken 45 min to 1 h after removal of the SLS chambers on day 1 (baseline). Test reactions on the back were photographed and evaluated according to the regular ICDRG criteria approximately 10 min after removal of the chamber and discs.[6]

Skin blood flow measurements

For a quantitative assessment of inflammation, skin blood flow was measured using laser Doppler flowmetry (moorVMS-LDF1, Moor Instruments Ltd, Axminster, U.K.). Prior to the measurements, the participants were instructed to rest for 15 min in the same position in which the measurements were taken, sitting with the exposure area of the forearms at heart level.[7] During the measurements, participants were asked not to move or talk, and to breathe normally. To secure the same measurement points over the study period, we used a template marking the two most peripheral points corresponding to the area exposed to metallic discs on an imaginary

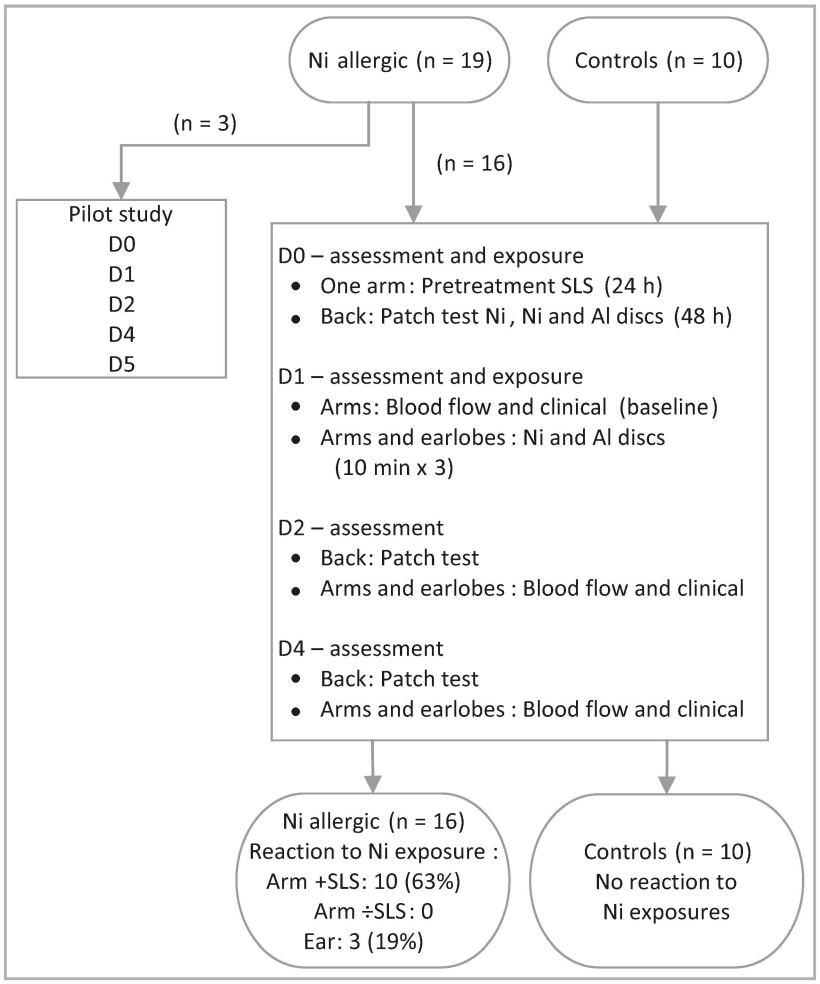

Fig 2. Flowchart of the study. D, day; SLS, sodium lauryl sulfate.

horizontal line through the test centre. Three consecutive measurements of approximately 20 s were performed, one in the centre of the exposure area and one on either side midway between the centre and the two marked peripheral points. For the earlobes, three consecutive measurements of approximately 20 s were made at the central exposure area.

Statistics

For the statistical analyses, the clinical evaluation scores were transformed to numerical values as follows: ÷ = 0, (+) = 0·5, + = 1·0, +(+) = 1·5, ++ = 2·0, ++(+) = 2·5, +++ = 3·0.[8] For analysis of blood flow measurements, the mean of a steady-state region of interest of each of the three consecutive measurements was used.[7] For monitoring change of the blood flow at one exposure area over time, the baseline values (after SLS occlusion and before nickel or aluminium exposure on day 1) were subtracted from the values at the two different evaluation time points (days 2 and 4).

The resulting blood flow changes at days 2 and 4 for the respective exposure area (SLS or non-SLS, nickel or aluminium) were added for the nickel-allergic group and the control group, respectively. The paired t-test was used to compare the change in blood flow between the nickel and the aluminium exposure areas. The same method was used for the numerical values from the clinical evaluation. A positive allergic reaction was defined as a greater increase in the clinical evaluation score at the nickel area compared with the aluminium area at the respective measurement day.

Normality assumptions for the paired t-test were fulfilled. Power analysis to determine the appropriate sample size was done aiming for 80% power and a significance of 5%. The study data were collected and managed using REDCap electronic data capture tools.[9] All statistical analysis were made in SAS, version 9·4 for Windows (SAS Institute Inc., Cary, NC, U.S.A.) and the graphs with GraphPad Prism version 6·07 for Windows (GraphPad Software, La Jolla, CA, U.S.A.).

Results

All 26 included participants completed all study days. One nickel-allergic participant was excluded from the analyses of the arms at day 4 because she developed dermatitis with suspected superinfection at the nickel exposure areas of the SLS-

pretreated arm, which impeded further evaluation. The study characteristics for each participant are shown in Table 1. Previous and current nickel patch test reactivity in the nickel-allergic participants can be found in Table S1 (see Supporting Information).

Ten of the 16 nickel-allergic participants (63%, 95% confidence interval 38·6–81·5) developed allergic nickel dermatitis on the SLS-pretreated skin, and none did on normal skin on the arms. For the nickel-allergic participants, the overall increase in clinical reactivity on the SLS-pretreated skin from baseline to the two measurement days (days 2 and 4) was significantly higher at the nickel vs. the aluminium exposure area (Fig. 3a). The blood flow increased, with 42·0 tissue perfusion units (TPU) more at the nickel- vs. the aluminium-exposed SLS-pretreated skin at day 2, and with 50·2 TPU more at day 4 for nickel-allergic participants (Table 2) (Fig. 3b). A small but significant increase in blood flow at the nickel vs. aluminium exposure sites was seen in normal skin of nickel-allergic participants from baseline to day 2, but not to day 4 (Table 2).

Three of the 16 nickel-allergic participants (19%, 95% confidence interval 6·6–43·0) reacted to metallic nickel on the earlobe, one of them at day 4 and the other two after the last study day, namely at day 5. The two allergic reactions that occurred on day 5 were assessed by a description of the reaction and a verifying photo received from the nickel-allergic participants. There was no change in blood flow in the earlobes at the nickel site compared with the aluminium site for nickel-allergic participants at any time point compared with baseline. The three nickel-allergic participants with earlobe reactions in this study (nos. 4, 7 and 15) had reported previous earlobe dermatitis. Two of them reported a history of hand dermatitis and one of atopic dermatitis (Table 1).

No allergic reactions or blood flow changes were seen in the control participants (Table 2). The key results of the study presented for each individual are listed in Table S2 (see Supporting Information). Photos of the exposure areas of one nickel-allergic participant and one control volunteer over the study period can be found in Figure 4.

Discussion

In this experimental study, we found that relatively short repeated skin contact with metallic nickel (3 × 10 min)

Table 1 Characteristics of the study population

				Self-reported			
Participant	Sex	Age (years)	No. of ear piercings	Atopic dermatitis[a]	Hand dermatitis[a]	Hand dermatitis ≤ 3 months[b]	Earlobe dermatitis ever
Nickel							
1	Female	56	2	−	−	−	+
2	Female	48	2	−	−	−	+
3	Male	66	0	−	−	−	−
4	Female	19	≥ 8	+	+	+	+
5	Male	65	0	−	−	+	−
6	Female	20	≥ 8	−	−	−	+
7	Female	69	2	−	−	−	+
8	Female	65	2	−	−	−	+
9	Female	55	2	−	+	+	+
10	Female	61	4–7	−	−	−	+
11	Male	56	0	−	+	+	−
12	Female	48	4–7	−	−	−	−
13	Female	44	2	+	−	−	−
14	Female	44	4–7	−	−	−	+
15	Female	62	2	−	+	+	+
16	Female	42	2	−	−	−	+
Control							
17	Female	63	2				
18	Female	24	2				
19	Female	66	2				
20	Female	34	2				
21	Female	58	2				
22	Female	25	3				
23	Female	27	3				
24	Female	34	4–7				
25	Female	57	2				
26	Male	24	0				

[a]Participants were asked if a doctor had ever told them that they had hand dermatitis or atopic dermatitis. [b]Participants were asked for presence of dermatitis within the last 3 months; dermatitis was reported only on the hands in this period.

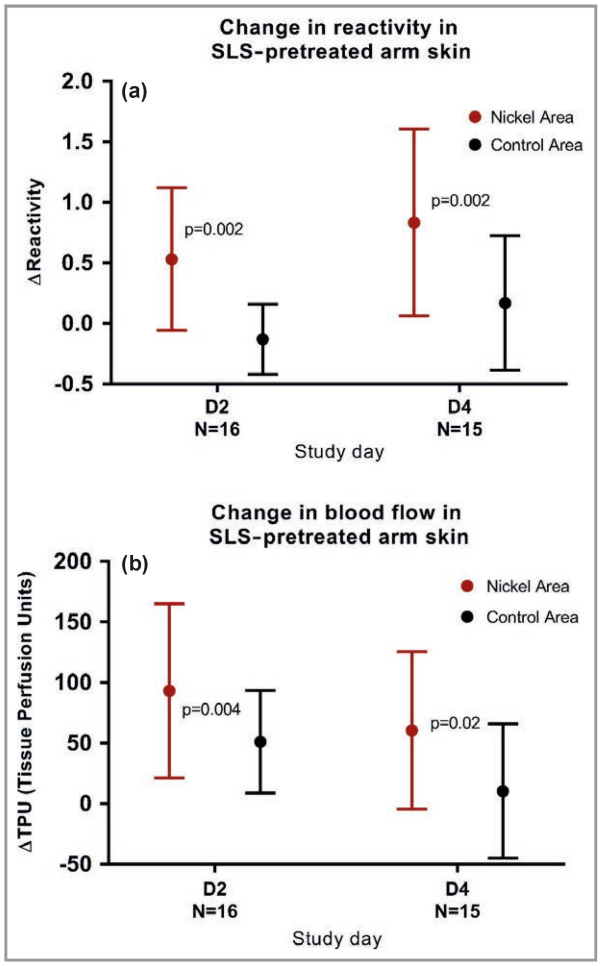

Fig 3. (a) Sodium lauryl sulfate (SLS)-pretreated arm skin in nickel-allergic participants. The change in clinical reactivity is given as a numerical value (mean with 95% confidence interval) from baseline to measurement days (D2, D4) at nickel and aluminium exposure areas. The numerical values were transformed from scores as follows: ÷ = 0, (+) = 0·5, + = 1·0, +(+) = 1·5, ++ = 2·0, ++(+) = 2·5, +++ = 3·0. (b) Change in blood flow on SLS-pretreated arm skin from baseline to the measurement days (D2, D4) at the nickel and aluminium exposure areas (mean with 95% confidence interval). A paired t-test was used to compare the change in clinical reactivity (a) and blood flow (b) between the nickel and aluminium sites.

caused allergic nickel dermatitis in irritated skin in the majority (63%) of nickel-allergic participants. This finding was supported by a statistically significant blood flow increase both 1 and 3 days after exposure to nickel compared with aluminium, and in the absence of clinical reactivity in non-nickel-allergic controls. Furthermore, we showed that some of the nickel-allergic participants (three of 16) developed dermatitis on earlobe skin after nickel exposure.

We created a theoretical worst-case scenario for provocation of allergic nickel dermatitis: irritated skin,[10,11] restriction of showering and emollients, slightly elevated ambient temperature,[12,13] pure nickel discs with newly prepared and untouched surfaces, and discs manually applied with friction and pressure.[14] Although these conditions are presumed to increase the risk of elicitation, they are not far from the real-life setting. Irritated skin is common in the general population, demonstrated recently by a lifetime prevalence of itchy skin rash and contact dermatitis in 52·3% and 15·5% of people, respectively.[15] Also, pressure and friction are common during skin contact with metallic items. A strength of the present study is that the set-up was controlled and reproducible: the metallic discs had equal surface properties, the temperature during exposures and measurements was uniform, all exposures and assessments were made by the same investigator, and all photos of the exposure areas were subsequently evaluated by a blinded investigator (T.M.).

