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PhD Thesis

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Nickel allergy

Low-dose nickel exposures and the involved immunology

Principal supervisor: Jeanne Duus Johansen

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PREFACE

This thesis is the result of the scientific work carried out between 2019 and 2023 at the National Allergy Research Centre, Department of Dermatology and Allergy, Herlev-Gentofte Hospital. The immunological study was conducted in collaboration with The LEO Foundation Skin Immunology Research Center, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen. The financial support provided by the Ministry of Environment and the Aage Bangs Foundation, is gratefully acknowledged.

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SCIENTIFIC PUBLICATIONS

This Ph.D. thesis is based on the following scientific publications:

I. Wennervaldt M, Ahlström MG, Menné T, Thyssen JP, Johansen JD. *Nickel release from metallic earrings: A survey of the Danish market and validation of the nickel spot test.* Contact Dermatitis, 2021, 85(2), 178–185. doi: 10.1111/cod.13832

II. Wennervaldt M, Ahlström MG, Menné T, Haulrig MB, Alinaghi F, Thyssen JP, Johansen JD. *Chromium and cobalt release from metallic earrings from the Danish market.* Contact Dermatitis, 2021, 85(5), 523–530. doi: 10.1111/cod.13917

III. Wennervaldt M, Ahlström MG, Menné T, Thyssen JP, Johansen JD. *Copper release from metals may mask positive nickel spot test results.* Contact Dermatitis, 2022, 86(5), 431–433. doi: 10.1111/cod.14049

IV. Wennervaldt M, Vaher H, Ahlström MG, Bischofberger N, Menné T, Thyssen JP, Johansen JD, Bonefeld CM. *Subclinical immune responses to nickel in sensitized individuals - A dose-response study.* (Manuscript)

ADDITIONAL PUBLICATIONS

Publications during doctoral studies not included in this thesis:

- Wennervaldt M, Ahlström MG, Menné T, Thyssen JP, Johansen JD. *Diagnostic workup of occupational allergic nickel dermatitis in a nurse with multiple nickel exposures.* Contact Dermatitis, 2019, 81(4), 311–313. doi: 10.1111/cod.13301

- Ahlström MG, Thyssen JP, Wennervaldt M, Menné T, Johansen JD. *Nickel allergy and allergic contact dermatitis: A clinical review of immunology, epidemiology, exposure, and treatment.* Contact Dermatitis, 2019 81(4), 227–241. doi: 10.1111/cod.13327

- Hoffmann SS, Wennervaldt M, Alinaghi F, Simonsen AB, Johansen JD. *Aluminium contact allergy without vaccination granulomas: A systematic review and meta-analysis*. Contact Dermatitis, 2021, 85(2), 129–135. doi: 10.1111/cod.13852

LIST OF ABBREVIATIONS

ACD	Allergic Contact Dermatitis
CEN	European Committee for Standardization
$CuSO_4$	Copper Sulfate
DPC	Diphenylcarbazide
DMG	Dimethylglyoxime
ESCD	European Society of Contact Dermatitis
ICP-MS	Inductive-Coupled Plasma Mass Spectrometry
LPS	Lipopolysaccharide
LOD	Limit of Detection
Ly96	Lymphocyte antigen 96
MHC	Major Histocompatibility Complex
MD-2	Myeloid Differentiation factor 2
$NiCl_2$	Nickel Chloride
$NiSO_4$	Nickel Sulfate
Pet.	Petrolatum
qPCR	Quantitative Polymerase Chain Reaction
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROAT	Repeated Open Application Test
TLR4	Toll-Like Receptor 4
T_{RM}	Tissue Resident Memory T cell
XRF	X-Ray Fluorescence

SUMMARY

Nickel allergy is the most prevalent contact allergy worldwide. Despite regulatory efforts in Europe, the prevalence of nickel allergy remains high especially among women even in adolescents.
The reasons for this are being debated but it is well-established that body piercings and earrings, are one of the primary risk factors for nickel allergy. This thesis comprises four studies that focus on these exposures and assess whether earrings available on the market exceed regulatory limits. The studies also validate the nickel spot-test as a screening tool, examine co-factors, and analyze immune stimulation by low doses of nickel to aid in the prevention of nickel allergy.

The first two studies investigated the release of nickel, chromium, and cobalt from 304 earrings randomly sampled from the Danish market. The earrings were spot tested for each metal and subjected to metal release testing using EN 1811. The test revealed that 28.3% of tested earrings released nickel, with 14.8% exceeding regulatory limits. Additionally, both chromium and cobalt were also found to be released from earrings, with the post component showing the highest release values. The dimethylglyoxime (DMG) spot test for nickel release has high specificity, albeit mediocre sensitivity. The sensitivity declines with lower levels of nickel, making it less useful in screening of earrings. The spot test for chromium (VI) was not able to identify any sources, while the spot test for cobalt release may have some clinical value.

The third study investigated the sensitivity and false negative rate of the DMG spot test. The study found that copper ions can effectively mask the positive results of the DMG spot test, potentially explaining its mediocre sensitivity. Nevertheless, the DMG spot test remains a valuable tool for identifying items with high nickel release, due to its low false positive rate, rapidness, and cost-effectiveness.

The fourth study focused on the clinical implications of nickel exposure by examining the immune response in biopsies from re-exposed skin. The study found that exposure to nickel in amounts equal to the regulatory limits can trigger mild clinical reactions and significant immune activation at the transcriptomic level. Interestingly, a similar immune response was found independently of clinical allergic dermatitis. The study highlights the importance of local memory in increasing the risk of dermatitis and suggests that the current regulatory limits for nickel exposure may not provide sufficient protection for individuals with nickel allergy.

In conclusion, there is an ongoing considerable exposure to nickel through earrings on the market, which may partly explain the continued problem of nickel allergy. The DMG spot test could be considered as a tool in policing regulations. Even low amounts of nickel were seen to cause immune stimulation in nickel allergic individuals on pre-exposed skin. The results suggest that the existing regulatory limits of nickel release may not provide adequate protection against nickel allergic dermatitis.

DANSK RESUMÉ

Nikkelallergi er den mest udbredte kontaktallergi i verden. På trods af at frigivelsen af nikkel fra forbruger produkter er reguleret i Europa forbliver prævalensen af nikkelallergi høj, selv blandt unge. Årsagerne til dette er debatteret, men det er anerkendt at kropspiercinger og øreringe er en af de primære risikofaktorer for nikkelallergi. Denne afhandling omfatter fire studier, der fokuserer på disse eksponeringer og vurderer, om øreringe tilgængelige på markedet overstiger de regulatoriske grænser. Studierne validerer også nikkel-spot-testen som et screeningsværktøj og undersøger immunstimulering ved lave doser af nikkel for at understøtte forebyggelsen af nikkelallergi.

De første to studier undersøgte frigivelsen af nikkel, krom og kobolt fra 304 øreringe, der var tilfældigt udvalgt fra det danske marked. Øreringene blev spot-testet for hvert metal og blev testet for metalfrigivelse ved EN 1811. Det blev fundet, at 28,3% af de testede øreringe frigav nikkel, hvoraf 14,8% overskred de regulatoriske grænser. Derudover blev både krom og kobolt frigivet fra øreringe, hvor stiften viste de højeste værdier. Dimethylglyoxime (DMG) spot-testen for nikkelfrigivelse har en høj specificitet, men en middelmådig sensitivitet. Sensitiviteten falder med lavere niveauer af nikkel, hvilket gør den mindre brugbar i screening af øreringe for nikkelfrigivelse. Spot-testen for krom (VI) var ikke i stand til at identificere nogen kilder, mens spot-testen for koboltfrigivelse kan have nogen klinisk værdi.

Det tredje studie undersøgte sensitiviteten og falsk negativ raten af DMG spot-testen. Studiet fandt, at kobberioner kan skjule positive resultater ved DMG spot-testen, hvilket potentielt kan forklare dens middelmådige sensitivitet. Ikke desto mindre forbliver DMG spot-testen et værdifuldt værktøj til at identificere genstande med høj nikkelfrigivelse, på grund af dens lave falsk positiv rate, hurtighed og omkostningseffektivitet.

Det fjerde og sidste studie fokuserede på de kliniske implikationer af nikkeleksponering ved at undersøge immunresponset i biopsier fra tidligere eksponeret hud. Studiet fandt, at eksponering for nikkel svarende til de regulatoriske grænser kan udløse kliniske reaktioner samt signifikant immunaktivering på transkriptomisk niveau. Interessant nok blev et lignende immunrespons fundet uafhængigt af klinisk allergisk eksem. Studiet fremhæver betydningen af lokal hukommelse, der kan give øget risiko for eksem og indicerer, at de nuværende regulatoriske grænser for nikkeleksponering muligvis ikke beskytter personer med nikkelallergi tilstrækkeligt.

Der en fortsat betydelig eksponering for nikkel gennem øreringe tilgængelige på det danske marked, hvilket delvist kan forklare det fortsatte problem med nikkelallergi. DMG spot-testen kan være et nyttigt redskab til at understøtte at reguleringen bliver overholdt. Selv lave mængder nikkel viste sig at forårsage immunstimulering hos personer med nikkelallergi på tidligere eksponeret hud. Resultaterne peger på, at de eksisterende grænser for frigivelse af nikkel muligvis ikke giver tilstrækkelig beskyttelse mod nikkelallergi.

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1. INTRODUCTION

Young women continue to become sensitized to nickel. For decades, the focus has been on preventive measures for nickel allergy, and regulatory values that limit nickel release from consumer items have been established in Europe. The most commonly reported single causes of nickel allergy are still jewelry used for body and ear piercings, despite the fact that these have been covered by the European nickel regulation since its implementation in 2001.[1–5] This raises the question of whether the regulatory limit values for nickel release are sufficiently protective, particularly for the unique and critical exposure of a pierced skin canal.

A lower threshold for the elicitation of nickel allergy from a piercing exposure has been described.[6] Aside from this, not much is known about the potential increased risk from post assemblies inserted into pierced body parts. Knowledge is also lacking as to whether the reactions to earrings reported by nickel-allergic individuals may partly be caused by co-sensitization and co-exposure to other common metal sensitizers such as chromium or cobalt. Another reason for the continued high incidence of nickel allergy may be that the regulation is not adequately enforced or respected so that women are exposed to, e.g., earrings that exceed the regulatory limits.[1]

The use of current methods being used to determine nickel release from items, such as the nickel spot test, have not been evaluated on small items such as earrings or on items that have a low nickel release. Yet, such knowledge is needed as the spot test is being used on a daily basis by nickel-allergic individuals to identify items that may cause nickel-allergic contact dermatitis (ACD). It is also of major relevance to know the reliability of the test for product self-control by importers and retailers and whether this test should be considered in the future as a first step when testing for conformity with the nickel regulation. Furthermore, exposure to low doses of nickel, that are currently considered safe, are possibly immunostimulant and without eliciting clinically visible dermatitis, may cause subclinical inflammation. This may keep local memory activated and over time with prolonged or repeated exposure manifest as ACD. Such exposures may be overlooked and are possibly a significant cause of the continued problem of nickel allergy.

The overall purpose of this thesis was to study potential deficits in the current preventive strategy of nickel allergy from consumer exposures exceeding regulatory limits; to explore the validity of the nickel spot test on small items with low nickel release; and to examine co-sensitizations to the potential immune stimulation from low doses of nickel. This may aid in improving prevention of nickel allergy.

1. Nickel

Nickel (atomic number 28) is a silvery metal with a shiny appearance and the fifth most common element on Earth. Nickel occurs in nature as oxides, sulfides, and silicates and often with cobalt.[7,8] Nickel ions are commonly found in the 2+ oxidized state (Ni^{2+}).

Nickel is hard but malleable, has good ductility, and is oxidation and corrosion resistant. In addition to being available at low cost, nickel has physical and chemical properties that are commercially valuable, which is why it is widely used in both industrially and in consumer goods.[7,9] As nickel is a common constituent in plating and various metal alloys, including most grades of stainless steel,[9] it is difficult to avoid skin contact with nickel in modern culture.[10]

2. Contact allergy

Contact allergy is a type IV delayed type hypersensitivity reaction, that is characterized by a cell-mediated response. Contact allergy consists of two distinct phases: the sensitization phase and the elicitation phase (Figure 1).[11,12] During the sensitization phase, or the induction phase, the innate immune system in the skin recognizes the hapten (allergen), and the antigen presenting Langerhans and dendritic cells present hapten-modified self-peptides to naive T cells in the draining lymph node via major histocompatibility complex class I or class II molecules on the cell surface. Allergen-specific T cells then starts to proliferate and differentiate, becoming effector and memory T cells, and migrate from the lymph node and are able to recirculate back to the skin. Upon e-exposure to the same antigen the specific memory T cells may initiate elicitation phase. The sensitization phase usually occurs over 10-15 days and is clinically silent.[12–16]

The elicitation phase usually occurs within 24-72 hours upon re-exposure. Hapten-protein conjugates are taken up by Langerhans and dendritic cells and activate the now-present allergen-specific T cells in the skin, which initiates an inflammatory response. The inflammatory response releases pro-inflammatory cytokines and recruits circulating T cells and several additional leucocytes, including neutrophils, monocytes, eosinophils, and/or mast cells, which further drive the allergic response.[12–15,17] This inflammatory response may cause tissue damage, which clinically manifests as allergic contact dermatitis (ACD) and includes symptoms such as erythema, infiltration, papules, vesicles, itch, and possible bulla formation. If the skin inflammation persists due to lack of treatment, continued exposure to the allergen, or concurrent skin inflammatory diseases, ACD can become chronic. Chronic ACD characterized by a dry, scaly, and fissured itching skin eruption.[11] The allergic inflammatory reaction persists for a couple of days and is downregulated by regulatory T cells. During the downregulation, effector T cells are gradually replaced by memory T cells. A subset of these memory T cells, called skin resident memory T cells (T_{RM}), does not recirculate but rather persists in the epithelia for several months after the exposure, which is why a rapid and enhanced allergic reaction may be seen upon re-exposure to the same area. T_{RM} is also be produced during the initial exposure and the sensitization phase.[18–21]

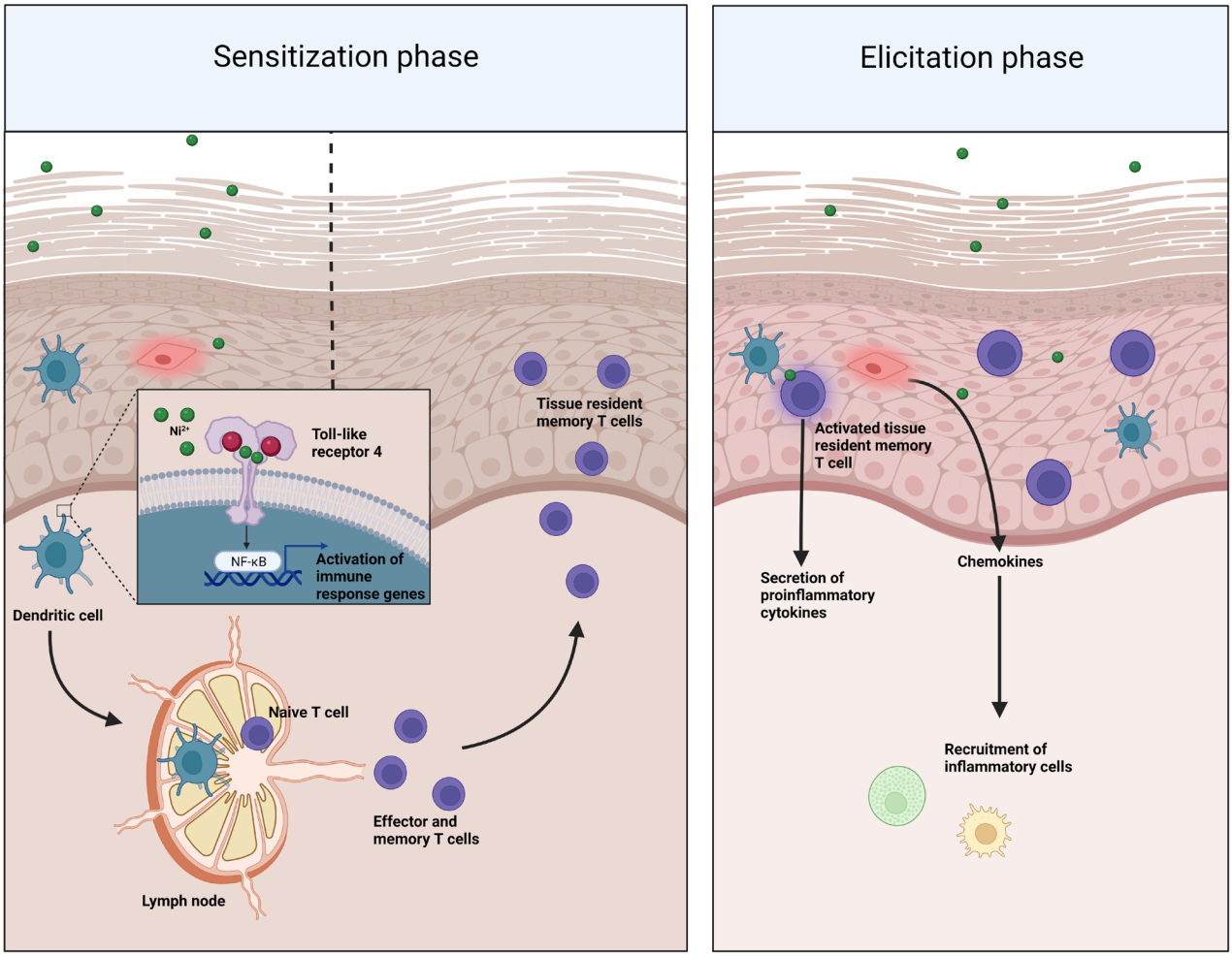

Figure 1. The two phases of nickel allergic contact dermatitis. Nickel ions enter the epidermis, and activate dendritic cells by binding to their toll-like receptor 4 at a non-typical pattern recognition site. The activated dendritic cells then migrate to the draining lymph node and present a nickel-modified self-protein to naïve T cells, which differentiate and proliferate as specific effector and memory T cells, some of which recirculate back to the initially exposed skin and persist as skin resident memory T cells (TRM). Upon re-exposure, activated dendritic cells may activate the antigen specific TRM in the skin, which will secrete several proinflammatory cytokines resulting in clinical dermatitis. In addition keratinocytes also respond to allergen exposure by secreting several chemokines, which will attract several inflammatory cells. Created with BioRender.

When the skin is exposed to nickel, the nickel ions bind specifically to toll-like receptor 4 (TLR4) present on dendritic cells. To initiate the activation of TLR4 and its subsequent signal transduction process, the association with lymphocyte antigen 96 (Ly96), also called myeloid differentiation factor 2 (MD2), is necessary. TLR4 stimulation induce production of various cytokines and are involved in inducing the migration of the activated dendritic cell to the draining lymph node, where it presents a nickel modified self-protein, which may trigger the T cell mediated immune response, as described.[12,14,22,23]

The TLR4 typically recognizes lipopolysaccharides (LPS), which are major components of the cell walls of several gram-negative bacteria. However, in the case of nickel ion exposure, TLR4 activation occurs at a site different from its primary pattern recognition site. Specifically, nickel ions bind through chelation to specific spatially localized histidine residues on the TLR4 homodimer, thus mimicking a LPS activation of the innate immune system. A similar activation has also been described for other metal ions such as cobalt and palladium. The homologue mice TLR4 lacks these specific

histidine residues, which may explain the comparatively weaker allergic response to nickel observed in mice as compared to humans.[24–26]

Patch testing is the gold standard for diagnosing contact allergies. A patch test is an occluded challenge test where an allergen is dissolved in a solute, typically petrolatum (pet.), water, or ethanol. The solution is placed in a chamber of aluminum or propylene and applied to the skin with adhesive tape.[11,27] The test is performed on the upper back as studies have reported higher skin reactivity in this area.[28,29] The chamber, or patch, is left on the skin for 48 h (day 2) and then removed. When the patch is removed, any skin reactions are read and scored, and additional readings are performed after 72 h (day 3) or 96 h (day 4) and 168 h (day 7). Skin reactions are scored on a 5-step scale according to the guidelines of the European Society of Contact Dermatitis (ESCD).[27] The scale covers the following: a negative reaction (0); a doubtful reaction (+?) with weak erythema or non-homogenous infiltration; a weak positive reaction (+) with erythema; homogenous infiltration, and possible papules; a strong positive reaction (++) with vesicle formation; and extreme positive reaction (+++) with strong erythema and possible bulla formation.[27] Depending on the tested allergen, concentration, and counterion, an irritant reaction may appear, which may be difficult to differentiate from an allergic reaction. Nickel sulfate 5% in petrolatum is used to diagnose nickel contact allergy and is a part of the European baseline series.[27] Although nickel chloride more closely resembles real-life exposure to nickel, nickel sulfate is used due to its reduced risk of irritant and unspecific reactions.[30]

3. Epidemiology of nickel allergy

Today nickel allergy is the most common contact allergy worldwide, with an estimated prevalence of 11.4% in the general population[31] and 14.5% in the European general population.[2] The allergy is four to ten times more predominant in women than in men,[10,31] with 22.2% of women and 5.2% of men affected in the European general population.[2]

Nickel allergy has been less studied outside of Europe, but is reported to have a high prevalence among patch-tested patients in North America (16.2%)[3,32] and Asia (25.7%),[33] and among adolescents in the Middle East (43%).[34] In a recent meta-analysis the prevalence of nickel allergy in the general population was estimated to be 11.4% (the meta-analysis included studies from Europe, North America, and Asia); 15.7% of women and 4.3% of men, when stratified by gender.[31] The prevalence also remains high among the younger generation, wherein 2016 a Swedish cohort study of 2,285 adolescents (ages 15-19 years) showed that 9.8% of girls and 4.9% of boys had a positive patch test to nickel sulfate.[35] A similar Danish cohort study in 2002, which included 1,501 adolescents (ages 12-16 years), reported similar findings with 13.7% of girls and 4.9% of boys having a positive patch test to nickel.[36]

Nickel dermatitis is frequently seen on the hands and the face and is often localized to an item with recurrent skin contact, such as items worn and metallic parts on clothing.[37] Currently, the only effective treatment for nickel allergy is to avoid contact with nickel-releasing items or to relieve the

symptoms of dermatitis with topical corticosteroids or, if the allergy is widespread, systemic immunosuppressive treatment.[11] Newer studies have begun to elucidate the underlying immunological mechanisms of nickel dermatitis,[14,15,19,22,24,38–42] but many questions remain regarding its pathogenesis.