The elicitation potential of short nickel skin contact has rarely been studied. Most studies on short repeated nickel exposure are repeated open application test studies. Fischer *et al.* found that repeated low-dose nickel exposure may be a more potent trigger of allergic nickel dermatitis than less frequent exposure to a higher single dose.[16,17] The exposures in the present study may more accurately represent most real-life exposures with nickel, as we used metallic nickel instead of nickel solution, and included factors such as pressure and friction. Also, the exposures were made in an exposure chamber with a temperature resembling an average Mediterranean midsummer day. Remarkable differences in the prevalence of nickel allergy have consistently been demonstrated between EU countries.[1] Several factors affecting the exposure to nickel are likely to contribute to the differences. Among them are fashion, compliance with the nickel restriction,[18] and possibly ambient temperature.[13]

The fact that we found enhanced clinical reactivity to nickel only on irritated skin in nickel-allergic participants and on skin with previous earlobe dermatitis (suggestive of allergic nickel dermatitis) is consistent with the current literature in the field. The elicitation threshold by a single occlusive nickel exposure on skin with a defective barrier has generally been found to be reduced compared with an intact barrier.[10,19] Furthermore, skin sites with previous allergic nickel dermatitis have been shown to have a local allergen-specific memory and a reduced threshold for elicitation of dermatitis.[5,20,21] The results of this study are in line with our previous finding that nickel-allergic individuals report dermatitis after relatively short direct contact with metallic items (21·4% after 10 min of contact).[22] Moreover, the increased occurrence of nickel dermatitis in patients with atopic dermatitis may point to the effect of an irritated and impaired skin barrier.[23]

Weaknesses of the study include the following. Firstly, there was no standardization of the pressure on the discs. Secondly, the blood flow measurement points were marked only peripherally on the skin, which increases the risk that the measurements were not made on exactly the same three points over the study days. Using the mean of three measurements reduces the uncertainty regarding this factor. Thirdly, no blinding could be done for the metallic disc exposures, clinical evaluations or blood flow measurements, as the same exposure pattern was used for all participants to avoid confusion.

Table 2 Blood flow in nickel-allergic and control participants

	Patients	Day	Baseline blood flow (TPU)[a]	Change in blood flow from baseline (TPU) (95% CI)[b]	P-value
SLS-pretreated arm skin					
Nickel-allergic participants	16	2	58·0	42·0 (15·3–68·8)	0·004
	15[c]	4	53·5	50·2 (7·5–93·0)	0·024
Controls	10	2	42·2	−14·3 (−30·5–1·82)	0·076
	10	4	42·2	−3·5 (−23·6–16·6)	0·7
Normal arm skin					
Nickel-allergic participants	16	2	24·2	3·8 (0·82–6·86)	0·016
	15[c]	4	23·7	2·3 (−1·72–6·23)	0·24
Controls	10	2	25·6	−1·1 (−6·43–4·30)	0·66
	10	4	25·6	−2·3 (−9·16–4·63)	0·48

TPU, tissue perfusion units; CI, confidence interval. [a]The mean of the baseline values of the aluminium and nickel exposure sites. [b]Difference between the nickel and aluminium test sites. [c]One participant was excluded at day 4 due to suspected superinfection.

COLOR

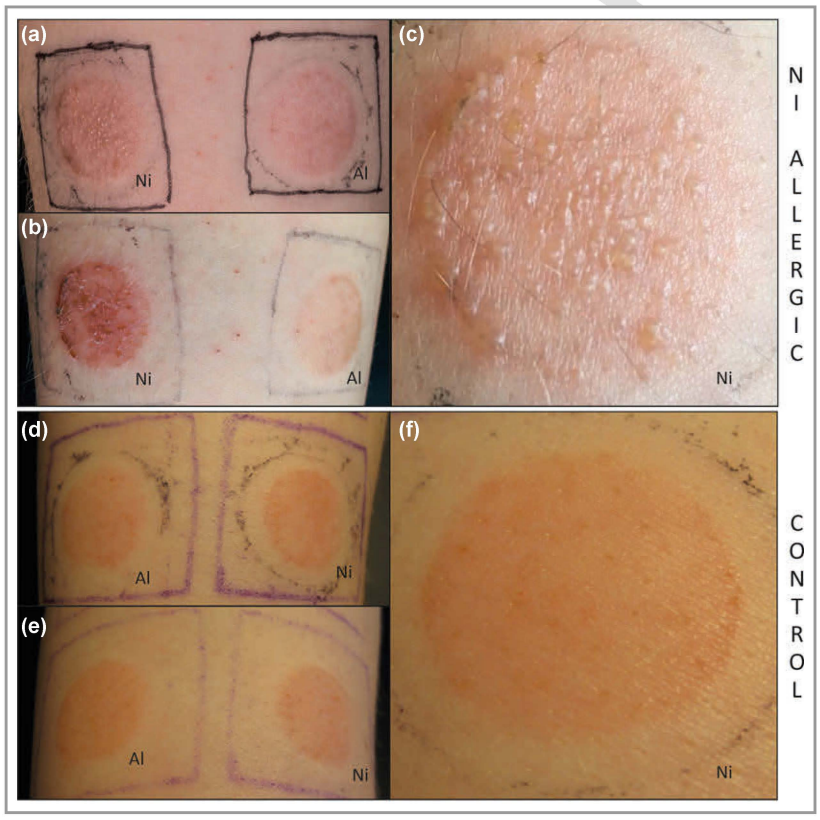

Fig 4. Photos of the reactions of one nickel-allergic participant (no. 4; a–c) and one control participant (no. 20; d–f) at day 2 (a, d) and day 4 (b, e) after exposure to nickel and aluminium discs on the sodium lauryl sulfate-pretreated arm. (c, f) Close-up of the nickel-exposed areas at day 2, corresponding to (a) and (d).

Fourthly, one could argue that the nonacceptance of emollients and water on exposure areas is far from the real-life setting. However, this was a compromise to produce a worst-case scenario, to meet the aim of studying the potential of relatively short repeated nickel exposure.

In the present study, we wanted to examine whether relatively short repeated nickel contact can elicit allergic nickel dermatitis. The duration of the exposures fulfilled the definition of prolonged contact according to the ECHA, in an attempt to enhance protection by the nickel regulation. In real life, skin contact with metallic items can be limited to sites with previous irritant or allergic contact dermatitis, which may be more susceptible to short-duration nickel contact. From the results we conclude that in skin with low-grade dermatitis, or previous dermatitis, relatively short skin contact with nickel can be sufficient to elicit allergic nickel dermatitis. The results may be valuable for nickel-allergic individuals and dermatologists in order to evaluate the possible risk of allergic nickel dermatitis after short contact with metallic items. From a regulatory perspective, the results of this study underline the

importance of regulating metallic items intended for short direct contact, in order to prevent allergic nickel dermatitis.

In conclusion, this experimental study shows that relatively short repeated skin contact with metallic nickel can elicit allergic contact dermatitis in irritated skin and on sites with previous dermatitis.

Acknowledgments

We are very grateful to Anne Marie Topp, Lene Toft-Jensen and Julie Sølberg for their help during the experiments and Anna Bünning Olsson for graphics support. Josefine Bandier granted us access to the laser Doppler flowmeter.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1. Pilot study and metal disc preparation.

Fig S1. Metallic disc placed centrally on the earlobe without marking of the skin.

Table S1. The reproducibility of nickel allergy in the nickel-allergic participants illustrated by patch test reactivity and reactions to metal discs on the back.

Table S2. Key results for each nickel-allergic participant.

Supporting material for study

"Short contact with nickel causes allergic contact dermatitis: an experimental study"

Pilot study

A pilot study with 3 nickel allergic participants preceded the study. The pilot study differed from the main study in four important ways; 1) the preparation of the discs were made by hand instead of by a sander with a process identical to the one described in (1), 2) the 3 blood flow measurement points were chosen differently in the test area, 3) the last study day (D5) occurred 96 instead of 72 hours post exposure. Changes 1) and 2) were made in order to optimize the test procedure with respect to time consumption and more accurate blood flow measurements. Change 3) was made for practical reasons.

Metal discs preparation

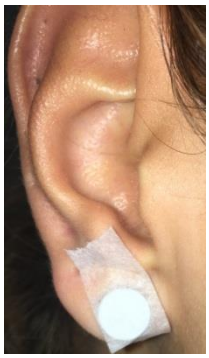
Discs were placed in a custom-made disc holder, and the surface of one side was grinded with wet sandpaper (P800; Schuller eh Klar GmbH, Vejle, Denmark) fastened on a 240 W sander (KA320EKA, Black & Decker, Slough, England) and the procedure was repeated with finer paper (P1200). The sander was applied to the discs on the lowest speed for approximately five seconds two times in perpendicular direction. The aluminium discs were prepared prior to the nickel discs and the area of preparation was carefully cleaned by gentle rubbing with mild detergent on nonwoven swabs (CURI MED, Abena, Aabenraa, Denmark) in tap water, followed by wiping with ethanol (70% v/v) on nonwoven swabs in between. Separate sand papers, disc holders and utensils were used for the two materials. In between and after sanding, the discs were rinsed in demineralised water (LIVA, Respekt Denmark, Jyderup, Denmark), gently dabbed with laboratory paper tissues (Lyreco lint free wipes; Lyreco, Marly, France), and left to dry on paper tissues overnight. The surfaces were not touched after preparation. The sander was dimethylglyoxime tested negatively prior to use, and was carefully disinfected (Triamin Disinfection, Wetwipe, Vallensbaek, Denmark) between preparation of different materials.

Supplementary Table 1. The reproducibility of nickel allergy in the nickel-allergic participants illustrated by patch test reactivity and reactions to metal discs on the back

	Nickel patch test			
Reactivity	**Previous (2015-2017)**	**Current *(present study)***	**Ni disc**	**Al disc**
No reaction	0	0	0	14
Doubtful	0	0	3	1
1+	0	2	2	1*
2+	14	11	7	0
3+	2	3	4	0

*No reaction to aluminium in metallic form (an empty Finn Chamber) and a standard preparation of aluminium chloride hexahydrate 2% in petrolatum.

Supplementary Figure 1. Metallic disc placed centrally on earlobe without marking of the skin.



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Supplementary table 2: Key results for each nickel-allergic participant

Participant no.	Patch test reactivity to Ni (current)	Clinical reactivity (numerical values based on scores) on different exposure sites [a]											Blood flow (TPU) on different exposure sites								
		Arm								Earlobe			Arm								
		D1 (Baseline)			D2 (SLS pretreated)		D4 (SLS pretreated)		Change in Ni reactivity [c]		D4		D1 (Baseline)			D2 (SLS pretreated)		D4 (SLS pretreated)		Change in Ni reactivity [c]	
		SLS pretreated(before Ni)	SLS pretreated (before Al)	Control (no SLS) [b]	Ni	Al	Ni	Al	D2	D4	Ni	Al	SLS pretreated (before Ni)	SLS pretreated (before Al)	Control (no SLS) [b]	Ni	Al	Ni	Al	D2	D4
1	++	0	0,0	0,0	0,0	0,0	0,5	0,5	0	0	÷	÷	44,0	40,3	26,5	74,3	60,6	55,8	36,8	10	15,3
2	++	0	0,0	0,0	0,5	0,5	0,5	0,5	0	0	÷	÷	15,3	20,8	21,9	30,8	37,8	16,9	25,4	-1,5	-3
3	++	0.5	0,5	0,0	0,5	0,5	0,5	0,5	0	0	÷	÷	44,9	86,0	19,5	71,2	78,4	32,0	36,4	33,9	36,7
4	+++	1.0	1,0	0,0	2,5	0,5	2,5	1,0	+2.0	+1.5	+	÷	167,7	172,2	17,2	254,3	222,3	295,5	86,0	36,5	214
5	+++	0.5	0,5	0,0	1,0	0,5	3,0	2,5	+0.5	+0.5	÷	÷	38,8	45,5	38,8	207,3	116,8	212,2	170,0	97,2	48,9
6	++	1.0	1,0	0,0	1,0	0,5	1,5	1,0	+0.5	+0.5	÷	÷	28,1	37,0	10,6	135,3	111,7	124,2	104,0	32,48	29,08
7	++	0.5	0,5	0,0	0,5	0,5	1,5	0,5	0	+1.0	+[d]	÷	23,5	26,1	22,1	83,5	52,6	109,3	40,0	33,5	71,9
8	++	0.5	0,5	0,0	1,0	0,5	1,5	0,5	+0.5	+1.0	÷	÷	31,0	33,6	17,5	90,4	103,5	81,3	65,8	-10,5	18,1
9	++	0,5	0,5	0,0	1,0	0,5	1,0	0,5	+0.5	+0.5	÷	÷	51,1	41,0	27,7	109,8	110,2	47,7	43,6	-10,5	-6
10	+	0	0,5	0,0	1,0	0,0	1,0	0,5	+1.5	+1.0	÷	÷	22,3	55,5	30,0	162,7	96,5	86,1	84,3	99,4	35,01
11	++	0.5	0,5	0,0	1,5	0,5	1,5	0,5	+1.0	+1.0	÷	÷	15,6	24,2	21,2	36,4	34,1	40,4	25,0	10,9	24
12	+++	1.0	1,0	0,0	2,5	0,5	2,0	0,5	+2.0	+1.5	÷	÷	52,3	88,0	23,9	252,7	118,8	243,2	89,9	169,6	189
13	+	1.0	1,0	0,0	0,5	0,5	0,5	1,0	0	-0.5	÷	÷	75,4	102,0	25,5	160,6	181,8	94,3	188,7	5,4	-67,8
14	++	0.5	0,5	0,0	0,5	0,5	0,5	0,5	0	0	÷	÷	23,9	18,7	23,3	47,1	35,0	22,6	19,3	6,9	-1,9
15	++	0.5	0,5	0,0	1,5	0,5	NA	NA	+1.0	NA	+[d]	÷	82,8	168,2	32,7	337,4	338,2	NA	NA	84,6	NA
16	++	0.5	0,5	0,0	1,5	0,5	2,5	0,5	+1.0	+2.0	÷	÷	48,7	131,2	29,4	202,7	209,8	129,0	60,2	75,4	151,3

[a] The clinical reactivity on arms given as numerical values transformed from scores as follows: ÷ = 0, (+) = 0.5, + = 1.0, + (+) = 1.5, ++ = 2.0, ++ (+) = 2.5, +++ = 3.0. For earlobes, clinical reaction were evaluated as being present (+) or not present (÷).