Preventing allergies can have extensive socio-economic benefits. A study conducted by the Danish Environmental Protection Agency in 2004 found that restricting the use of nickel in consumer products may have resulted in significant health savings in Denmark. The study estimated that if the use of nickel had not been restricted, the number of new cases of nickel allergy would have been twice as high over a 20-year period. This would have resulted in additional healthcare costs and lost productivity due to missed workdays. The restrictions on nickel were estimated to have led to health savings of approximately DKK 9.7 billion (€1.3 billion) in Denmark (based on 2002 price levels) during the 20-year period.[43] Likewise, restrictions on chromium VI in leather products can lead to socio-economic savings. According to the European Chemicals Agency's Risk Assessment Committee (RAC) and Scientific Committee on Emerging and Newly Identified Health Risks (SEAC), limiting hexavalent chromium exposure from leather items could result in healthcare cost savings of approximately €4.4 billion over a 20-year period across the EU.[44,45] This highlights the importance of restricting the use of allergenic metals in consumer products to protect public health and promote socio-economic benefits and well-being.

4. Sources of nickel exposure

In the late 1800s, nickel allergy was an occupational disease that affected workers in the plating and mining industry, but the emphasis shifted in the 1900s when the allergy became more common in women and consumer exposures were identified.[10,46] In the 1960s it was discovered that 86.5% of the primary sensitizing exposures came from consumer items and the handling of nickel-releasing items at home.[47] Occupational nickel exposure is still an issue today, but the clinically relevant exposure may be difficult to identify due to various sources and potentially simultaneous irritant exposures.[10,11,37] Common occupational exposures are found in the industrial setting, construction work, and in the service and healthcare sectors.[37,48,49]

Consumer nickel exposures are largely related to current fashion.[10,37] The first cases of nickel allergy in consumers were published in the 1930s and came from spectacles, wristwatches, and in particular, suspenders,[50–52] which were deemed the primary sensitizing exposure.[52] Today, stocking suspenders are less frequently used and body piercings have become more popular, resulting in nickel dermatitis from earrings and body piercings becoming more common.[1,3,53] In modern society, it is difficult to avoid topical contact with nickel as it is ubiquitous in metallic items. To be allergenic, the nickel ions must be released, or migrate, from the metal to the skin. Thus, an item containing nickel is not necessarily a source of nickel exposure if the quality is sufficient to confine the nickel ions to the metal. Common exposures include but are not limited to jewelry, body piercings, wristwatches, hair clips, buttons, zippers, belt buckles, spectacles,[1,54,55] and keys,[1,561,56] while toys,[57,58] mobile phones,[59] coins,[55,60,61], utensils,[62] tools[63,64] and spectacles[50] may also play a role.

In general, risk factors for the sensitization and elicitation of nickel allergy include the amount of nickel ions on the skin,[65,66] frequency of exposure,[67,68] occlusion,[6,69,70] exposure time, skin barrier function, and penetration-enhancing factors.[71–74] Other factors such as nickel accumulation in the skin from short, repeated exposures,[75–77] impaired or bypass of the skin barrier[6,78] (piercing exposure), and prolonged low-dose exposures may be of importance.

Today the primary cause of nickel allergy is the use of body piercings that release nickel. Several studies have found a strong correlation between the presence of body piercings and nickel allergy and that the number of piercings may be of importance.[1–3,6,53,79–82] Nickel is released from body piercings in amounts that may elicit nickel ACD, as demonstrated by challenge tests.[83–86] Several market studies have shown excessive levels of nickel being released from various consumer items, including jewelry and earrings.[87–90] The amount of nickel released from many of these items does not comply with the enforced nickel regulation in Europe, which limits nickel release from consumer items.[91] Exposure to items with excessive nickel release is likely one of the main risk factors contributing to the continued high incidence of nickel allergy among adolescent women.[1] To prevent new cases of nickel allergy, the focus should be on identifying and effectively regulating critical exposures. This requires that screening measures for nickel release are efficient and have practical applications.

5. Skin penetration and nickel exposure

To be allergenic, nickel ions must reach the viable epidermis through absorption via the upper skin cell layers. Several factors influence penetration, such as dose, pH, oxidation, occlusion, skin barrier integrity, and differences in epithelial thickness between anatomical sites.[92–95]
The diffusion of nickel ions is mainly intercellular but can also be transcellular or appendageal, such as through hair shunts.[92,93] Nickel ions diffuse slowly through the skin with a lag time of around 50h.[96] This is in contrast with elicitation reactions that occur earlier in time, and it seems that these mechanisms are not fully understood. Nickel ions accumulate in the stratum corneum, where 90% of all nickel from a single exposure is found to be bound.[92] This is assumed to be due to the presence of histidine-rich filaggrin proteins in the stratum corneum, which can strongly chelate nickel ions, and is likely also the cause of the slow passive diffusion.[97] The absence of filaggrin, as in the case of filaggrin-null carriers, causes a higher penetration of nickel ions through the stratum corneum.[98]
Nickel chloride is more rapidly absorbed than nickel sulfate under occlusion. Without occlusion, nickel penetration is strongly reduced, and for nickel sulfate permeation is hardly detectable.[96]
However, the elicitation threshold for nickel sulfate is roughly the same for patch testing and the total accumulated dose from a repeated open applications test (ROAT). Interestingly, no significant difference was found in the dose-response rate if the ROAT was performed over one, two, or three weeks, but was mainly dependent on the total accumulated dose.[70] This might stem from an accumulation of nickel ions in the stratum corneum, which saturates filaggrin-histidine bindings and allows for more rapid penetration to the epidermis. This emphasizes the risk of frequent, repeated, and/or low-dose skin exposures, which individually may be considered safe, but might deposit and accumulate to a dose above the elicitation threshold, as has been highlighted and discussed in recent studies.[75–77]

While occlusion is an important factor, so is an altered skin barrier function. Atopic dermatitis and irritant exposures may increase skin absorption.[30,99] *In vitro* studies on human skin have shown that the penetration rate was increased 10-100 times if the skin was abrased with a needle prior to exposure, compared with intact skin.[100] The piercing exposure from an earring or body piercing initially bypasses the skin barrier during the re-epithelization and has direct release to the blood plasma. After epithelization, there is a risk of accumulation of doses from prolonged and frequent exposure. This is, however, currently being largely overlooked in risk assessment.

6. Threshold

Safe levels for nickel exposure are difficult to determine as exposures are not uniform and several factors influence the inherent risk of the exposure[6,10,66,68] as described earlier. More recent research, has revealed that frequent contact can lead to the deposition of nickel ions in the skin,[75–77] potentially causing an accumulation that could surpass the threshold for eliciting or sensitizing reactions, even with low-dose exposures.

In 1987, it was proposed that nickel release should not exceed 0.5 µg/cm^2/week from consumer items.[101] This would later be the basis for the Danish nickel regulation and became the limiting regulatory value enforced in the European nickel regulation on items with direct and prolonged skin contact.[91,102,103] This value was, and still is, considered sufficiently protective for most nickel-allergic individuals although a minority might react to exposure below this value.[62] Most elicitation threshold studies on nickel allergy have been performed by patch test applications on the back of naive skin. However, with newer studies showing that previous allergic dermatitis will enhance the allergic reaction to repeated exposure,[19,21] these studies might be too conservative. The elicitation threshold varies between individuals and over time.[104] As the current European regulatory value has largely been extrapolated from patch test results, studies elaborating upon the effect of low-dose nickel exposure, deposition, and accumulation are warranted.

Sensitization studies are scarce as they are considered unethical in humans, and no sensitization threshold has been established.[10] The threshold for initial sensitization is generally considered higher than the elicitation threshold.[105] Factors such as genetic predisposition and irritated or inflamed skin,[10,106] along with the mentioned risk factors, such as concentration, frequency, occlusion, exposure time, and altered skin barrier function, determine the risk of sensitization. The sensitization and elicitation threshold from piercing exposure is lower than that of intact skin.[83,84]

7. Methods for identifying and quantifying nickel release

Nickel must be released as a nickel ion from a metallic item to be allergenic. A release or migration of metallic ions from a metallic item occurs due to corrosion or oxidation of the metal, dissolution of the surface oxides as in contact with sweat, or by physical removal such as in touch and friction.[75,107] Released nickel ions can be identified from an exposure source using different methods, such as the dimethylglyoxime (DMG) test[108] or quantified by chemical analysis following submersion in artificial

sweat, standardized as the standard reference test EN 1811 by the European Committee of Standardization (CEN).[109]

The current gold standard for measuring metal release from an item is the artificial sweat test. An item of interest is submerged in artificial sweat for one week, and the metal released to the sweat is measured by mass spectrometry, preferentially inductive-coupled plasma mass spectrometry (ICP-MS). The amount released is divided by the surface area of the item and reported as µg/cm^2/week.[109] The artificial sweat test, with an analytical focus on nickel, has been adapted and defined as the European standard reference test EN 1811 to test items for compliance with the European nickel regulation.[91,110] Although the test provides an advanced and precise quantification, it is time-consuming and expensive and has been criticized for poor reproducibility.[111] Furthermore, the test measures the passive diffusion of nickel ions and does not factor in friction during skin contact, which reportedly enhances nickel release.[75] Additionally, the surface area calculations are difficult and prone to errors, and the release rate of nickel ions from an item has been demonstrated to be initially very high, followed by a rapid decline.[112] Thus, the test's duration of one week might on one hand give a conservative result as the surface is not allowed to re-oxidate during testing,[37] and on the other hand, fail to display the high initial release.

The DMG test also called the nickel spot test, is a colorimetric test based on the chelation of the dimethylglyoxime molecule with free nickel ions, which creates a complex that is bright red (Figure 2). Use of the DMG test to screen items for nickel release is described the technical report TR 12471 published by CEN,[113] though it does not test for legislative conformity. The test is performed by adding 1-2 drops of a 1% DMG and 10% ammonia solution on a cotton swab. The swab is then rubbed against the item of interest for 20 seconds. If a bright red discoloration appears, the item is positive and is considered to be a risk factor for nickel allergy.[6,108,114] The DMG test is a rapid, low-cost, and commercially available test. Due to its ease of use, the test is used in the majority of studies on nickel exposures on many different types of items and is largely responsible for current knowledge on the diversity of nickel exposures. In the validations done so far, the test has very high specificity and mediocre sensitivity, with an estimated detection limit of 0.5 µg Ni/cm^2,[108] however its performance on small surface areas and low dose release has rarely been investigated. Compared to its widespread use, validation studies of the DMG test are scarce. The DMG test can also be used on the skin to detect nickel deposition and accumulation from multiple exposures.[115,116] Longer-term accumulated nickel exposure can also be measured in fingernails.[117]

Figure 2. Left: chelation of nickel with two DMG molecules to form a nickel DMG-complex. Right: A positive DMG test shown by the bright red discoloration after the spot testing of a key.