[b] Mean of nickel- and aluminum site corresponding to sites on SLS pretreated arm used

[c] Change in nickel reactivity calculated as the difference in change (of clinical reactivity or blood flow) from baseline at each measurement day at the nickel test site compared with aluminum test site. For full explanation see statistics section.

[d] Reaction on day 5

NA: Not Applicable due to dermatitis with suspected superinfection at the nickel test site on the SLS arm

Paper III: Nickel deposition and penetration into the stratum corneum after short metallic nickel contact: an experimental study

- Nickel skin deposition and SC penetration capable of eliciting allergic nickel dermatitis was found after metallic nickel skin exposure of 3 x 10 minutes.
- Nickel penetrated the SC within one hour of exposure.
- Up to 72 hours post-exposure, nickel was recovered from the skin surface and from the outer layers of the SC and could clearly be differentiated from aluminium-exposed areas.
- Nickel skin surface amount correlated with nickel penetration in the SC in the normal and irritated skin of all participants.
- Significantly more nickel was found both on the skin surface and in the SC in normal skin of participants with nickel allergy compared with control individuals immediately and 24 hours post-exposure.
- A large variation in skin nickel deposition and resulting SC penetration was measured in participants with nickel allergy
- A significant correlation between declining PCA levels and increasing nickel penetration into the SC was found for normal skin of participants with nickel allergy.
- Three 10-minute immersion periods in artificial sweat with the metallic nickel discs used in the study released 70% of the nickel release measured after one week (1.82 µg/cm^2 vs. 2.60 µg/cm^2).

Nickel deposition and penetration into the stratum corneum after short metallic nickel contact: an experimental study

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Conflict of interests: The authors declare no conflicts of interests pertinent to this study.

Funding: This study was funded by the Environmental Protection Agency under the Ministry of Environment and food of Denmark, by Aage Bang Foundation and Karolinska Institute faculty funding

Running title: Nickel penetration after short skin contact

Manuscript word count: 4074

Abstract

Background

Knowledge on skin deposition and penetration of nickel into the stratum corneum (SC) after short contact with metallic items is limited.

Objective

To quantify nickel skin deposition and penetration into the SC after short contact with metallic nickel.

Methods

Sixteen nickel allergic participants and 10 controls were exposed to three pure nickel- and one aluminium disc on each volar forearm for 3 x 10 minutes. Before exposure, one forearm was irritated with 0.5% sodium lauryl sulfate (SLS) under 24-hour occlusion. Immediately, 24 and 72 hours after metallic disc exposure, outer SC layers were removed by adhesive tapes and the nickel content was measured.

Results

Nickel deposition and SC penetration capable of eliciting allergic nickel dermatitis was found immediately and after 24 hours. Significantly higher nickel amounts were found on normal skin and in the SC of nickel allergic participants compared with controls both immediately and after 24 hours, and on irritated skin immediately after exposure.

Conclusions

Nickel deposition and SC penetration is considerable after nickel skin exposure of 3 x 10 minutes. Combined with the allergic responses from the same exposures reported previously, this study highlights that short skin exposure to nickel releasing items may cause allergic nickel dermatitis.

Word count: 200

Search letters: contact allergy, tape stripping, nickel allergy, nickel skin dose, nickel release, nickel penetration, stratum corneum

Introduction

Nickel remains the most prevalent cause of contact allergy worldwide. The sustained high prevalence of nickel allergy in Europe (1) is often blamed on prolonged skin contact with nickel releasing items, while the potential role of nickel exposure as a result of short and daily skin contact with nickel releasing items has largely been overlooked.

The dose of available nickel deposited onto the skin surface is essential for nickel penetration into the stratum corneum (SC) and viable epidermis, which in turn can lead to induction of nickel allergy and allergic nickel dermatitis. Recent studies have shown that the rate of nickel release from metallic items is particularly high immediately after contact with artificial sweat and that even short skin contact can result in deposition of nickel onto the skin (2–5). These studies have been conducted in both controlled laboratory settings by using metallic items and in occupational settings where exposure to metallic items has occurred. Penetration of nickel ions from different nickel salts into the SC has also been studied in humans (6,7). However, knowledge on penetration of nickel ions into the SC after short contact with metallic items is missing.

The aim of this study was to quantify nickel skin deposition and subsequent penetration into the SC after relatively short contact with metallic nickel (3 x 10 minutes) in normal and irritated skin of nickel allergic participants and controls.

Materials and methods

Study population and design

The study has been described in detail elsewhere (8). A flow chart of the study design is found in figure 1. Briefly, a clinical experimental study was performed including 16 nickel allergic participants from the Department of Dermatology and Allergy at Herlev and Gentofte Hospital and 10 control subjects recruited by advertisement. Before the study, one of the volar forearms was randomized for skin irritation, and on day 0, four exposure areas of each arm were marked. To induce skin irritation, the four areas were pretreated for 24 hours with 0.5% sodium lauryl sulfate (SLS) (99% purity SLS, Sigma-Aldrich, St. Louis, Missouri, United States) under occlusion. All participants were exposed to three pure nickel discs and one aluminium disc (negative control disc) on each forearm for 3 x 10 minutes. The exposures were done in an exposure chamber with a temperature of 29.2±0.7 °C (range 28.8-29.4 °C). The discs were applied manually by the same investigator (M.G.A.) in three 10-minute contacts, separated by 10-minute intervals, with three different metallic discs for each exposure area. Each disc was applied with initial 90 degrees of rotation forth and back two times, to create friction between the skin and the metal surface. Participants were not allowed to take showers or use emollients during the study period. Before recruitment, the study was approved by the local ethics committee (H-16050296) and the Danish Data Protection Agency and all participants gave written informed consent. The study was registered at ClinicalTrials.gov (NCT03309215).

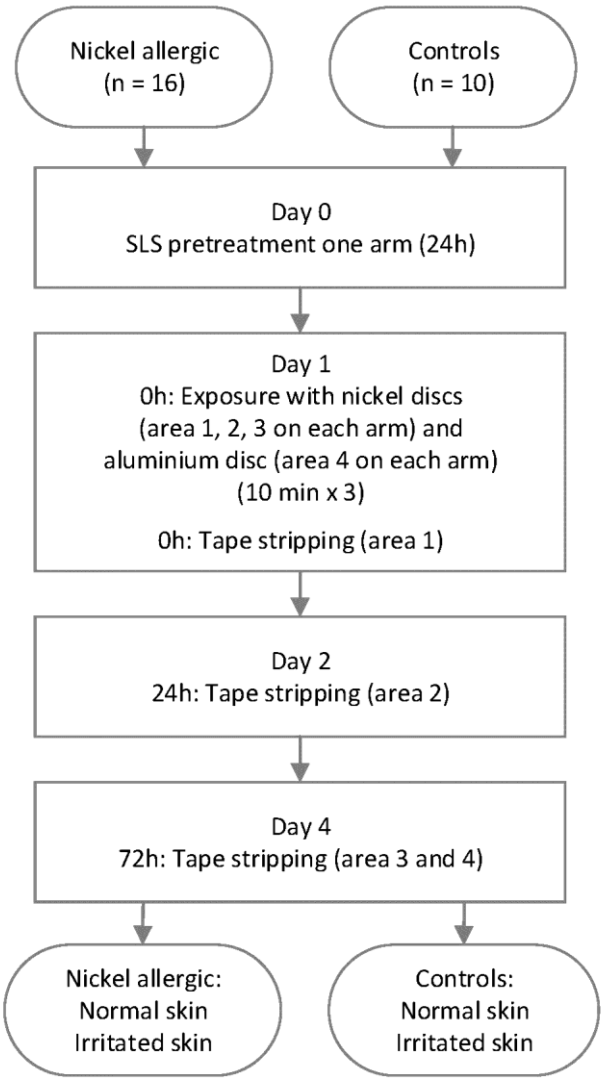

Figure 1. Flow chart of study, displaying nickel exposure and sampling (separate tape stripping for PCA is not included in the overview).

Tape stripping of the SC

Immediately (0-1), 24 and 72 hours after exposure with the metallic discs, sequential tape stripping was used for measurement of nickel penetration into the SC in one of the nickel exposed areas on each arm (normal and irritated skin). The aluminium exposed skin on each forearm was tape stripped 72 hours after exposure and was used as a negative control (Fig.1). Each area was tape stripped once using fifteen consecutive tapes (3.8 cm^2, D-Squame®; Monaderm, Monaco, France). Tapes were placed on the most central part of all test areas, and after use of a pressure applicator (225 g/cm^2) (D-squame®; Monaderm, Monaco, France) for approximately 10 seconds, the tape was gently removed with a quick uniform movement with a plastic tweezer. In addition, eight consecutive tape strips were taken from normal skin of both upper inner arms for analysis of 2-pyrrolidone-5-carboxylic acid (PCA). The method for tape stripping of the inner arms for PCA

analysis was identical, except that the skin around the first strip was marked. A new tweezer was used for each test site. Optical density with D-Squame Scan 850A (Heiland Electronic; Wetzlar, Germany) was used on each tape to normalize for the variable amount of protein content. After protein measurement, all tapes were stored separately in 2 mL micro tubes (Thermo Fisher Scientific, San Diego, USA) at room temperature and transferred to the laboratory at Karolinska Institute for nickel analysis.

Determination of nickel in tape strips

Based on the experience from analysis of samples from pilot exposures (8), we analyzed seven tape trips from each test site for nickel content. For the extraction of nickel in tapes, the micro tubes with tape strips were filled with a volume of 2 mL of 67% HNO_3 (Normatom, VWR, Leuven, Belgium), completely covering the tape. After 72 h, 1 mL of the acid extract was transferred to a new micro tube (1.5 mL, Sarstedts AG & Co, Nümbrecht, Germany). Prior to analysis, 150 µL of the acid extracts were transferred to a 12 mL PP-tube (Sarstedts, Nümbrecht, Germany) pre-filled with 4.85 mL Milli Q water (Millipore, Solna, Sweden, 18.2 $M\Omega cm^{-1}$), 5.0 mL 2% HNO_3 (67% Normatom, VWR, Leuven, Belgium diluted in Milli Q water) and indium added as internal standard (5 ppb, prepared from stock solution 1000 µg/mL Spectrascan, Teknolab AB, Kungsbacka, Sweden) . Standard solutions of an 8-point (0, 0.1, 1, 5, 10, 50, 100, 500 µg/L) calibration curve were diluted from a nickel stock solution (1000 µg Ni/mL, Spectrascan, Teknolab AB, Kungsbacka, Sweden), using 2% HNO_3 with the addition of indium as internal standard (5 ppb). Samples were analyzed in kinetic energy discrimination (KED) mode with helium as collision gas and argon as carrier gas, using an iCAP Q inductively coupled plasma mass spectrometry (ICP-MS) system from Thermo Scientific (Waltham, MA, USA). Nickel was monitored at masses 58 and 60 and indium at mass 115. The method detection limit (MDL) was evaluated using the results from 72 blank tapes and set to 0.08 µg/L (3 x STD of blank tape samples). The instrument limit of detection (LOD) was calculated for each run as 3 x STD using 3 acid blanks, and ranged from 0.000771 to 0.122639 µg/L.

Estimation of the nickel exposure concentration

To study nickel release from the nickel discs used on the skin, discs were immersed in artificial sweat for 10 minutes three times (3 x 10 minutes) and for one week (168 h). Samples were kept in a heating cabinet (Memmert, Schwabach, Germany) at 30°C during the respective immersion time period. Additionally, a wipe test was performed to mimic the nickel release from nickel discs at skin contact (full description in supplementary material). The released concentrations of nickel in artificial sweat and wipe extract were determined by ICP-MS. Method limit of detection was 0.0073 µg/L for nickel.

Filaggrin (FLG) genotyping

For all participants, buccal mouth swaps were taken for filaggrin genotype analysis. The filaggrin gene (*FLG*) mutation status for R501X, 2282del4 and R2447X were determined by multiplex analysis of buccal swabs at Herlev- and Gentofte Hospital, previously described in detail (9).

Analyses of 2-pyrrolidone-5-carboxylic acid (PCA)

To quantify filaggrin degradation products, the tape stripping technique was performed on both inner upper arms. PCA from tape strip number 3 was analyzed by ultra-performance liquid chromatography (UPLC) at the Department of Autoimmunology and Biomarkers at Statens Serum Institut (full description in supplementary material). The sample preparation was performed as described by Kezic et al (10).