Measurements of nickel content in an item can be done by X-ray fluorescence spectrometry (XRF).[58,118,119] An XRF spectrometry analysis is a non-destructive test method that determines the elemental composition of an item by irradiating it with high-energy X-ray beams and capturing the characteristic emitted fluorescent radiation. It has been utilized for screening but does not provide information on whether the nickel ions are released.

8. The nickel regulation

In 1994, an EU Nickel Directive was introduced to limit consumer nickel exposure in European countries. Fully implemented in 2001, and later included in the European chemical regulation, REACH annex 27, limits the release of nickel to 0.5 µg/cm2/week for nickel-containing items intended to be in prolonged and direct contact with the skin and to 0.2 µg/cm2/week for any post assemblies to be inserted into pierced body parts.[91,110] The regulatory limit of 0.5 µg Ni/cm^2, which was established in 1990 as part of Danish regulations,[120] is based on studies conducted by Menné et al.[86] The limit was established with the goal of reducing nickel allergy and was deemed to be easy to assess using the DMG test, which had an estimated detection limit of 0.5 µg/cm^2.[114] With the introduction of the European Nickel Directive, the EN 1811 was established to assess legislative conformity for items.[109] While the regulation proved effective and the prevalence of new nickel-allergic individuals has declined significantly in some European countries since its implementation, new cases of nickel allergy are considerable and remain a general health problem.[1,121–124]

The release of nickel from piercing post assemblies has been covered by the European nickel regulation since its implementation in 2001. Originally, the nickel content was restricted to <0.05%.[86] In 2005 the regulation was amended and introduced a lower nickel release limit of 0.2 µg/cm^2/week to replace the restriction of the nickel content for piercing post assemblies.[103] The change from limiting the nickel released instead of the nickel content was made to recognize that the sensitizing factor is the release of nickel ions from items to the skin. This effectively allows the use of various

grades of stainless steel with a high nickel content if the production quality and surface finish confine the nickel ions to the metal.

Due to the complexity of measuring the surface area and the reproducibility of analytical measurements, an adjustment factor of 0.1 was introduced in 2005, effectively allowing 10 times higher nickel release.[103] This has later been amended and changed to a "measurement of uncertainty," which since 2016 allows a nickel release of ≤0.35 μg/cm^2/week for piercing post assemblies and ≤0.88 μg/cm^2/week for other items with prolonged skin contact[109] (Table 1).

	Legislative limit (REACH)[110]	**Interpretation (EN 1811:2011+A1:2015)**[109]
Items with prolonged skin contact	0.5 μg/cm^2/week	≤0.8 μg/cm^2/week
Piercing post assemblies	0.2 μg/cm^2/week	≤0.35 μg/cm^2/week

Table 1. Current European regulatory limits of nickel release in consumer products and interpretation of test results for compliance.

As the prevalence of nickel allergy remains high, including in adolescent females, the regulation may not be sufficiently protective.[88,125,126] This may be due to either the regulation not being respected, the limits being too high, or the inadequacy of the extent of items covered. There is a need for more efficient surveillance of nickel release from items on the market as well as further studies on the risk of low nickel-releasing items and the causative exposures leading to nickel allergy.

9. Nickel allergy and piercings

To date, there are limited clinical provocation studies on body piercings with known nickel release and symptoms of nickel allergy. One study reported that a grade of high-quality stainless steel commonly used in piercing post assemblies, with nickel release within the limits of the regulation, did not elicit nickel dermatitis in any of the 25 nickel-allergic individuals tested, despite indications of nickel have been released and deposited onto the skin. However, 2/25 subjects developed erythema and itching within 48 h.[85] Two other studies have tested different stainless steel piercing alloys, in which 4/10[127] and 6/6 nickel-allergic individuals[84] elicited nickel dermatitis to piercing post assemblies with nickel release (Table 2).

	n/N	**Nickel release from tested earring**	**Comments**
Fisher, A. A. (1984)[127]	4/10	0.05-15.0 μg	Total nickel release
Räsänen, L. et al. (1993)[84]	6/6	0.15-104.59 μg/cm^2/week	Measured in plasma
Ingber, A. et al. (2003)[85]	0*/25	0.11-0.21 μg/cm^2/week	Measured in unused earrings

Table 2. Studies on the elicitation potential of wearing nickel-releasing earrings. n/N: Participants with developed dermatitis/tested nickel-allergic participants. *Two participants had transient erythema and itch for 48 h.

The study from Ingber et al.[85] concluded that AISI 316L stainless steel can safely be used for piercing post assemblies as no participants had an allergic reaction. It can, however, be problematic to draw a definitive conclusion from a zero-event study as a statistical probability cannot be neglected, nor can a confidence interval be calculated from zero numerators. In a zero-event study, the "rule of three" can be applied as an approximation for the 95% confidence interval. The rule of three states that the 95% upper confidence limit for the true proportion is 3/N.[128,129] The 95% confidence interval of the rate of developing nickel dermatitis to 316L stainless steel is then calculated to 0-14% (3/25*100%). The importance of introducing this uncertainty is emphasized in the study by Räsänen et al.,[84] where dermatitis from two earrings with low nickel release (0.15 and 0.17 µg/cm^2/week) was demonstrated and because there are few studies in the field upon which to assess the risk of low-dose piercing exposure.

The decision to lower the limit in piercing post assemblies compared to other items was based on a European Committee contracted study on the risk of nickel sensitization from piercing exposure. The study found an approximate doubling of the release rate of nickel ions from stainless steel to blood plasma compared to artificial sweat as used in the EN 1811.[130] As nickel ions are mainly released to the blood plasma during the epithelization of a body piercing, the limit was lowered to 0.2 µg/cm^2/week for all piercing post assemblies and amended in the European nickel regulation in 2005.[78,91,130] The study does, however, state that there is insufficient information available to make a complete risk assessment.[130]

The regulatory migration limit for piercing post assemblies is hence a calculated approximation of the safety of nickel-releasing piercings and clinical evidence is needed to support this safety margin. While it is generally recommended among piercing practitioners for the initial piercing stud that is worn during epithelization to be of "biocompatible" materials, such as "surgical grade steel" (316L), titanium, plastic, or glass,[131] there are no specific legislative requirements.

10. Other metal allergies

Simultaneous sensitization to nickel and other metals is not uncommon, and concomitant sensitization to cobalt and chromium is relatively frequent. It is thought that this is not due to cross reactions but due to common exposures, as these metals occur in the same sources.[37,46] Both chromium and cobalt are often included in common metal alloys. Studies have shown both chromium and cobalt ions are released from various consumer items, creating a potential risk of sensitization or elicitation.[58,90,132–136] Chromium and cobalt are potent sensitizers and are, after nickel allergy, the most prevalent metal allergies.[137–139] It is estimated that 0.8-1.8% of the general population are sensitized to chromium and 2.2-2.7% are sensitized to cobalt.[31,140] Historically, chromium sensitization has primarily been related to cement work and exposure to leather items.[141] The causative exposures for cobalt allergy have been difficult to determine and are largely unknown.[142,143]

Currently, a spot test for chromium and for cobalt exist. Similar to the DMG spot test, these test function as colorimetric tests with molecule chelation to a free metal ion. The diphenylcarbazide (DPC) test can detect chromium ions,[133] and disodium-1-nitroso-2-naphthol-3,6-disulfonate (Nitroso-R salt) can detect cobalt ions.[144,145] Chromium commonly occurs in two oxidation states (Cr(III) and Cr(VI)), of which the DPC test is only able to detect hexavalent chromium (Cr(VI)) ions.[133] While both tests have been used to identify chromium and cobalt exposures,[58,132,133,146] their use has been criticized due to questionable sensitivity and specificity.[147–149]

Copper is a very common constituent in many metal alloys, including brass and bronze, and contact with copper is frequent in everyday society. Copper is a weak sensitizing agent,[150] and despite being an increasingly common exposure, sensitization to copper is rarely reported.[151] Sensitizations to other metals such as aluminum, beryllium, gold, mercury, palladium, and titanium are not uncommon but have an albeit relatively low prevalence.[11,27,152] Many women report rash from wearing earrings.[1–4] This is interpreted to be due to nickel exposure/nickel allergy. However, it is unknown to which extent other common metal allergens may at least potentially play a role. For this reason, we investigated the content and release of chromium and cobalt.

2. OBJECTIVES

The overall aim of this PhD project was to assess the safety of nickel-releasing piercing post assemblies on the market in terms of contact allergy and to investigate whether low doses of nickel could induce a subclinical immunological response prior to visible allergic contact dermatitis. Furthermore, we aimed to evaluate and validate the DMG spot test as a screening tool for detecting nickel release from metallic items.

Manuscript I: To examine the proportion of earrings available on the Danish market with excessive nickel release according to REACH regulation and to validate the DMG spot test as a screening tool for nickel release.

Manuscript II: To examine the content and release of chromium and cobalt in earrings available on the Danish market.

Manuscript III: To show whether and to what degree the presence of copper may interfere with the DMG spot test.

Manuscript IV: To determine whether low-dose nickel exposure can cause allergic nickel dermatitis and to investigate a potential subclinical immunological activation prior to the elicitation of nickel dermatitis.

3. MATERIALS AND METHODS

The studies presented in this thesis were mainly conducted at the Department of Dermatology and Allergy, Copenhagen University Hospital, Herlev-Gentofte Hospital, Hellerup. The experiments for Manuscripts I, II, and III were based on a random market sample of earrings with different analytical foci, regarding metal release and validation of screening tests for the same. Manuscript IV consists of a clinical provocation trial of recruited participants. In addition to the clinical results, skin biopsies were taken for immunological analysis, which was performed at The LEO Foundation Skin Immunology Research Center, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

1. Market sampling

A total of 304 earrings were randomly sampled from the Danish market from March to May 2020. The majority of earrings were bought from physical visits to stores in the Copenhagen Capital Region, while some were bought from online retailers situated in Denmark. While it is difficult to match consumer buyer trends, we sought to perform a diverse random sampling to avoid introducing a sampling bias. The types of stores were diverse, ranging from smaller ethnic-oriented shops to larger commercial chains. The stores were 15 fashion stores (n = 86), 7 accessory stores (n = 40), 4 variety stores (n = 44), 2 supermarkets (n = 52), 2 beauty retailers (n= 8), 1 jeweler (n = 5), and 4 online accessory retailers (n = 69). All stores, including the online retailers, were situated in Denmark. The price range was 2.5 to 50 EUR per earring, with a mean price of 12.9 EUR. Only earrings with a perforating metallic part were included. We did not include very expensive earrings (>50 EUR) due to the inherent cost of the study and because these earrings are often marketed as being of a single pure metal, i.e., silver or gold. Earrings explicitly noted and marketed as “nickel-free” were also excluded.

For analysis, the earrings were divided into the following components categories: post, lock, decorative part, and dangle charm (Figure 3). The post and the lock component have direct contact with the pierced skin canal when worn, the decorative part has direct prolonged skin contact, and the dangle charm did not have prolonged skin contact. If necessary for the analysis, the earring was dismantled into components prior to analysis.

Figure 3. Components of an earring.

Due to the inherent differences in the many types of earrings (Figure 4), many earrings lacked a lock and/or decorative part or parts were of non-metallic materials such as glass or plastic. Components were only tested if they visibly consisted of metals. This resulted in a distribution in metallic components of 304 posts, 37 locks, 18 decorative parts, and 33 dangle charms subjected to analysis with XRF and spot tests.

Figure 4. Presentation of earrings sampled from the Danish market, showing a vast difference in materials and components among the different types of earrings.

The majority of earrings were bought in identical pairs, in which case only one part was used for analysis, as they were assumed identical. This sampling of 304 earrings was the basis for the analysis in Manuscripts I, II, and III.

2. X-ray fluorescent spectrometry

Firstly, all metallic components were subjected to X-ray fluorescent (XRF) spectrometry analysis with a handheld XRF spectrometer (X-MET8000 Series, Uedem, Germany). As the components are very small, earring components were dismantled with a side cutter if necessary. The XRF device is fitted with a camera that allows for exact location measurements. Measurements were done with 10 seconds exposure time on the factory default settings for alloy measurements as recommended by the manufacturer. To increase accuracy, measurements were done in triplets and the mean value is presented as the results. Results are given in weight percentage of each measured constituting element.

3. Spot testing for nickel, chromium, and cobalt

The DMG spot test solutions were 1% DMG in ethanol and 10% ammonium hydroxide in water and were prepared by the hospital pharmacy in the Capital Region of Copenhagen. While our experimental solutions were separate, the commercially available DMG spot test is usually sold as one premixed bottle. During testing, the samples were handled as little as possible in order not to disrupt the surface layer and unintentionally wipe off released nickel ions. The samples were only cleaned by gently wiping them with a cotton cloth, if they contained visible debris, which could affect a potential test discoloration.

The DMG test was performed by adding two drops of each solution to a cotton swab and then rubbing it against the test surface area for 20 seconds. The result can be read immediately after testing. A pink-red discoloration indicates a positive result and a nickel release above the estimated detection limit of 0.05 $\mu g/cm^2$.[63,108] No change in color indicates a negative result and no detectable nickel release within the sensitivity of the test. A discoloration other than a reddish hue indicates a doubtful result, and no conclusion can be made. In our testing, a doubtful reaction was retested, and if the test remained doubtful, it was registered as negative.

Based on a comparative analysis of results from the XRF and DMG test, as described in Manuscript I, the XRF results were used as a guide for which samples to test with DPC and Nitroso-R for chromium and cobalt release. If a sample contained chromium (n=166), all components from the sample were tested with the DPC spot test. As cobalt was found as a trace element in the majority of samples, only samples with >0.1% were tested with the Nitroso-R spot test (N=79). Additionally, a random selection of samples with a cobalt content of 0.002-0.1% was tested (n=20).

Several days after the DMG testing, the components were tested with the spot test for chromium, DPC, and then with the spot test for cobalt, nitroso-R. If a component was positive in one spot test, the sample was washed with de-ionized water, after the test, to avoid cross-contamination. At least 1 day passed between the two tests, for the same component, to allow the surface to re-stabilize.

Both the DPC and Nitroso-R spot tests were prepared according to previously published methods,[133,144,145] at the Department of Dermatology and Allergy, Herlev-Gentofte Hospital, and solution preparation is described in further detail in Manuscript II.

For both DPC and Nitroso-R, a cotton swab was soaked in the test solution and rubbed on the tested component for 30 seconds. DPC test results were read after 2 minutes to allow for the reaction to occur and produce a purple/red coloration if positive for Cr(VI). Nitroso-R can be read immediately and produces a yellow/red coloration if positive. For both tests, no change in color was regarded as negative. Similar to the DMG testing, a discoloration other than the positive color indication is regarded as doubtful, and the component was retested. If the result remained doubtful, the test was regarded as negative.

All testing was done according to previously published studies.[108,114,133,135,148,153]

4. EN 1811

Earrings with a positive DMG spot test or elevated nickel content, as measured by XRF, were selected for the EN 1811 analysis for nickel release in artificial sweat. However, due to a limited number of test samples (n = 100), we had to exclude 36 earrings with a low nickel content in the post.

The EN 1811 measures nickel per definition and we added measurements of chromium and cobalt to this analysis. The analysis was performed at ILAC, UKAS, and CPSC accredited institution Eurofins | BLC Leather Technology Centre Ltd. (Kings Park Road, Moulton Park, Northampton, NN3 6JD, UK) according to BS EN 1811:2011 +A1: 2015.[109] The released metal was measured by ICP-MS and results were divided against the surface area of the component and presented as $\mu g/cm^2$/week.

5. Follow-up analyses with a focus on copper

Many samples were found to contain copper in the XRF analysis. Similar to nickel, copper ions commonly occur in the 2+ oxidation state and are also able to chelate with the DMG molecule. However, the DMG-copper complex is brown-yellow as opposed to the DMG-nickel complex, which is bright red. Thus, there was the possibility of a copper interaction that might interfere with the results when DMG spot testing for nickel.

We had a subsample of 25 earrings analyzed by EN 1811 with an added measurement of copper release. The EN 1811 analysis was performed by Eurofins | BLC Leather Technology Centre Ltd as previously described. The subsample had measured copper content in the XRF analysis and had not previously been tested with EN 1811. Additionally, we prepared a dilution series of $NiCl_2$ and $CuSO_4$, to experimentally investigate a potential interference. The dilutions ranging from 0.1%-10% of either solute were prepared at the Department of Dermatology and Allergy, Herlev-Gentofte Hospital, and are described in further detail in Manuscript III. A total of 0.5 mL of each $NiCl_2$ concentration was mixed with 0.5 mL of each $CuSO_4$ concentration to create a matrix of 18 different solutions with

varying concentrations of each solute. From each solution, 10µL was added to an 8mm filter paper. To start the reaction, we added 5µL 10% DMG solution in ethanol to the filter paper. Lastly, an earring with considerable simultaneous copper and nickel release as measured by EN 1811 was retested with the DMG spot test, according to the previously described method.

6. Recruitment of the study population

Patients with a 2+ positive patch test for nickel tested at the Department of Dermatology and Allergy, Herlev-Gentofte Hospital from 2017 to 2020 were invited to participate. Contact information for this group was acquired through a database extraction of the nationwide clinical database for contact dermatitis, with permission from the Danish regions' clinical quality development program (RKKP). A total of 249 invitations were sent, and of the responders, 11 were included in the study. Control participants were recruited through advertisements on social media and the public websites http://forsøgsperson.dk and http://forskningnu.dk. Control participants were recruited simultaneously with included allergic participants and were sought to be age-matched as much as possible. Two of the included control participants had a positive patch test to nickel in the preliminary patch test (cf. 3.7 Clinical study design) and continued their participation in the allergic group, resulting in a total of 13 included allergic participants (12 female, median age 47.2) and 13 control participants (9 female, median age 30.0).

All participants had to be 18-70 years of age, and exclusion criteria were pregnancy or breastfeeding, systemic immunomodulatory treatment or topical treatment of test area, prolonged exposure to ultraviolet radiation such as sunbathing or solarium within the past 14 days prior to trial start. Additionally, the control participant could not have had a positive reaction to nickel in a patch test.

7. Clinical study design

The study was designed as a double-blinded clinical controlled trial based on the patch test method and the recent knowledge of persisting skin resident memory T cells (T_{RM}) after allergic dermatitis. It consisted of two patch test provocations with nickel sulfate on the exact same area with 3-4 weeks rest in between (Figure 5).

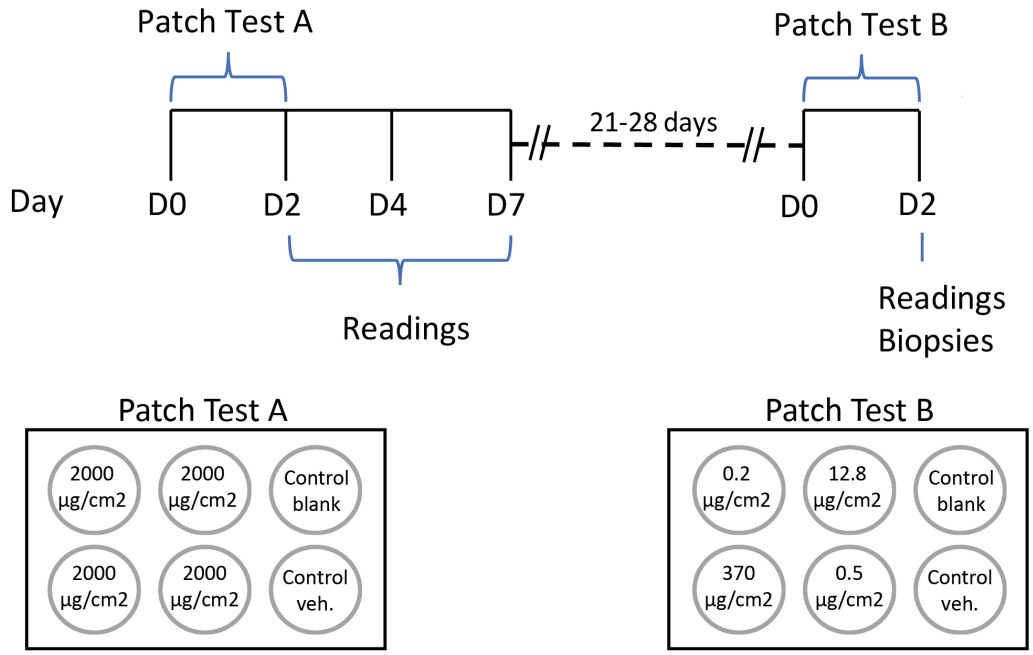

Figure 5. Schematics of the study method. Two patch tests were performed with 21-28 days rest between. Patch test A included the diagnostic dose of nickel sulfate in pet., while patch test B included an experimental dose range in aq. Both patch test included a blank control and a vehicle control.

A preliminary patch test with diagnostic doses of 5% nickel sulfate in pet. (patch test A) was placed on a suitable uniform area on the lower back of the participant at day 0 (D0) (Figure 6). The area was marked with a skin marker and photographed, and distances to the spine, scapula, hip bone, and potential birthmarks or scars were measured and noted. The patch was removed after two days, and potential allergic reactions were read and scored (D2). The skin reactions were read again on day 4 (D4) and day 7 (D7). Participants in the nickel allergy group had to show an allergic reaction, while the control group had to not react in order to be allowed to continue in the study. The skin area was left to heal for 21-28 days, during which time the participants were instructed to frequently redraw the skin marking and avoid the use of topical treatments in those areas. This preliminary patch test served to both induce T_{RM} in the exposed areas, which will enhance and quicken an allergy response upon repeated exposure, and to verify or refute allergy to nickel in the two participation groups.