Statistics

For presentation of data and statistical analysis, nickel in the first 2 tape strips on each sampling occasion was interpreted as situated on the skin surface and was thus analysed separately. Nickel in tape 3-7 was considered to indicate penetration into the SC (11). The participants were divided into four groups, depending on nickel allergy- and skin status (Nickel allergic/control, Irritated/normal skin). *FLG* mutation status was categorized into `wild type´ or `null mutation´, the latter including any of the three mutations tested for. A mean value of the PCA from tape strip number 3 of both upper arms was used in the analysis. Non-parametric analyses were used. The probability value $p<0.05$ was considered significant. Mann Whitney U test was used for comparison of nickel doses between nickel allergic participants and controls subjects, and for test of differences in nickel doses in participants with/without mutation in the *FLG*. To test for trend between patch test reactivity and nickel penetration into SC or nickel deposition on skin surface, Kruskal-Wallis test was used. Spearman correlation was used to test for correlation of PCA levels and nickel penetration into SC or deposition on skin surface. For comparison of nickel in normal and irritated skin or the nickel- compared with the aluminium control area on the same participants, Wilcoxon signed rank test was used.

REDCap electronic data capture tools were used for data collection (12). Statistical analysis and graphs were made in SAS, Version 9.4 for Windows (SAS Institute Ics., Cary, NC, USA), GRAPHPAD PRISM version 6.07 for windows (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel (Excel 2010; MicrosoftCorporation, Redmond, WA).

Results

All participants completed the study. The study population, their clinical reactions and blood flow measurements have previously been described in detail (8). In brief, we showed that 62.5% of nickel allergic participants reacted with allergic nickel dermatitis on irritated skin, and 18.5% on normal skin with previous dermatitis, whereas none of the controls had any clinical reactions or blood flow increase. Study population characteristics can be found in Table 1.

A substantial amount of nickel was deposited onto the skin after the exposures (3 x 10 minutes). Nickel was found in all the analysed tapes from the nickel exposed areas; immediately, 24 and 72 hours after exposure (Fig. 2). At all three time points, the highest amount was present in the first tape, ranging from 8.7 $\mu g/cm^2$ (range: 5.9-14.4) in normal skin of controls to 13.3 $\mu g/cm^2$ (range: 7.4-44.2) in normal skin of nickel allergic participants immediately after exposure. Nickel content in tapes decreased in the same pattern in tapes from deeper SC layers for the four groups. The variation in nickel penetration was largest immediately after exposure, and was higher in nickel allergic participants.

The proportion of nickel penetrated into the SC (tape 3-7) and nickel on skin surface (tape 1-2) is shown in Fig. 3 (11). In normal skin, the proportion of nickel in SC/on surface was similar in nickel allergic participants and controls at all time points, except for a lower ratio in controls immediately after exposure. However, the actual amount of nickel on the skin surface in normal skin was significantly higher in nickel allergic participants compared with controls both immediately (mean difference: 13.4 $\mu g/cm^2$) ($p \leq 0.03$) and 24 hours after exposure (mean difference: 2.0 $\mu g/cm^2$) ($p \leq 0.01$). In irritated skin, the amount of surface nickel was higher in nickel allergic participants, but only just after exposure (mean difference: 10.7 $\mu g/cm^2$) ($p \leq 0.05$).

The amount of nickel on the skin surface (tape 1-2) correlated with the amount that penetrated into the SC (tape 3-7) in both irritated and normal skin of all participants (normal rs=0.91) (irritated rs=0.94) ($p<0.0001$) (Supp. Fig. 1a, 1b). Also, a correlation was found between nickel amount on the skin surface in normal and irritated skin of all participants (Supp. Fig. 2). The amount of nickel that penetrated into the SC in normal skin was higher in nickel allergic participants compared with controls both immediately (median difference: 2.2 $\mu g/cm^2$) ($p=0.047$) and after 24 hours (median difference: 0.54 $\mu g/cm^2$) ($p=0.006$) (Fig. 4). No difference in amount of nickel that penetrated into SC of irritated skin was found between the groups. When comparing nickel penetration into the SC between normal and irritated skin of the same nickel allergic participants, a higher amount was found in normal skin immediately after exposure (median difference: 1.85 $\mu g/cm^2$) ($p=0.02$), but there was no difference in deposition of nickel on the skin surface.

Nickel penetration into the SC (tape 3-7) in normal skin decreased over time to 18-22% and 10-15% of the amount found in the SC immediately after exposure, after 24 and 72 hours respectively. Seventy-two hours post exposure; nickel was still present in the SC at the nickel exposed areas. At this time point, significantly more nickel was found on the surface of the nickel

exposed skin compared with the aluminium exposed skin (median difference: 0.68 µg/cm^2) (p<0.0001) and in SC (median difference: 0.28 µg/cm^2) (p<0.0001).

Mean amounts of nickel release from nickel discs after immersion in artificial sweat for different time periods, are presented in Table 2. The total amount of nickel released during three immersion periods of 10 minutes each (1.82 µg Ni/cm^2) corresponded to 70% of the release after one week (2.60 µg Ni/cm^2). One simulated participant exposure using a wipe moistened with artificial sweat, resulted in released amounts of nickel orders of magnitude higher (165.7 µg Ni/cm^2) than what was measured after immersion in artificial sweat for one week. This is reflected by the nickel amount in the seven tapes taken immediately after exposure; 7-83 µg Ni/cm^2.

Three participants were heterozygous *FLG* mutation carriers, whereas one was compound heterozygous mutation carrier. There was no correlation between nickel on skin surface or nickel penetrated into the SC at any time points and *FLG* mutation status, self-reported hand dermatitis or a history of atopic dermatitis. No trend could be found between patch test reactivity and nickel on skin surface or penetration into the SC. In normal skin of nickel allergic participants, there was a significant negative correlation between 2-pyrrolidone-5-carboxylic acid (PCA) and nickel penetration immediately after exposure (rs = -0.54) (p=0.03).

	Ni allergic n= 16	**Controls n=10**	p *
Gender, n			
Women	13	9	NS
Men	3	1	
Age at test (years) Median (25/75)	55.5 (44.0-63.5)	34 (25.0-58.0)	NS[a]
Atopic dermatitis ever [b]	2	0	NS
Hand dermatitis ever [b]	4	0	NS
Filaggrin gene mutation carriers [c]	2	2	NS

[a] Mann-Whitney U test was used

[b] Participants were asked if a doctor ever had told them that they had atopic dermatitis or hand dermatitis

[c]Genotyping was done for the three most common loss-of-function mutations in the filaggrin gene (501X, 2282del4 and R2447X). The two nickel allergic participants and one control were heterozygous in one FLG gene, one control was compound heterozygot.

* Fisher´s exact test was used.

Table 1. Characteristics of study population.

Procedure	µg Ni/cm² (mean)	STD
Release in artificial sweat		
168 h	2,60	0,31
3x10 min	1,82	0,70
1x 10 min	0,30	0,04
Wipe		
1x simulated participant exposure	166	37

Table 2. Mean nickel release per unit surface area from nickel discs in artificial sweat for one week (168h), 3 x 10 minutes (using three different nickel discs) and 10 minutes respectively. Further, mean amount of nickel deposited onto a wipe moistened by artificial sweat, from one nickel disc that was rotated 90 degrees forth and back two times, simulating participant exposure. All results are based on triplicate experiments.

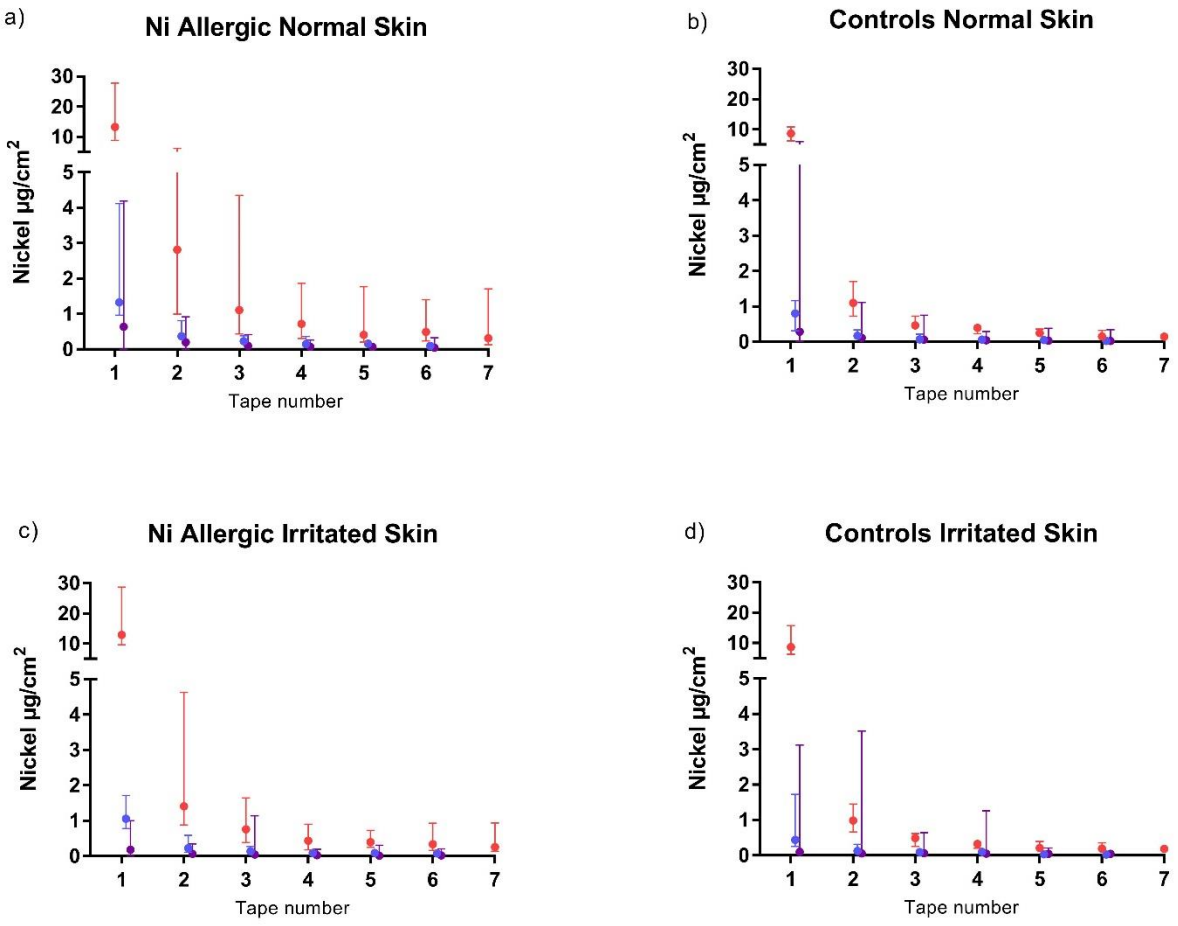

Figure 2. Measured nickel amount per tape (µg/cm²) at different time points post exposure (0, 24 and 72 hours) in normal or irritated skin of nickel allergic participants (a, c) and controls (b, d). Results are indicated by bars for interquartile range; markers for median value; colours for time points: red 0h, blue 24h, violet 72h.

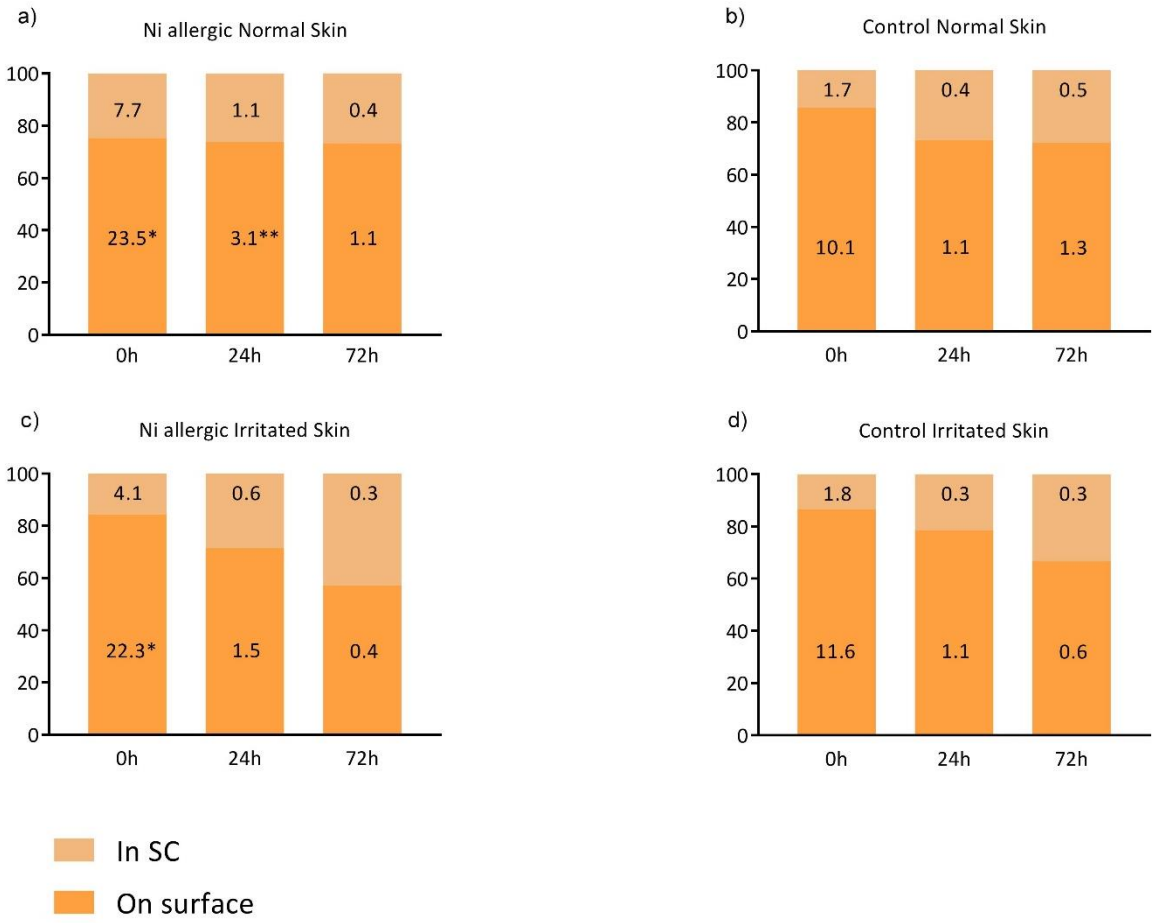

Figure 3. Relative comparison of nickel on the skin surface (Tape 1+2) and amount penetrated into the stratum corneum (SC) (Tape 3-7) at different time points (0, 24 and 72 hours) post exposure for nickel allergic (n=16) (a,c) and control (n=10) (b, d) participants in normal skin (a, b) and skin irritated by sodium lauryl sulfate (c, d). The actual mean amount of nickel (µg/cm^2) is noted on the bars, differences between nickel allergic and control participants tested with Mann Whitney U test. *p≤0.05 **p≤0.01.

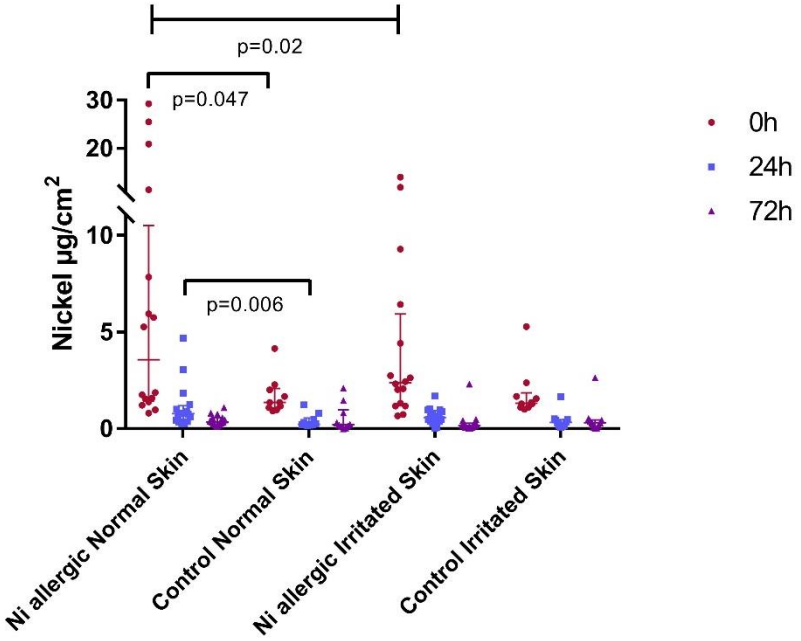

Figure 4. Penetrated amount of nickel (µg/cm^2) measured in tape 3 to tape 7 at different time points post exposure (0, 24 and 72 hours) for the 4 groups (Nickel allergic/Controls, Irritated/Normal skin). Each dot represents one participant. Bars: Interquartile range, lines: median. At different time points post exposure with nickel discs, Mann Whitney U test was used to compare nickel amount between nickel allergic and control participants (significant differences noted by half tick-down lines) and Wilcoxon signed rank test was used to compare nickel in normal and irritated skin of the same individuals (significant differences noted by capped line).

Discussion

Principal findings

This is the first study to quantify nickel penetration into the SC after relatively short duration of skin contact with metallic nickel. A large amount of nickel was deposited onto the skin and penetration into SC took place already within 1 hour. Nickel was recovered from the skin surface and the outer layers of SC up to 72 hours after exposure, emphasizing sustained exposure from nickel on the skin surface. Unexpectedly, higher amounts of nickel were found on the skin surface and in the SC in normal skin of nickel allergic participants compared with controls, both immediately after and 24 hours after exposure. There was a large variation within the group of nickel allergic participants; some individuals had a much higher level of nickel on the skin surface, resulting higher penetration into the SC. An interesting negative correlation between nickel penetration into the SC and PCA concentration was found in nickel allergic participants.

Interpretation

Studies of nickel on the skin surface and subsequent penetration into the SC, following real-life exposure to nickel releasing metallic items, are very limited. Although it has been claimed that short contact with metallic items is harmless in the context of nickel allergy, studies have demonstrated rapid deposition of nickel onto the skin following contact, e.g. coin handling (5). Based on these findings, it has been suggested that short and repeated skin contact with metallic items may lead to considerable nickel build up in the skin. In a recent study, where fingertip skin was stroked against metallic nickel for three seconds, 4.7 µg/cm^2 nickel could be detected on the skin surface with the acid wipe method. In the same study, the amount of nickel deposited on the skin from nickel alloys and pure nickel, was more dependent on contacts with newly abraded surfaces than the actual number of repeated contacts with the same surface (3). We found higher but comparable nickel skin doses (mean: 10.1-23.5 µg/cm^2) after 3 x 10 minutes of exposure with newly abraded metallic nickel discs. In our previous questionnaire study of 342 nickel allergic individuals, a large proportion reported allergic nickel dermatitis after relatively short contact with metallic items (21.4% ≤ 10 minutes and 30.7% ≤ 30 minutes of contact) (13).

The large variation in nickel skin deposition observed in nickel allergic participants is interesting. Considerable inter-individual variation in nickel skin deposition after skin contact with nickel containing metallic items has previously been demonstrated in two studies that included individuals without nickel allergy (3,14). The present study is the first to demonstrate a difference in skin deposition between persons with and without nickel allergy in a controlled set-up. One possible explanation could be differences in the amount and composition of sweat which was not measured in this study, as sweat may affect nickel release and accumulation of nickel in the SC. ´Rusters´ in industry were described in the 1960´s, being workers with a tendency to cause corrosion on metal surfaces, due to high sweat chloride content (15,16). The skin topography,

affecting friction and contact area, may also be important, but has rarely studied in this context (17).

To estimate the penetration of a contact allergen in the skin (μg/cm^2) is of importance, as the penetration is a requirement for sensitization or elicitation of allergic contact dermatitis. Penetration of nickel into SC has previously been studied using different nickel salts in aqueous solutions after hours of contact, mostly in vitro (7,18,19). By using an aqueous solution with a nickel salt, the actual applied nickel ion dose and counter-ion are known, thus the skin absorption can be calculated. These studies provide important knowledge about the kinetics of nickel ion penetration and associated rate-determining factors. In addition, SC penetration has been studied after application of nickel powder, where both particles and released nickel ions were involved (20–22). However, it is important to keep in mind that most short and daily contacts with nickel occur with metallic nickel items. The mechanism of nickel transfer to the skin surface in these contacts is also governed by pressure and friction.

The primary rate-limiting factor for nickel skin absorption is the SC (19,23). A limited amount of the applied nickel dose is supposed to penetrate through the SC; one study found that less than 1% of nickel chloride penetrated the SC within 96 hours of exposure (18). In the present study, the nickel amount was highest in the first tape and decreased in tapes from deeper layers. This is consistent with the observation that nickel accumulates superficially in the SC after exposure with nickel salts (6,7,19,24,25). One study is of particular interest when comparing the results of nickel penetration. Hostynék et. al. used tape stripping to measure the penetration of nickel in forearm skin in vivo after 30 minutes to 24 hours of exposure to an aqueous solution of different nickel salts (7). Important differences compared with the present study were occlusion of the test areas, surface decontamination prior to stripping and another type of tape. However, similarly to our results, they found that the main nickel dose was located on the skin surface and that nickel concentration decreased with the number of tapes within 24 hours after exposure. If we exclude the first 2 tapes in our study, the amount of nickel in the following tapes were similar to those found 30 minutes after open application of a single liquid dose of nickel chloride (concentration: 19.8 μg/cm^2). While there is little insight in nickel skin penetration, it is likely that the continuous proliferation and shedding of corneocytes will help to remove nickel that has been bound in the upper layers, in turn limiting the tendency of nickel ions to reach the viable layers. However, excessive exposure, either prolonged or repeated, will lead to high nickel concentrations and in turn allergic nickel dermatitis.

There was no difference in immediate nickel skin deposition between normal and irritated skin of nickel allergic participants, but more nickel was found in the SC of normal skin. This finding may indicate that nickel had been absorbed into the viable epidermis of irritated skin already 0-1 hour after exposure, which is also supported by the fact that most nickel allergic participants (63.5%) developed allergic nickel dermatitis in irritated skin (8). It is not known to what extent rapid

shunting of nickel ions via sweat ducts, pores, etc., occurs and this cannot be quantified by tape stripping (23).

In this study, a high SC nickel penetration correlated with low PCA concentration in nickel allergic participants. PCA is an important marker for the relative amount of amino acids in the natural moisturizing factor (NMF). PCA and other amino acids origin from the decomposition of SC proteins. The best known are filaggrin but many others participate. The active known nickel chelating element in NMF is the amino acid histidine. It is known that this amino acid vary in parallel with PCA in the NMF (10). In this study, concentration of PCA was used as an estimation of acquired filaggrin deficiency to supplement filaggrin genotyping (26).

No correlation was found between atopic dermatitis, hand eczema or *FLG* mutations and deposition/penetration, although the power of these analyses was low due to a limited number or participants with these conditions. Previous epidemiological studies have proposed higher risks of nickel allergy in individuals with *FLG* mutations (27–29). Our study indicates that histidine levels in the outer SC influence nickel penetration.

The duration of exposure to metallic discs of this study corresponded to the definition of "prolonged contact" in the EU nickel restriction (*"10 minutes on three or more occasions within two weeks, or 30 minutes on one or more occasions within two weeks").* (30). From the results we conclude that the duration of contact is not crucial for deposition of high amounts of nickel onto the skin surface. Ten minutes three times is sufficient to result in nickel deposition onto skin, penetration into the SC and allergic nickel dermatitis (8). In accordance with others, we found that nickel ion release in artificial sweat after short duration led to a considerable proportion of the total release after 168 hours of immersion (2,3). It cannot be estimated from our experimental study how longer, shorter, or no time intervals between nickel exposures may affect the dose of nickel in skin.

Strengths and weaknesses

The study was carefully controlled in many aspects, most importantly the metallic discs had equal surface properties and the exposures with the metallic discs and the tape stripping procedure were performed by the same investigator. Further, the temperature during exposures was controlled, as it may influence nickel release and SC penetration (19,31).

Due to the set-up with skin exposure to metallic discs, a boundary condition was that the exact nickel exposure dose remained unknown. Although the rotations of the discs were made in the same manner, the pressure and rotation could not be fully standardized, and the friction of the skin differed due to skin texture and moisture. Further, it is known that skin temperature and the amount and composition of sweat vary between body parts and individuals. The nickel exposure dose, by release from nickel discs, was estimated by a wiping procedure, although it may be overestimated as the wipe was moistened in artificial sweat. Variations in measured nickel surface

doses at later time points may partly be attributable to difference in compliance with restrictions during the study period (emollients and wash) and differences in clothing. Another weakness was that we only used pure nickel in this study. In real-life, nickel alloys are more commonly used in metallic items, although pure nickel is used in nickel-plated items and some coins. Finally, we did not assess the possible influence of lipid bilayers or paracellular penetration in hair follicles and sweat glands.

To obtain a quantitative measurement of protein removed in each tape strip (indirect measure of the mass of skin cells removed), infrared densitometry was used. However, interference between nickel and protein in tapes was monitored; hence the results are not presented. Previous research indicate that a constant amount of SC is removed after the first 2 tape strips, for a given test person, tape and skin site (7).

Conclusion

This study shows that relatively short skin contact with nickel, corresponding to what is covered by the current EU restriction of nickel, gives rise to substantial doses of nickel both on the skin surface and penetration into the SC, capable of eliciting allergic nickel dermatitis (8). Some nickel allergic individuals apparently `glued´ nickel onto the skin after exposure, which lead to great differences in nickel penetration compared with controls. The current inclusion of items intended for relatively short skin contact will likely result in more efficient prevention of nickel allergy in EU countries.

Acknowledgement

We thank Anne Marie Topp, Lene Toft-Jensen and Julie Sølberg for their help during the experiments. Further we are grateful to Amelie Wenz and Céline Reverchon for assistance in the laboratory.

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Supplementary material:

Nickel deposition and penetration into the stratum corneum after short metallic nickel contact: an experimental study

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Detailed description of the experimental procedures

Determination of nickel in tape strips

Evaluation of tape extraction recovery

The extraction method was evaluated in samples from 9 of the participants, by the spiking of samples with 20 µL rhodium (Rh) (0.5 ppm, diluted from 1000 µg Rh/mL stock solution, Spectrascan, Teknolab AB, Kungsbacka, Sweden) prior to the extraction of tapes. The recovery of rhodium, monitored at mass 103, was found within the accepted range of 80 to 120%.