After the skin area was healed, participants were subjected to a second patch test (patch test B; D0), with an experimental dose range of nickel sulfate in distilled water. The patch test was placed on the exact same location as the first, which was identified by a combination of the participant's own marking, photograph, and noted measurements as have been described. The patch test was removed after two days (D2), and potential allergic reactions were scored by MW and the assisting nurse. The reactions were scored on a 9-step experimental scale based on the ESCD guideline criteria[27] with added steps for weaker reactions.[21,70] Doses were color-coded to ensure a blinded scoring; however, the order of the doses in patch test chambers was not randomized.
After the areas were scored, a 4mm punch biopsy was taken from each tested area with a total of six per participant.

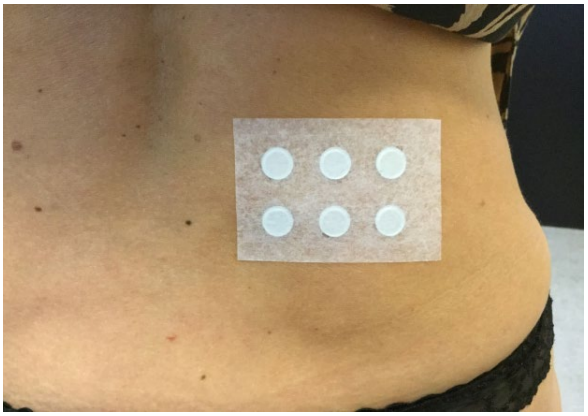

Figure 6. Location of the patch test area. The area was marked and distances to spine, hipbone, scapula, and birthmarks were measured to allow for a re-test of the exact same area.

The participants were split into two cohorts subjected to different transcriptomic analyses. Ten participants, five nickel-allergic and five healthy controls, had their biopsies analyzed with the multiplex transcriptomic analysis Nanostring nCounter. These biopsies were stored in RNA*later* for 24 h at 4°C and then at -20°C until use, as per the manufacturer's instructions. The biopsies from the remaining 16 participants were analyzed by qPCR and were immediately snap-frozen in liquid nitrogen in a dry cryotube and stored at -80°C until use.

8. Patch test

The patch test procedure was performed according to established guidelines for the diagnosis of contact allergies.[27] For each chamber either 20 mg of solution in petrolatum (pet.) or 15 µL of solution in distilled water (Aq.) was used in 8mm Finn chambers (Figure 5). In the preliminary patch test (patch test A) the diagnostic dose of 5% nickel sulfate in pet. was used, which corresponds to 2000 µg/cm^2. A total of 20 mg petrolatum was here used as vehicle control, while another control chamber was left empty (blank).

As the concentrations in the experimental dose range (patch test B) were much lower, we opted for distilled water as the solute, to allow for a more uniform mixture and thus exposure. We used 15 µL of distilled water as vehicle control, and another control was empty.

The experimental challenge doses were chosen after a study by Fischer et. al on the dose-response relationship of occluded patch-testing with nickel sulfate in nickel-allergic individuals.[6] In this meta-analysis, it was estimated that 370 µg Ni/cm^2 could elicit dermatitis in 95% of nickel-allergic individuals and 12.8 µg Ni/cm^2 could elicit dermatitis in 50%. The aim was to include doses close to the elicitation threshold of nickel dermatitis. Additionally, we included 0.5 µg/cm^2 and 0.2 µg/cm^2 of nickel as these are the limiting values as defined in the European nickel regulation.[91]
The solutions were made in-house with nickel(II) sulfate hexahydrate ≥98% (CAS no. 10101-97-0; Merck KGaA, Darmstadt, Germany).

9. Multiplex transcriptomic analysis

Nanostring nCounter analysis is a multiplex accurate and consistent, direct counting of molecules. Samples are run on a panel with up to 800 specific reporter probes which hybridize with a target molecule and are counted by the reporter's fluorescent signal. We used the method to investigate the mRNA expression of 594 immune-related genes as specified by Nanostring's "Human Immunology V2" panel (additional details in Manuscript IV). RNA isolation and nCounter analysis were performed by contract research laboratory BioXpedia A/S, Palle Juul-Jensens Blvd. 82, 8200 Aarhus, Denmark.

A total of 60 skin biopsies from 10 participants, stored in RNA*later,* were mailed on dry ice. Biopsies were homogenized and RNA was purified and further concentrated by speed-vac. As the RNA concentration was low in several samples, we opted for a low RNA-input panel. This panel amplifies target mRNA by cDNA conversion and works with as little as 1 ng RNA for analysis.

Results were normalized against positive controls and internal reference genes as were included in the panel. Seven samples were excluded for further analysis due to high normalization factors and low mRNA content. Normalization and quality control were mainly done by BioXpedia A/S through nSolver 4.0 (Nanostring, R 3.3.2) and the geNorm algorithm.[154] Additionally BioXpedia A/S performed an initial statistical analysis including differential expression analysis. These data were further analyzed and visualized using R 4.2.1 and GraphPad Prism 9.

10. qPCR

A total of 96 skin biopsies from 16 participants were homogenized using a Precylles 24 homogenizer (Bertin Instruments). Total RNA was then extracted and purified with the RNeasy mini kit (Qiagen) as per the manufacturer's instructions. The concentration and quality of the RNA were measured using a Nanodrop 2000 (Thermo Fisher Scientific). cDNA was synthesized from the purified RNA using oligo-dT (TAG Copenhagen, Frederiksberg, Denmark), RevertAid Reverse Transcriptase, and RiboLock RNase Inhibitor (Thermo Fisher Scientific). qPCR was performed using 5x HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne) and measured on a Lightcycler 480 (Roche Diagnostics) according to the manufacturer's instructions. The 28 PCR reporter primers used in this process were selected based on the results of nCounter transcriptomic analysis and subsequent differential expression analysis. High counts of transcriptomic reads, significant upregulation in several comparison groups, and relevance to the literature on nickel dermatitis were decisive factors in their selection. Results were normalized against internal reference gene EEF1A1 by the ΔΔCt method calculated in Microsoft Excel.

11. Ethical statement

Data on the nickel-allergic participants (Manuscript IV) from the National Database of Contact Allergy were withdrawn with approval from the Danish Clinical Quality Program – National Clinical Registers. The Danish Data Protection Agency approved methods for data handling and analysis (P-

2020-582). The clinical trial and all participant information were approved by the Danish Capital Regional ethics committee (H-1908328), and participants signed a written informed consent prior to inclusion. Additionally, the study has been maintained at clinicaltrials.gov (NCT04438330). The market studies (Manuscript I, II, III) did not require any ethical approvals.

4. MAIN RESULTS

The major conclusions of each study are summarized in this section. The thesis concludes with the original manuscripts.

1. Manuscript I

A total of 392 components from the sampled 304 earrings were analyzed with the XRF spectrometer and the DMG spot test. From the XRF analysis, nickel content was found in 39.8% (121/304) of sampled earrings. Nickel was mainly found in the post component (38.8%; 118/304). In the components containing nickel, the mean constitutional percentage was 6%-11.2%. Copper was the pre-dominant constituting metal found in >90% of earrings, though there was a large variability in the elemental constituents. Other constituting metals were iron, silver, chromium, zinc, titanium, gold, aluminum, zirconium, cadmium, and lead. Trace elements of other metals were also found.

The DMG test for nickel release revealed that 9.2% (28/304) of the sampled earrings had one or more positive components. A total of 6.6% (20/304) of the post components were DMG positive, 5.4% (2/37) of the locks, and 25.5% (13/51) of the decorative parts.

In the subsample of 100 earrings analyzed with EN 1811, a total of 86 released nickel. The posts were the most frequent component to release nickel, with 74 positive posts. Generally, the results were skewed towards the limit of detection (LOD) of 0.02 $\mu g/cm^2$/week, with a median nickel release of 0.22 $\mu g/cm^2$/week and some high outliers reaching a maximum release of 180 $\mu g/cm^2$/week. These results were compared to the upper or lower limits set by European regulation for each component, depending on whether the component had direct contact with a pierced skin canal or with the skin. This revealed 45 (14.8%) of sampled earrings had nickel release in levels exceeding the regulatory limits.

A sensitivity and specificity analysis were performed to validate the DMG spot test, with the results from the EN 1811 defining the true positive at different thresholds. This was done for each individual component tested with both tests (n = 179) regardless of component type. The sensitivity of the DMG spot test declines with a lower threshold for positive nickel release. At >0.5 $\mu g/cm^2$/week the DMG spot test had a sensitivity of 61.1%, and at ≥0.2 $\mu g/cm^2$/week the sensitivity was 45.2%. For all components with any level of nickel release, above LOD of EN 1811, the sensitivity was 29%. The specificity of the DMG spot test was calculated to 97.8%.

There was a moderate linear correlation between the nickel content, as measured by the XRF, and the nickel release, as measured by the EN 1811 (Spearman's correlation, r = 0.592, n = 179, $P < .001$). The XRF analysis was found to have a sensitivity of 85.1% if used as a predictor for nickel release, with a specificity of 36.9% among the 179 components tested with both methods.

2. Manuscript II

According to XRF analysis, chromium was present in 54.6% (166/304) of the sampled earrings, and cobalt was present in 72.0% (219/304). Both metals were common in the post component (53.3% and 72%). Chromium was a commonly constituent metal with median content values of 0.4%-11.8%, while cobalt mostly was found as a trace metal with median content of 0.02%-0.07%.

All 166 earrings containing chromium tested negative in every component for chromium release with the DPC spot test for Cr(VI). Among the 99 earrings chosen for Nitroso-R testing for cobalt release, only one lock component was found positive. It is noteworthy that only one lock in a pair of identical earrings tested positive.

The EN 1811 analysis, with added measurements of chromium and cobalt release, showed chromium release from 59 of the sampled earrings (59/100). The release was predominantly found from the post component (n = 43), which also had the highest release values (110 $\mu g/cm^2$/week). The median chromium release in all 83 positive components was found to be 0.06 $\mu g/cm^2$/week (0.04-0.11, 95% CI).

Cobalt was released from 29 (29/100) of sampled earrings. Similarly, most positive components were post components, though a single lock was a high outlier with a value of 1.5 $\mu g/cm^2$/week. The median cobalt release in all 39 components was found to be 0.06 $\mu g/cm^2$/week (0.04-0.13, 95% CI). There was a moderate correlation between the release of chromium and the release of cobalt, shown by a bivariate Spearman correlation analysis ($r = 0.48$, $P < .01$), though there was no significant correlation with released nickel.

3. Manuscript III

The DMG solution showed a red discoloration at 0.05% $NiCl_2$, which became more distinct at higher concentrations of $NiCl_2$. The discoloration was progressively discolored to brown-yellow by increasing concentrations of $CuSO_4$ for all tested concentrations of $NiCl_2$. At 0.05% $NiCl_2$, a small amount of added copper (0.05% $CuSO_4$) caused the red coloration to fade. When the concentration of $NiCl_2$ was increased to 0.1%, the red coloration was barely visible in the presence of 0.5% $CuSO_4$. At 0.5% $NiCl_2$, the red coloration remained visible but became weaker with higher concentrations of $CuSO_4$.

An earring component with simultaneous copper and nickel release (43 and 0.87 $\mu g/cm^2$/week) was identified from the EN 1811 analysis. The release of nickel is close to the regulatory limit value for nickel release and close to the detection limit of the DMG test. The earring was DMG spot tested and showed a brown discoloration without any red and was registered inconclusive.