Estimation of the nickel exposure concentration: nickel release and wipe tests

The nickel release test was performed by immersion of metallic discs (3 cm in diameter, total area 16 cm^2) in artificial sweat (0.5% (w/v) NaCl, 0.1% (w/v) lactic acid and 0.1% (w/v) urea, pH 6.5) for 10 minutes three times (3 x 10 minutes) and for one week (168 h) (1). The bottom of 125 mL flasks (HDPE, Azlon®, VWR International, Radnor, PA, USA) was covered with glass beads, one disc was placed on top of the beads and 16 mL of pre-heated (30°C) artificial sweat solution was added, resulting in a surface area/volume ratio of approximately 1. Samples were kept in a heating cabinet (Memmert, Schwabach, Germany) at 30°C during the different immersion periods. After immersion, the metallic disc was removed from the artificial sweat using plastic forceps. The artificial sweat solution was acidified to a concentration of 2% HNO_3 and stored in fridge until chemical analysis.

Additionally, a wipe test was performed to mimic the nickel release from nickel discs at skin contact. A nickel disc was applied to a cellulose wipe (injection wipes; Paper-Pak Sweden AB, Sundbyberg, Sweden), moistened with 1 mL of artificial sweat, and manually rotated 90 degrees fourth and back two times (by the investigator who applied the discs in the experimental study), mimicking the procedure of the experimental skin exposure. The wipe was placed in a 125 mL flask, 16.6 mL of 1% HNO_3 was added and the wipes were extracted for 45 min on a shaker at 200 rpm. After extraction, the wipe was removed from the flask that was stored in the fridge until chemical analysis.
All nickel release and wipe tests were performed in triplicate with sample discs prepared according to the procedure for experimental skin exposures (2). Gloves were used in all steps of the experimental laboratory work and the lab material was acid cleaned. The released concentrations of nickel in artificial sweat and wipe extract was determined by ICP-MS. Method limit of detection was 0.0073 µg/L for nickel.

Filaggrin (FLG) genotyping

For all participants buccal mouth swap was taken for filaggrin genotype analysis. The filaggrin gene (*FLG*) mutation status for R501X, 2282del4 and R2447X were determined by multiplex analysis of buccal swabs at Herlev- and Gentofte Hospital, previously described in detail (3).

Analyses of 2-pyrrolidone-5-carboxylic acid (PCA)

To quantify filaggrin degradation products, the tape stripping technique was performed on both inner upper arms. PCA from tape strip number 3 was analyzed by ultra-performance liquid chromatography (UPLC) at the Department of Autoimmunology and Biomarkers at Statens Serum Institut. The sample preparation was performed as described by Kezic et al (4). Chromatographic separations were carried out with a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent delivery system, and an auto sampler. The detection of PCA was carried out with the usage of UV detector set at 210 nm (ACQUITY UPLC™ UV detector). The chromatography was performed on a 100 × 2.1 mm Waters ACQUITY BEH C18 1.7 µm column. The injection volume was 20 µL. The column was eluted with a linear gradient from 100% 0.1 M KOH to 100% 20 mM ammonium formate, containing 1.5 mM tetrabutylammonium hydroxide and 1% acetonitrile at pH 7.3 over 18 min at 100 µL/min. The elution gradient was linearly increased from 0.1% β to 60% β in 3 min, then increased to 100% β in 10 min and kept isocratic for 2 min. Total run time, including the conditioning of the column to the initial conditions was 18 min.

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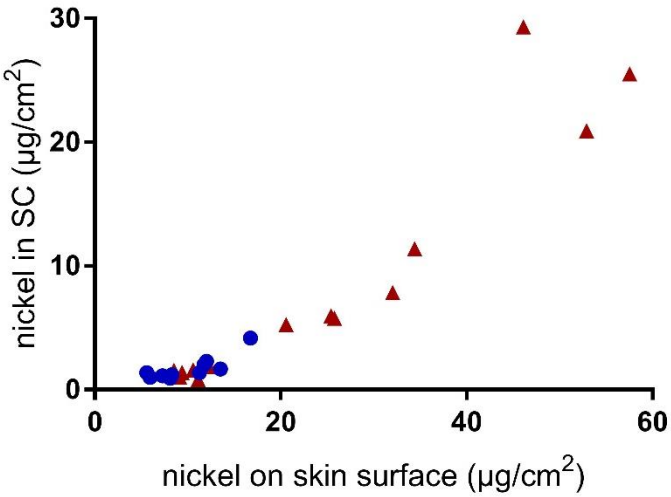
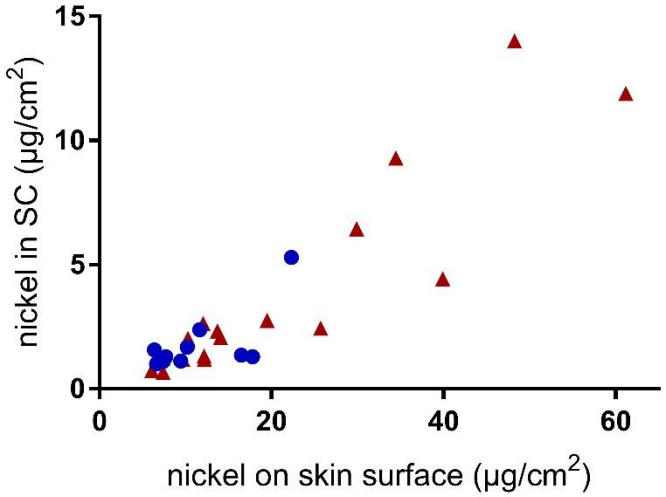
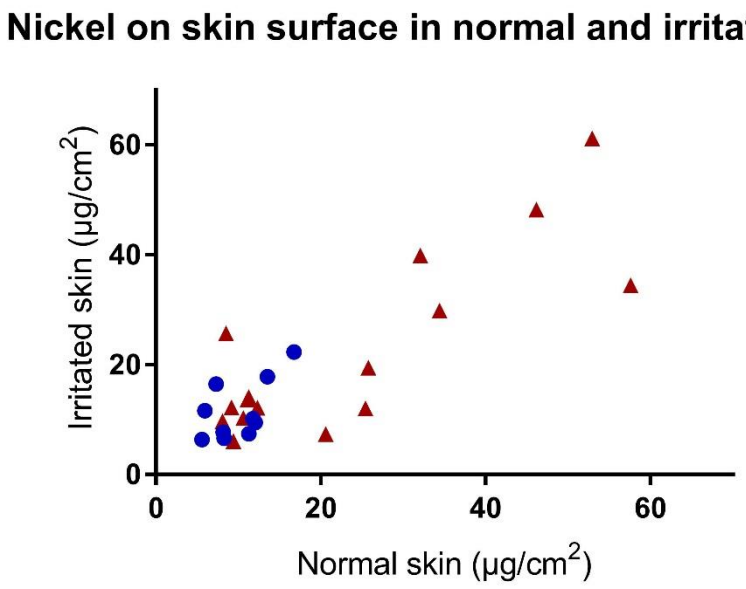
a)



b)



Supporting Figure 1. Correlation between the amount of nickel in stratum corneum (SC) (tape 3-7) (y-axis) and nickel on the skin surface (x-axis) (tape 1-2) within one hour after exposure to nickel discs for all participants in A) normal and B) irritated skin (Spearman correlation, (rs normal skin= 0.91) (rs irritated skin=0.94) (p<0.0001). Red triangles mark nickel allergic participants and blue circles control subjects.



Supporting Figure 2. Correlation between nickel on skin surface (µg/cm^2) in normal (x-axes) and irritated (y-axes) skin within one hour after exposure to nickel discs in all participants (rs=0.63) (p=0.0006). Red triangles mark nickel allergic participants and blue circles control subjects.

5. CONSIDERATION OF METHODOLOGY

In the following, the strengths and weaknesses of the thesis not covered or described only briefly in Papers I–III are elaborated.

Overall, participants with nickel allergy in this thesis were recruited from the same tertiary dermatology clinic and all had previously been tested positive to nickel sulphate 5% in pet. Although inter-observer variability of patch test readings cannot be excluded, readings were performed by a limited number of nurses experienced in the field who followed the European Society of Contact Dermatitis guidelines for patch testing and interpreting results (71). Further, in Study II, re-testing was performed, and all readings were performed by the same investigator (MGA).

5.1 Questionnaire study

Questionnaire studies serve a purpose when aiming to describe a large cohort of individuals, limiting cost- and time consumption. This is one of the largest studies performed in a population with nickel allergy. Nevertheless, several limitations in the interpretation of our data must be considered.

5.1.1 General limitations

In the present study, the questions were constructed by the participating researchers based on our knowledge in the field and assumptions on causality and may therefore be biased. Further, misinterpretation of the questions and the response categories is of general concern in questionnaire studies. Pre- and pilot testing of the questionnaire may have reduced the risk of misinterpretation of questions by the respondents. Further, cognitive interviewing parallel to the questionnaire was performed, which has been shown to be useful in detecting errors of interpretation (95). Preferably, interviewing would have been done for the whole study population; however, this was not possible due to lack of time.

Retrospective questionnaire studies entail the unavoidable risk of recall bias. Recall is affected by cognitive ‘errors’ or heuristics. For example, past experiences are judged almost entirely on how they were at their peak and how they ended (96). Further, recall may decrease over time (97), which potentially reduces the reliability of some answers compared with others (e.g. first occasion vs. most recent dermatitis).

To limit the risk of selection bias, it is crucial that the respondents are representative of the population of interest. A high participation rate is related to high representativeness (98). A general consensus is that a minimum of half the sample should have completed the survey instrument to limit response bias (99). In this study, the participation rate was 63.2% and was increased by three distributions of the questionnaire. Further, the questionnaire was sent in paper form, which has been demonstrated to lead to higher response rates compared with web-based questionnaires (100,101). To elaborate on response bias, we compared the sex and age of respondents with non-respondents. Non-respondents were found to be younger than respondents, which is a common phenomenon. Nevertheless, this limits information from this study regarding current exposures and exposure relationships after the EU restriction on nickel came into force. The response rate was calculated by dividing the number of usable responses returned by the total number of patients identified in the database (342/541). This approach has generally been preferred to subtracting the number of undeliverable questionnaires from the initial sample to acquire the denominator, which would have generated a slightly higher participation rate (342/524; 65.3%) (102). It must be remembered that the study population chosen for this study consisted of dermatitis patients from a tertiary dermatological department of a university hospital in Copenhagen and is thus a subpopulation of patients with nickel allergy in Denmark. The findings of this study are not representative of all patients with nickel allergy in Denmark because our study population may be older with generally more severe skin disease. There may be a latency period for the young nickel allergic individuals to be referred to this department, which would explain the low number of patients aged 25 years or younger in this study. The overweight of women compared with men in this study was expected as the prevalence in women is much higher than in men. However, this makes the study results in men less robust.

5.1.2 Reporting of metal dermatitis

In this study, 14% of patients with nickel allergy did not report metal dermatitis (e.g. lifetime event from skin contact with a shiny metallic item such as earrings or ear studs, watches, buttons or metallic fastenings). It is well known that some persons who are sensitized to nickel have not experienced dermatitis after contact with metallic nickel-releasing items (16), although this may partly be explained by recall bias. For those who confirmed dermatitis upon contact, the possibility of this reaction being caused by another metal or irritation cannot be excluded. Concomitant reactivity to nickel and other metals is relatively common in dermatitis patients (103). To strengthen the results concerning metallic dermatitis, we could have included a control

group with dermatitis patients from the same clinic with a negative patch test to nickel sulphate. A control group would have been useful to get an idea of the number of unspecific/irritant reactions to metallic items.

Regarding the reporting of metallic items, a list of 15 items commonly reported in the literature was compiled (21,44,104,105). The list was not exhaustive, and other exposures could be added in free text. Nevertheless, overlooked exposures are a potential bias. It was surprising that most respondents reported more than one item causing dermatitis on their first and most recent occasion. It may be that stating that multiple answers were allowed led to patients not being sure about the first and second dermatitis and so reporting both. Further, it would have been desirable to elucidate to what extent problematic items were related to occupational use, which was not distinguishable with the questionnaire construction. In addition, new potential sources of allergic nickel dermatitis were not listed and may therefore not be discovered from the results of this study.

Another concern is the lack of information on origin and year of purchase of the reported items. Although this is an obvious limitation, a previous study of items causing allergic nickel dermatitis in dermatitis patients with nickel allergy from the same clinic found that the items were almost exclusively bought in Europe after the implementation of the EU restriction on nickel (44).

Misinterpretation may be of special concern regarding the question of minimum skin contact duration needed for dermatitis to occur. This may be so for several reasons. First, we do not know whether patients are reporting actual dermatitis and neither do we know the strength of their reaction. Some patients reported very rapid dermatitis reactions within two minutes of contact, and contact urticaria has been reported in the literature (106). Second, the length of contact may be difficult to estimate, especially the distinction of exposures lasting for only minutes. Third, the repeatability of the exposure was not assessed and may be important for the elicitation of a reaction, not only as a result of skin memory (107).