4. Manuscript IV

All participants in the allergic group had an allergic (+) reaction to the preliminary patch test A (nickel sulfate 5%, 2000 µg/cm$_2$), confirming their nickel allergy (Figure 7), while there were no reactions among the control participants. In patch test B, out of the 13 nickel-allergic individuals tested, 12 (92%) had a clinical reaction of at least +? to the highest dose of 370 ug/cm^2 (corresponding to 0.925%), with 10 (77%) reacting with + or ++. Eight (62%) of the nickel-allergic individuals had a clinical reaction of +? to 12.8 µg/cm^2 (0.0320%), two (15%) had a +? reaction to 0.5 µg/cm2 (0.00125%), and one (8%) had a +? reaction to 0.2 µg/cm^2 (0.0005%). Notably, a dose that is 10,000 times lower than what is typically used for diagnostic testing was sufficient to trigger an allergic response. The allergic responses were generally of clinically mild degree, but they were clearly distinguishable from the control exposures.

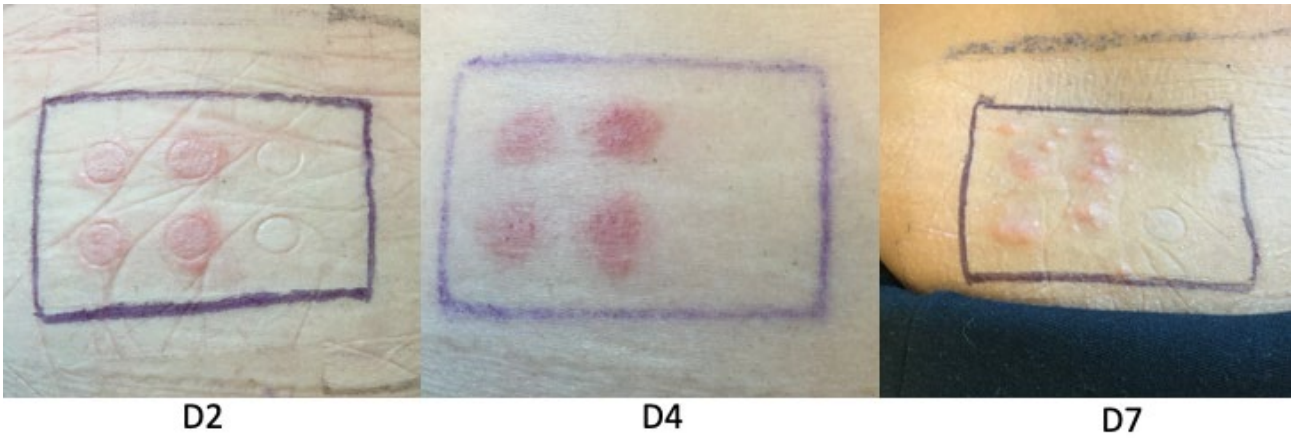

Figure 7. Nickel dermatitis after exposure to 5% nickel sulphate pet. in the preliminary patch test (patch test A). Pictures are from day 2, when the patch was removed, and at day 4 and day 7 from different participants.

Biopsies were collected from each exposure site, and immune-related transcripts were analyzed by Nanostring nCounter and qPCR to examine the possibility of a subclinical immune response to nickel exposures.

In the nickel-allergic individuals exposed to low doses of nickel, significantly differentially expressed genes (DEGs) were identified at skin areas exposure of 370 µg/cm^2 of nickel, 12.8 µg/cm^2 of nickel, and 0.2 µg/cm^2 of nickel. Interestingly, an activation of the immune response was seen to 0.2 µg/cm^2, despite none of these participants having a clinical reaction to this exposure. of response was consistent across all areas of skin that were re-exposed to nickel, with a total of 81 shared DEGs observed across all exposures. Notably, the response was particularly pronounced at a nickel concentration of 12.8 µg/cm^2. Furthermore, there was considerable overlap in the significant DEGs between different exposure doses, suggesting that the immune phenotype response is consistent, regardless of the clinical presentation, and is independent of the specific exposure level. These differential expression changes were found to be mainly driven by an upregulation of cytokines and chemokines. Few significant DEGs were found in skin upon re-exposure to nickel in the exposure groups of healthy participants. Only one DEG was common for all exposures, Ly96 (also called MD2) which is recognized as essential in nickel allergy.

5. CONSIDERATIONS OF METHODOLOGY

This section of the thesis will discuss the potential weaknesses and biases in our methods and their implications for the validity and reliability of our findings. Aspects are discussed concerning the market studies (Manuscript I, II, III) and the clinical trial (Manuscript IV).

1. Market studies

Random market sampling

The data for the market studies (Manuscript I and II) were obtained from a random sampling of available earrings on the Danish market. As mentioned, it is difficult to match consumer buyers' trends. Thus, the sampling and following results are descriptive of the Danish market and might not be directly translatable to what is worn by consumers. To avoid, or minimize, sampling bias, we adopted a systematic approach where up to 20% of the available offering of unique earrings from the stores was bought up to a maximum of 20. For stores with a smaller offering, of less than 10 unique earrings, half their selection was bought. The purpose was to maintain representation from smaller stores and limit the influence of a single larger store while attaining a diverse market sampling. There was no preference in types of stores or earrings and the intention was to choose as randomly as possible.

We had considered obtaining information on the frequency of earring sales from individual stores and where the earrings were produced. With data on sales, it could be possible to weigh the results of release based on an estimation of consumer trends. Data on origin could reveal problematic or positive import routes. Getting this information was ultimately dropped because it would be either too challenging or unreliable.

The selected stores were mainly situated in the Copenhagen Capital area due to logistic reasons and would it have been favorable to have obtained a more geographically diverse sampling.
We had to exclude flea markets, street markets, and street stands, because they could not provide the needed receipts for financial accounting with funding partners. These stands would, however, be very interesting to have examined, as many earrings and piercing are sold here. Perhaps the availablity of such stands stems from unregulated import or the existence of antiques that were created before nickel legislation had been enforced.

Subsampling and EN 1811

The subsample analyzed with EN 1811 was chosen based on whether nickel was a constituent. There was an analytical focus on nickel release, but we had added measurement of chromium on cobalt. The subsample for EN 1811 analysis was limited to 100 samples and was thus not fully representative of the full sample size. The samples were selected according to whether they had nickel content as measured by the XRF analysis or a positive DMG spot test. Due to the limited size of the subsampling, 36 samples with measured nickel content, albeit low, were not included. While it

would have been preferential to analyze the full market sample, these selection criteria were chosen to cherry-pick the samples which may have nickel release. As 36 samples were not included the results might be slightly conservative.

In the validation of the DMG test, the results were compared to EN 1811 results. This was done on an individual component level and was not dependent on the size of the market sample or the subsampling. Despite the subsampling being cherry-picked for potentially higher nickel release, it would have little to no influence to include the full sample size. Under the assumption that components with no measured nickel content would not have measurable nickel release, testing the remaining samples would only result in a higher number of true negatives or false positives. However, as samples with a positive DMG test were included, there would be no increase in false positives and a higher number of true negatives does not change the specificity calculations.

As the selection criteria focused on nickel, it does introduce a selection bias when analyzing the results for chromium and cobalt. As the subsample is not random, it is not representative of the full sampling. The results presented are likely conservative as some samples with high chromium and cobalt content were excluded in favor of those with high nickel content. We aimed to show that chromium and cobalt are released from earrings at clinically relevant levels, but these results are too conservative to scale to represent the full Danish market. For the validation of the spot test, it is negligible as this was done on components tested only with the two tests and as the only positive sample was included in the subsampling.

Additionally, we did not look further into the types of alloys. The elemental analysis with XRF revealed a very diverse metallic composition among the earrings and components, and it would be quite difficult to correctly identify and report the many different possible alloys. The diverse composition could likely be due to the poor quality of metal used in the earrings. While it would be an interesting addition to stratify the results based on the alloy type, the XRF results were not consistent enough to make this possible.

As the EN 1811 analysis was done by a third party, who did their own dismantling into components, there could be a slight discrepancy in the categorization of earring components. In any case dismantling was done as described in the in standard EN 1811.

2. Experimental study

Participants and criteria

The recruitment of the allergic group from the same tertiary clinic may have introduced selection bias, as these participants may not be representative of the general population. Additionally, the presence of comorbidities such as alopecia and history of atopic dermatitis in some participants may impact the results.

The healthy control group was age- and sex-matched to the allergic group, but there was a greater interest among younger males to participate. As a result, the control group ended up with a slightly higher proportion of males and a slightly lower median age than the allergic group. This demographic difference may have potentially impacted the results due to age-related differences in immune responses, although there were no statistically significant differences between the groups.

While the exclusion criteria were aimed at limiting other direct immune activations, other factors such as environmental exposures and lifestyle factors were not further elaborated upon. Originally, the study was scheduled for the winter months to reduce potential interference from UV rays, but due to the COVID-19 pandemic, it was postponed and conducted during the summer months. Participants were instructed to avoid direct sunlight on the area for the duration of the study, though this could not be fully controlled, and may have introduced a potential interference from UV rays, which may have affected the results.

Study design

The scale used to score the clinical reactions is based on the diagnostic guidelines established by the ESCD.[27] The scale has been extended to increase sensitivity and capture mild allergic reactions, and it has been successfully used in other publications.[21,66] While scores of 1-3 would be considered doubtful reactions in a diagnostic setting, the experimental setup is more controlled, and since nickel allergy was confirmed, the reactions were indeed allergic despite being mild. However, as the scale is numeric, it can be debated whether a score of 2 (weak erythema, no papules) represents a stronger allergic reaction than a score of 1 (few papules, no erythema). Additionally, the skin was skin at day 2, and it is possible that the clinical reaction would continue to develop over the next days as nickel ACD generally peak at day 4.

While doing their own marking during the rest period was thought to be difficult, most participants were extremely thorough, and with few exceptions their drawings perfectly matched our measurements and photos. For the few exceptions, the area was found using only photos and measurements.
Only a subset of participants (5 out of 13) was analyzed using the Nanostring platform, while the remaining samples were validated using qPCR. However, validating the Nanostring results in another cohort using a different platform posed a significant challenge. To enhance the validation process, it would have been preferable to split the biopsy samples and use both Nanostring and qPCR on the same samples. Additionally, the samples selected for Nanostring analysis were not based on clinical reactions, which could have been a relevant selection criterion. Furthermore, none of the participants in the qPCR analysis group exhibited strong clinical reactions (++) to the nickel exposure, and no participants demonstrated clinical reactions to the lower doses (0.2 and 0.5 $\mu g/cm^2$) in the samples utilized for Nanostring analysis.

Another potential limitation was the use of multiple exposure arms (four exposures per sample), which created numerous potential comparisons for the differential expression analysis between

groups. The study's many arms and relatively low sample size could have led to statistical power issues. P-values were reported unadjusted, and this may have increased the risk of a type 1 error. Furthermore, some samples had to be discarded from the Nanostring analysis due to low mRNA content and high normalization factor. These biopsies were stored in RNAlater as recommended by BioXpedia A/S. However, it has been discussed in our laboratory whether RNAlater can penetrate and stabilize tissue in a 4mm biopsy sufficiently. This may have contributed to some samples having a poor mRNA content.