5.1.3 Data interpretation

In this study, we interpreted initial dermatitis as the event of sensitization, which may be controversial. Products responsible for the first clinical sign of contact dermatitis, correspond to the early elicitation phase in a sensitized person. This information is the closest one can get in a

clinical study to the sensitization event (108). This concept has been used for fragrance allergy caused by consumer products and deodorants identified as risk products; subsequent risk assessment (based on experimental data) gave the same result. Despite the risk of this approach not holding in each individual case, we considered it reasonable in the study of a cohort of this size where no alternative exists. The division of patients into subgroups, especially the group reporting the most recent dermatitis within the last 5 years, could have been done differently. Nevertheless, the causes of metal dermatitis in the whole patient group were very similar to those in the subgroups; accordingly, dividing the subgroups differently would not have markedly changed the results.

5.2 Experimental study

This is the first study to investigate the elicitation potential of short-duration skin contact with metallic nickel. The main strength of the study was the controlled set-up and the high participation rate (100%). This was achieved through a thorough planning phase and a prior pilot study.

5.2.1 Study population

Two groups were included in this study: participants with (16) and without (10) nickel allergy. With this set-up, the potential irritative risk of metallic nickel-releasing discs could be elucidated. Further, possible differences in nickel skin uptake between the groups could be investigated. When interpreting the results, it is important to note that the groups differ not only with respect to nickel-allergy status. Participants with nickel allergy were recruited from a tertiary dermatologic clinic and they may have had other or more severe skin diseases and be more generally ill compared with control individuals, who were recruited from Internet advertising. Nonetheless, we did not find any significant differences between groups regarding sex, median age, self-reported atopic dermatitis or hand dermatitis, or FLG mutation carrier status. Our study population was relatively small, and the sub-analyses contained too few participants to reach sufficient power. However, the size of the two groups was sufficient with respect to the main comparisons and conclusions.

5.2.2 Materials and preparation

The concentration of SLS was chosen to evoke the highest number of weak irritant reactions (109), and thereby to create a slightly impaired skin barrier in most participants. Hence, we could compare elicitation of allergic nickel dermatitis and penetration of nickel into the SC in normal and irritated skin of the same participants. Although one-time occlusive SLS exposure is

commonly used for research purposes, it only mimics acute irritant reactions, while the more common situation in real-life is chronic irritant or allergic contact dermatitis (73). A strength of the study is that we followed the applicable guidelines regarding use of SLS: high purity SLS (>99%) in water solution applied within a minute of preparation, recommended volume and time of occlusion (73) and storing of the solution in low temperatures between use (110). Application of SLS was done in Finn Chambers over 24 hours, which also causes occlusion of the skin. Occlusion affects the skin barrier in a complex way (111) that obscures the interpretation of the results of irritated skin. To remove the effect of occlusion from the irritated skin, we would have needed an occluded test area without SLS pretreatment. Instead, we prioritized including normal skin, which we considered more relevant. We cannot rule out the effect of occlusion in this study.

The metallic discs (ø: 30 mm) were circular and had a larger diameter than the tape strip (ø: 22 mm). However, the largest Finn Chambers found on the market for SLS application were smaller than the tape strip (ø:18 mm). Each tape strip was placed centrally in the exposure area. In this study, only one nickel test material was included (metallic nickel), chosen due to its large deposition of nickel onto skin compared with various alloys (24). It would have been interesting to include nickel-containing alloys, such as copper-nickel or nickel-silver, as the nickel release and skin deposition from these materials differ (24,69).

A strength of this study was the standardized and reproducible disc preparation. All discs were prepared within approximately the same time interval prior to exposure, and all discs were untouched after preparation. In contrast to studies where aqueous nickel solutions have been used for skin exposure, the exact nickel exposure dose is not known. The nickel-release tests and simulated patient exposures demonstrated substantial variation between triplicate metallic nickel discs used in the study (Paper III, Table 2). Previous studies have shown that nickel skin deposition from the same type of metal differs regardless of efforts to standardize it (24,67); accordingly, these uncertainties can be seen as a condition of these types of study.

5.2.3 Study design

For test areas, it would have been desirable to randomize not only the forearms for SLS pretreatment but also all forearm exposure areas. This was initially tried in the pilot study and found to be associated with a risk of inaccurately placed discs during the many exposures. Because of the same pattern of disc placement on all participants, exposures could not be

blinded. Although we attempted to mark test areas centrally on each forearm between the wrist and the antecubital fossa, as this area has been shown to have comparable skin properties (112,113), the marking of test areas was not exact and depended on local skin factors in each participant.

To create the most favourable environment for developing allergic nickel dermatitis, the exposures with metallic discs were done in a heated exposure chamber. In addition to heat, humidity would have been desirable because this factor increases sweating and thereby nickel release and penetration (51,114,115).

For exposures, separate discs were used for each 10-minute exposure to increase nickel skin deposition (24,67,68)**.** The rotation of the metallic discs on the skin surface was done by hand, and it was sometimes difficult to maintain the exact placement of the disc in the test area. This was especially true of the earlobe exposures and is a weakness of this study. Different approaches to further standardize the exposure procedure were initially tried without success.

5.2.4 Readings

Readings were done by the same researcher throughout the study. Increased patch test reactivity of the skin sites due to extensive simultaneous nickel exposures cannot be excluded. An example of reactions of one participant with nickel allergy can be seen in Fig. 8.

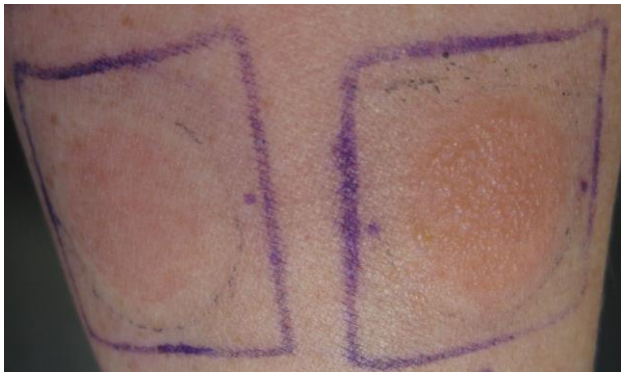


Figure 8. Photos of reactions on aluminium (left) and nickel (right) exposed areas one day after exposure (Day 2) on SLS pretreated skin of one participant with nickel allergy.

5.2.5 Laser Doppler measurements

To quantitatively measure skin inflammation to support visual scoring of the test areas, LDPM was used. Further, blood flow has been shown to increase before visual reactions can be seen and decline prior to fading of the reaction (81). Current guidelines regarding measurement and analyses of the output were followed (79). The fact that blood flow measurements were made on

the same skin areas over time limits spatial variability (116). The error from repeated measurements with the LDPI method is expected to be approximately 5–10% (116). Further, as tape stripping of the skin has been shown to affect perfusion, blood flow measurements preceded the tape stripping procedure on Day 4 (82). An effort was made not to touch the skin prior to/during measurements because touch may cause hyperperfusion (82). The DC, the amount of reflected light, was constant during measurements of exposure areas on the forearms, indicating constant pressure. However, during earlobe measurements, touching the skin areas was inevitable due to the limited space and the anatomy of this site. Therefore, we interpreted the results with caution regarding earlobe blood flow.

5.2.6 Tape stripping

The tape stripping technique was chosen for the analyses of both nickel and PCA in the SC as it is minimally invasive and leaves no permanent scars in contrast to skin biopsies. Moreover, as the major barrier to nickel absorption is the SC, much information can be gathered by investigation of solely this layer. D-Squame was chosen as this is one of the most used tapes (84,117,118). The consecutive tapes were applied to the same skin area to advance deeper into the SC (Fig.9). To minimize variation, duration and weight of applied pressure on each tape were standardized (119). Each tape strip was analysed separately, as recommended (120). Nickel penetration from the upper SC was chosen as previous studies demonstrated close to non-detectable levels of nickel retention in the lower SC for nickel chloride salt (51,92). Fifteen tape strips were collected and analysed in the pilot study; however, in the main study, only the first seven tape strips were analysed. This was a result of very low amounts of nickel retention after tape number seven in the pilot study; it was also a compromise to limit cost- and time consumption.

A drawback of the tape stripping method is that is does not measure nickel in skin appendages; accordingly, information on rapid nickel diffusion is missing. Comparison of nickel- vs. aluminium-exposed skin was possible only on the last day of the study but would have been preferable on all days. Again, this was a compromise due to space limitation and the desire to measure nickel over time. It would have been particularly interesting to have a baseline of nickel on the skin surface before exposures. Another challenge is that contamination between the adjacent test areas cannot be precluded. We do not consider this of major importance since nickel measurement values were high.

We faced some challenges when analysing the amount of collected SC by optical density. First, inhomogeneous SC was collected from irritated skin, which is why only tape strips from normal skin would yield reliable measurements (121). In addition, we found an interference of the metal on the tapes in the measurements; accordingly, we did not use them in any analysis. To our knowledge, optical density has been used on tape strips from metal exposed skin once before, after skin contact with a garment containing silver (91). No interference of the silver particles and optical density was described. Another method for protein determination from tape strips involve tape extraction which was not an option as nickel was to be measured from the same tapes. (118). On normal skin, seven tape strips are probably not enough to remove the whole SC. On irritated and occluded skin, however, we often reached the glistering layer, which has been described to be a sign of complete SC removal (122). This finding was strengthened by values below the detection limit by optical density in the subsequent tapes. The fact that different depths of SC were reached on normal vs. irritated skin complicates the comparison between the two skin areas.

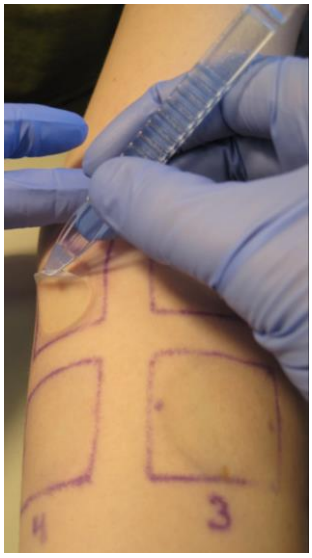


Figure 9. Photo of tape stripping of normal skin on Day 2.

5.2.7 Chemical analyses

Total amounts of nickel on tapes were analysed by ICP-MS. The method to extract metals from the tapes, dilute extracts and validate measurements was developed with our collaborators at Karolinska Institutet, Solna, Sweden. A modification of a previous method used for another tape type was elaborated (123). For example, the recovery of tape extraction was evaluated (by spiking of rhodium) from nine participants and found within the accepted range (80–100%). Initially, we experienced difficulties related to the acrylate-based glue from the tapes, which

clogged the nebulizer of the ICP-MS; however, the method was optimized during measurements of samples from the pilot study.

ICP-MS allows simultaneous, multi-elemental analysis of primarily metal isotopes at a very low detection limit (124). In this study, Ni^{58} and Ni^{60}, the most abundant isotopes of nickel, were used for quantification of total nickel concentrations. Sample preparation (digestion, extraction and dilution) was performed in acid (HNO3), which does not give rise to any substantial interferences. Possible matrix effects were overcome by matrix matched calibration (preparation of the calibration standards in the same solution as the samples) and by internal standard correction of results. A strength of the analysis was the collection of field blanks, i.e. on each tape stripping day, three non-used tapes were saved and stored separately in the same type of tubes as the exposure tapes. Measurement of nickel amount in these 72 blank tapes was used to determine the `matrix background´ and for calculation of the method's limit of detection.

A drawback of the experimental and analytical approach is that ICP-MS measures only the total nickel content in the samples, no information about the present state in the skin is given (free nickel ion, bound to proteins, nickel oxide particles etc.). Such differentiation would have required another methodology. Although this limitation must be taken into consideration in the quantitative measurements, the many allergic reactions in participants with nickel allergy demonstrate the potential of the nickel transferred to the skin in the present study. Another limitation of this method is that it is time consuming and expensive. X-ray fluorescence have the advantage of rapidly providing data (quantitative and qualitative) on the chemical composition of materials (63), but the background noise from tape, glue and skin cells would reduce the possibility to quantify nickel in tapes at low levels, which was a pre-requisite for our study.

Ultra-performance liquid chromatography and UV detection were used to analyse PCA (125). PCA is one of the main degradation products of filaggrin. Since epidermal amounts of filaggrin and its degradation products not only depend on filaggrin genotype, but also on local inflammation and exogenous stressors, PCA was quantified to supplement FLG mutation analysis. In the pilot study of three participants, PCA in eight tapestrips from both upper arms was measured. Based on the results of the correlation between the arms, tape strip number three was chosen for the main study. A mean value from the left and right side was used for analyses. Liquid chromatography and UV detection of PCA from the same type of tapestrip has previously been shown to be a suitable biomarker of the FLG genotype.

6. DISCUSSION

6.1 Questionnaire study

6.1.1 Sources of exposure

In light of high prevalence of nickel allergy and continuing sensitization to nickel in Europe, continuous efforts to identify current causes and exposures are warranted. Information on nickel exposure stems mostly from market surveys of nickel release from metallic items, very few have addressed self-reported problematic exposures in nickel-allergic populations. It was not surprising that the reported current problematic sources of nickel exposure were almost unchanged over the years. The leading items were mostly used by women (earrings, other jewellery), and women are sensitized to nickel to a much higher extent than men (17,18,126). With some exceptions, our findings confirm `risk items´ previously shown to release substantial nickel amounts in market surveys (5,20,21,36) and identified by individuals with nickel allergy (44). However, it was unexpected that within the past 5 years 51% of all individuals with nickel allergy had experienced dermatitis after contact with a metallic item. This is a strong indicator of continued and clinically relevant nickel exposure 10 and 20 years after implementation of the EU nickel restriction and the Danish nickel regulation, respectively. In our view, this is unacceptable, and a revision of the restriction needs to be considered.

Notably, earrings were found to be the leading cause of nickel allergy and allergic nickel dermatitis in all patients and in all subgroups. The special risk of piercing with regard to nickel allergy is generally acknowledged (127) and lower values of nickel release from all items inserted into pierced parts of the body are set in the EU nickel restriction. Nevertheless, the studies forming the basis of the safe limit of nickel release were performed on intact skin, and the safe limit for post assemblies is unknown.

6.1.2 Exposure duration

To our knowledge, this is the first study presenting self-reported critical time durations in relation to development of nickel allergy and dermatitis in individuals with nickel allergy. Despite the limitations in the interpretation of exposure duration, a high response rate was found to this question. It is very interesting that more than one fifth of nickel-allergic patients reported dermatitis within 10 minutes of exposure and more one third within 30 minutes of exposure, which are the limits complying with the restriction.

Our findings are in line with recent findings of rapid deposition of nickel following contact with metallic materials (24) and with results from the experimental study of this thesis. Although items intended for short-duration skin contact were not the most commonly reported regarding allergic-nickel dermatitis, such items (keys, coins, scissors, tools and lighters) were reported to some extent in the patient subgroups. This may imply that although not causing most cases of nickel allergy and dermatitis, they may partly explain the remaining nickel-allergy problem. In addition, since these items are used by both sexes, they may also partly explain the remaining prevalence in boys and men (17). It is possible that items intended for short-duration skin contact may be underreported as a cause of allergic-nickel dermatitis, due to their continual, low-dose exposure of a diffuse nature (63). Further, the combination of these exposures with irritants may lead to difficulties in elucidating the causality. It is worth considering that not being aware of a risk limits the chances of detecting the cause.

6.2 Experimental study

6.2.1 General findings

This is the first clinical experimental study on
1) elicitation of allergic-nickel dermatitis after skin contact of a relatively short and repetitive nature corresponding to the minimum durations used in the EU nickel restriction
2) nickel penetration in the SC after skin contact with nickel-containing metal in a controlled setting with participants with and without nickel allergy.

In relation to the 2014 definition of prolonged contact by the ECHA, the relevance of metallic exposures lasting 10 and 30 minutes has been debated. Moreover, with respect to the forthcoming publication by the ECHA of an extended list with items to be covered by the definition, the nickel industry has argued that an item needs to be in contact with the skin for a longer and continuous period of time to be of any clinical relevance. The industry has proposed that the definition of prolonged contact should be amended to a duration of two hours for one occurrence or 30 minutes for three occurrences within two weeks (128). Three 10-minute exposures in this study were sufficient to deposit enough nickel onto the skin to elicit allergic nickel dermatitis in irritated (63%) and normal skin (19%) previously affected by dermatitis in individuals with nickel allergy. In addition, nickel penetration into the SC was demonstrated in both nickel allergic and control individuals and the amount between normal and irritated skin was correlated. Further, penetration in the outer layers of the SC was shown to be rapid and

measurable within an hour of contact. The results regarding the clinical reactions, blood flow and the quantification of total nickel on the skin surface and in the SC were consistent. Thus, our findings prove the importance of relatively short repeated nickel contact in relation to allergic nickel dermatitis.

6.2.2 High and varying skin surface doses

Our findings are both unexpected and worrying. However, they open up for new considerations regarding possible risk exposures. Short repeated contact with common metallic items may contribute considerably to nickel exposure. It has generally been thought that short metallic exposures lead to low-dose nickel deposition. Even though this is probably true, the nickel skin dose will depend on the local condition of the skin and the surrounding environment, generating large variations in different situations. In this study, nickel skin deposition far above the limit of nickel release in the EU nickel restriction (0.5 µg/cm^2) was found in all participants after short repeated contact. In some participants, the skin surface dose was up to 100-fold above the restriction limit. A few other studies have shown that nickel skin deposition can be high from different materials after skin contact duration of seconds and minutes (24,43,67). These studies had a limited study population and included only healthy volunteers or workers. No study has comprehensively investigated skin surface dose, amount of SC penetration, blood flow and elicitation potential of short metallic exposure in both individuals with and without nickel allergy. Notably, we found differences in nickel skin deposition between those with and without nickel allergy. Interindividual differences in nickel deposition have been demonstrated previously (24,67), but the correlation between high deposition and nickel allergic individuals is new. Skin deposition was very high in a subgroup of individuals with nickel allergy, in both normal and irritated skin. This finding is very interesting as it points towards the risk of developing nickel allergy possibly differing between humans. No reason for this finding could be concluded with the parameters measured in this study, but we consider the possible influence of differences in the amount and composition of sweat as one partial explanation.

6.2.3 Nickel stratum corneum penetration

Considerable nickel amounts were found superficially in the SC after relatively short repeated exposure. This is in accordance with previous studies of different nickel salts and nickel powder (50,92,129–132), yet the novelty in our finding is the use of metallic nickel in contrast to aqueous nickel solutions and the short time exposure. Similar to the exposure of $NiCl_2$ on human forearm skin in another study, nickel from the metallic nickel discs had penetrated into the SC within an hour of exposure (92). Despite the differences in the exposure methods, the results on

penetration profiles are similar. Nickel was also found to accumulate in the skin up to 72 hours after exposure, which is of importance regarding potential nickel build up in the skin, which can lead to elicitation over time. A novel finding was the significantly higher nickel SC penetration in those with compared with those without nickel allergy related to a higher skin deposition. In general, FLG mutations are associated with nickel allergy (55) and increased risk of nickel allergy in individuals with FLG mutations may be a result of compromised chelation of nickel in the SC (52). In this study, an interesting significant correlation was found between declining PCA levels and increasing nickel penetration into the SC in the normal skin of participants with nickel allergy. PCA is a degradation product of filaggrin, varying in analogue with urocanic acid, which is degraded from the active nickel binding amino acid histidine. Our findings support the hypothesis that nickel penetration is faster in patients with FLG mutations due to a decrease in filaggrin and its degradation products (133). However, we did not take the exposure leading to sensitization (sensitized by piercing or by contact with intact skin) of nickel allergic individuals into account in this study. A larger and different set-up would be needed to study the filaggrin bypass-theory.

Skin irritation caused by SLS has been shown to increase the penetration of hydrophilic substances (62). Increased nickel SC penetration in irritated skin could not be measured in the present study. Nevertheless, the many allergic reactions in irritated skin in the study (62.5%) indicate that nickel permeation into the viable layers of epidermis had occurred. We suggest that an indirect measure of a higher penetration in irritated skin in the pertinent study is the finding of higher nickel amounts in the SC of normal compared with irritated skin, and similar nickel surface doses. Partly, penetration may be explained by appendageal diffusion, which can occur within 1–5 minutes (59) and is known to elude tape stripping.

6.2.4 The elicitation potential of short exposures

In the present study, individuals with nickel allergy developed dermatitis only on irritated skin (forearms) or on normal skin where previous dermatitis had occurred (earlobes). On irritated skin, reactions are most likely explained by increased permeability of irritated skin. Dermatitis is common in the general population, affecting 52.3% in a lifetime (18) and hand dermatitis affects 15–20% of the general population. Skin contact with metallic items intended for short-duration skin contact is probably most common on the hands, and these exposures in combination with irritants may contribute to the elicitation and maintenance of hand dermatitis. Such items should

also be considered as potential players in some patients where no obvious continuous long-lasting nickel exposure is found.
On currently normal skin where previous dermatitis had occurred on the earlobes, dermatitis developed after several days, i.e., on the last day of study or after the study finished. Thus, the penetration may not have been increased at these sites. The mechanisms may well be explained by local memory on the sites, resulting in a lower allergen exposure needed to provoke elicitation (107).

We believe that the conditions created in this study regarding skin irritation, restricted showers and elevated temperature in the present study are not unrealistic. Further, the exposure pattern may resemble short skin contact with metallic nickel-releasing items in real life. Therefore, defining prolonged contact is not enough to protect against elicitation of allergic nickel dermatitis and probably not against sensitization of nickel allergy.

6.2.5 Nickel release in artificial sweat

Immersion tests over 168 hours (EN 1811) are used to test metallic items for compliance with the EU nickel restriction. In accordance with others, we found that nickel release in artificial sweat after a short duration led to a considerable proportion of the total release after 168 hours of immersion (24,68). In this study, 70% of the total nickel after one week of immersion was found after three 10-minute periods. Further, in simulated patient exposures where friction was included, the released nickel amount was orders of magnitude higher. This implies that the reference test method EN1811 does not represent actual nickel exposure after short and repeated nickel skin contact. Our results are in line with a study where comparisons of nickel release and nickel skin deposition for three seconds were made. The authors concluded that friction and wear are more decisive than chemical dissolution or corrosion-induced nickel release for the resulting nickel skin deposition after short and repeated contact (24).

7. CONCLUSIONS

The high persisting prevalence of nickel allergy and continued sensitization of young persons in the general population in Europe despite the EU nickel restriction is concerning. Metallic items are covered only if they are intended for prolonged skin contact, i.e., a duration that is estimated as critical for the development of allergic nickel dermatitis. In this thesis, new light has been shed on current causes and the relevance of short repeated nickel exposure regarding nickel allergy and allergic nickel dermatitis.

We conclude that the duration of contact is not pivotal for deposition of high levels of nickel onto the skin surface. Three 10-minute periods are enough to deposit nickel amounts sufficient to provoke allergic nickel dermatitis. Some persons may be more at risk than others of developing nickel allergy due to extensive skin nickel deposition after skin contact. Self-reported critical contact durations were consistent with experimental results on nickel skin deposition, SC penetration, skin inflammation and elicitation of allergic nickel dermatitis in patients with nickel allergy. Nevertheless, the main sources of nickel allergy and allergic nickel dermatitis remain the well-known problematic items such as earrings, jewellery, buttons on clothing, wrist watches, zips and belt buckles.

8. FUTURE PERSPECTIVES

In Europe, literature indicates that the prevalence of nickel allergy has stabilized at a high level (134). The explanation is probably multifactorial and there are several unanswered questions. In the present thesis, I have shown that relatively short and repeated skin contact with metallic items may play a role in this complex interplay, but that well-known problematic items intended for longer duration are probably the main players. To further elucidate the causality of short daily contacts with metallic nickel-releasing items, I propose some future perspectives regarding research and the consequences of our results.

Nickel is used in a wide range of products and materials and its use is rapidly increasing (Nickel Institute https://www.nickelinstitute.org/). Nickel contact is inevitable in everyday life, but to limit the nickel allergy problem in Europe, the use of nickel must be considered to a higher extent than at present. The use of nickel in earrings is a European health concern, justifying further preventive actions. To address this issue, revision of the restriction should be considered so that nickel-containing materials will no longer be permitted in piercing post assemblies. Other materials with no hazard of contact allergy are available. For this approach to be advocated, however, exposure-response relationships need to be studied in a systematic way. Further, since the major problematic items found in this study are supposed to be covered by the restriction, surveillance must improve of the compliance with the restriction. Market surveys of some items covered have repeatedly demonstrated nickel release above the limit of the restriction after implementation of the EU nickel restriction. Patients with nickel allergy and dermatitis should be recommended to screen metallic items before use to limit skin nickel exposure. Another factor that has influenced the risk of the items covered is the changing interpretation of the restriction over time. It is concerning that despite the unchanged limits of nickel release after 2004, accepted release has differed considerably over time.

The most recent update of the EU nickel restriction in 2014 gives a scientifically based definition of the concept `direct and prolonged contact with the skin´. Although this phrase has been difficult to translate to the real-life situation for both authorities and industry, our results imply that the definition of minutes of contact is not too rigorous. Clarification of the definition will further strengthen the positive effects of the restriction.

An interesting finding was very high nickel amounts in some individuals with nickel allergy, resulting in high nickel SC penetration in the same persons. This novel finding needs to be followed up on a larger scale, where possible determining factors, such as quantification of sweat and its pH, SC lipids, TEWL, skin hydration and skin topography, are included. The implication of this finding may ultimately lead to new understanding of the development of allergic nickel dermatitis occurring only in some individuals.

The importance of pressure and friction in determining nickel transfer onto the skin during exposure is clear from the comparison of nickel release from immersion in artificial sweat with the wipe test of the nickel discs in this study. Evaluation of risk of items intended for skin contact of seconds or minutes cannot be estimated from the standard reference sweat test (EN1811), as also outlined by others (66). Therefore, EN1811 should be supplemented by shorter time durations. Further, in the screening of risk items, the dimethylglyoxime (DMG) test is appropriate as it includes friction.

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