The samples were taken after 48 hours to allow for immune activation to occur, which could be considered the early stage of a type IV allergic reaction. However, as this was a re-exposure study, it was assumed that the reaction would occur faster than usual. Further investigation of participants at later time points could have resulted in more reactive participants or a different immune phenotype. Though, a higher immune response was seen at 12.8 µg/cm^2 than 370 µg/cm^2, which may suggest an earlier stage of the immune response at 12.8 µg/cm^2, though further studies are needed on the kinetics of nickel ACD.

It is worth noting that the study results are based on transcriptomics, which may not always translate to biological activity at the protein level. Therefore, additional validation using protein-level analysis could provide a more complete picture of the biological effects of nickel exposure. Finally, the study utilized two control exposures, one blank and one vehicle control. The purpose was to subtract a potential vehicle response and isolate the nickel response. Preliminary analysis showed that the vehicle control was more consistent and thus the blank control was largely omitted.

Our study aimed to investigate the effects of low dose exposures on pre-exposed skin, where a memory response is expected. Although we did not directly perform any direct measures of the formation of local memory, our assumption was based on previous reports of increased clinical response to re-exposures[21,155] and the presence of T_{RM} after ACD.[18,19]

6. DISCUSSION

The current studies aimed to assess the safety of nickel-releasing piercing post assemblies on the market with respect to contact allergy and to investigate whether low doses of nickel could under certain conditions induce a subclinical immunological response before visible allergic contact dermatitis. In addition, we aimed to evaluate and validate the DMG spot test as a screening tool for detecting nickel release from metallic items.

1. Nickel exposure from earrings on the market

As the prevalence of nickel allergy remains high even among the adolescent generation, it is crucial to identify the critical and causative exposures. Most of our knowledge on nickel exposure comes from market studies of consumer and occupational items. Despite the regulation of nickel on the European market, studies show that the legislation is not entirely respected. In our study (Manuscript I), we found that at least 28.3% (n = 86) of the 304 tested earrings from the Danish market released nickel. Of these, 14.8% (n = 45), showed nickel release exceeding the regulatory limits. This corresponds to almost every seventh earring available on the Danish market from a random sampling. These findings are consistent with previous market studies, which have reported excessive nickel release from 10.0%-18.4% of tested earrings across European countries.[156–158] Notably, the portion of earrings with excessive nickel release seems to be steady, as shown by a similar study conducted in 2009 on earrings from the Danish market, which found 14.7% with excessive nickel release.[88] These results indicate the need for better enforcement of the current regulation is needed. It is unknown whether manufacturers, distributors, and retailers are unaware of the regulation or if they simply ignore it, more efforts should, nonetheless, be made by enforcers, either through informative measures or random screening procedures.

To conform the current regulation, an object must pass the EN 1811, which is the responsibility of the manufacturer or retailer. Previously, the cheap and rapid DMG spot test could be used to test for legislative conformity in Denmark, but it was replaced by the EN 1811 due to the latter's higher sensitivity.[103] However, the EN 1811 is time-consuming and expensive, which may be problematic for small manufacturers or retailers and lead to a reduction in testing. We found that the DMG spot test identified 28 earrings that released nickel, as opposed to 86 identified by the EN 1811. The sensitivity and specificity of the DMG spot test on objects that released >0.5 µg nickel/cm^2/week were 61.1% and 90.9%, respectively, which is close to previously reported values.[108] As earrings are recognized as a critical exposure, they are regulated according to a lower limit of 0.2 µg/cm^2/week. We found that the sensitivity of the DMG spot test decreased to 45.2% at this threshold, making the spot test less suitable for testing earrings for nickel release. It is important to emphasize that a negative DMG spot test result does not rule out the possibility of nickel release. Moreover, the DMG test is commonly utilized at home by individuals who have nickel allergy. It is critical that these individuals are informed that only a positive result can be trusted because a negative result cannot disregard potential nickel release. However, despite its mediocre sensitivity, the DMG spot test may have great potential due to its high specificity and thus low rate of false positive results. Comparatively, the EN

1811 is circumstantial to use in screening procedures. While screening with the DMG spot test will not identify all items that have excessive nickel release, a potential increase in testing might allow for more items to be identified and taken off the market. While we conclude that the DMG spot test would not be suitable for testing earrings for excessive nickel release, it would likely identify the most critical items with high nickel release, as we also report a tendency of dose-dependency in DMG spot testing (further elaborated in Manuscript III).

A potentially beneficial amendment to the legislation would be a preliminary screening with the DMG test prior to EN 1811. This would facilitate rapid and cheap market surveillance for enforcers, while limiting the unnecessary cost of additional EN 1811 testing while maintaining the high sensitivity of the EN 1811. This would essentially make it a requirement to pass two tests for legislative conformity, which may be problematic.

Additionally, we found that the XRF has some predictive value with a sensitivity of 85.1% for identifying items with nickel release, although with a low specificity of 36.9%. This means that many items contain nickel but do not release it although an item must likely contain measurable nickel to have nickel release.

While we did find that the five earrings bought from a recognized jeweler chain store consisted primarily of silver as was advertised, some had a trace amount of chromium and/or cobalt (<1%). These were not tested by the artificial sweat test, as the tested subsample was selected based on nickel content, but they were, however, negative in the DPC and Nitroso-R spot tests. It is generally assumed that 'pure' metals or metallic products from more costly or luxurious brands are not the source of allergic exposure, but no studies have been conducted to support these assumptions as these studies are inherently quite costly.

2. False negatives of the DMG spot test

While the DMG spot test is widely used in market studies,[118,157–160] it has been criticized for its mediocre sensitivity.[161,162] Being a colorimetric test, the result of the DMG spot test is indicated by a change in color. A bright pink/red denotes a positive result, while any other change in color denotes a doubtful result, and no change in color denotes a negative result. The doubtful result is uncertain as a potential formation of red coloration may not be distinguished. Often a doubtful result is retested, and if it remains doubtful, it is registered as negative. In Manuscript III we sought to investigate the doubtful reactions and potential false negatives of the DMG spot test. We found that copper ions can effectively mask the positive results of the DMG spot test, a possible explanation for the mediocre sensitivity of the test. This is due to the brown discoloration and compete binding to available DMG molecules caused by copper. We found that the DMG test is dose-dependent, with more intense red coloration at higher nickel levels. The masking effect of copper was more prominent at higher copper levels and lower nickel levels. At the detection limit of the DMG spot test of 0.5 nickel μg/cm^2, a slight addition of copper (0.05% CuSO4) masked the positive result, making the test inconclusive.

While this was investigated in an experimental setup, we found a doubtful (brown discoloration) DMG spot test on an earring with nickel release of 0.87 µg/cm^2/week and copper release of 43 µg/cm^2/week. Other metals such as palladium or cobalt may also be able to mask the DMG spot test result, as these also make 2+ charged ions and are capable of chelating with the DMG molecule. We emphasize that items with a doubtful test result should be registered as inconclusive to avoid false-negative results.

These findings may be used to optimize the use and sensitivity of the DMG spot test for nickel release. Specifically, given the significant levels of copper release observed in our tested earrings, a pre-treatment step to eliminate copper ions or the addition of a copper-affinity binding molecule to the DMG test solution may increase the sensitivity and effectiveness of the test. The newly updated technical report on the use of the DMG spot test as a screening tool for nickel release[163] has added an optional post-hoc test with dithiooxamide to confirm nickel release and suggests that doubtful results be tested with EN 1811.

The DMG test has also been adapted to test for nickel deposition on the hands,[164] a method that has successfully been used to identify occupational nickel exposure from multiple exposure sources.[115] Our results indicate that caution should be taken in interpretation of negative test results in this procedure, as the deposition from multiple exposure, may likely include other metal ions, that may interfere with the DMG nickel chelation.

3. Chromium and cobalt release from earrings

Chromium and cobalt are other important metal allergens. We found that both chromium and cobalt were widely present in earrings on the Danish market (Manuscript II) and were often released, with a median release of 0.06 µg chromium/cm^2/week and 0.06 µg cobalt/cm^2/week. These levels are low but may potentially act as a co-factor in driving clinical symptoms. Few studies have assessed the release of chromium or cobalt from earrings and piercing jewelry. Our cobalt release findings were slightly higher than those in a similar German survey.[134] Despite release levels being relatively low, they are within clinical relevance and may be sufficient to elicit allergic dermatitis on a case basis.

We found that all samples were negative for chromium release in the DPC test, while only one sample (0.3%) tested positive for cobalt in the Nitroso-R test. The DPC spot test is only able to detect Cr(VI). Although we were unable to differentiate between the oxidation states in the artificial sweat test, it is likely that the measured levels of chromium were primarily Cr(III), which would not be detectable by the DPC spot test. The DPC test has been successfully used on leather,[133] while another study found all 848 jewelry items tested negative for chromium release.[148]

We found that the lock component that tested positive for Nitroso-R released 1.5 µg cobalt/cm^2/week. This suggests that the Nitroso-R spot test is useful in screening objects for cobalt release that is of certain clinical relevance. A similar study conducted in 2010 found that 1.8% of 354 jewelry and hair clasps released cobalt, as determined by the Nitroso-R spot test.[135]

4. Nickel re-exposure and subclinical activation

For the nickel regulation to be effective, the regulatory limits must be sufficiently protective. As mentioned, the limiting values have been extrapolated from clinical results from patch testing on naive skin.[86,103] However, this method does not take into consideration the new knowledge regarding the formation of local memory by induction of T_{RM} after ACD. Moreover, the development of more sensitive methods for detecting immune response should be utilized to ensure that the regulations are as effective as possible.

Although the regulatory limiting values of 0.5 and 0.2 μg/cm^2/week are generally considered safe for nickel allergic individuals, our findings show that re-exposure to these doses may elicit nickel ACD. Additionally, we observed a significant subclinical immune activation at the transcriptomic level, which brings into question the safety of exposure to these levels for nickel-allergic individuals.

Few studies have investigated low-dose nickel exposures. In 1999 Nielsen et al. found a significant increase in local vesicle formation and blood flow after repeated daily exposure to 0.01% nickel chloride (comparable to 0.4 μg/cm^2), suggesting frequent low-dose environmental exposures to nickel could contribute to and maintain dermatitis and/or hand eczema in patients with nickel allergy.[165] Fischer et al. conducted a study in 2007 to determine the lowest threshold for eliciting a reaction in nickel-allergic individuals.[70] They found that the lowest threshold for patch testing was 0.5 μg/cm^2, while in a repeated open application test (ROAT), a dose of 0.035 μg/cm^2 twice daily over a week elicited a reaction in 4 out of 18 participants. It is notable that the threshold for repeated exposures was found to be lower, which might be explained by other studies which have shown that nickel accumulates in the skin during repeated exposures,[94] which likely enhances the risk of elicited dermatitis.[75] The induction of local memory is likely to attribute to this enhanced response, which may be more pronounced at lower levels. Hence it might not be optimal to perform these exposure studies on naive skin.

We found that 92% (12/13) of the participants had ACD to 370 μg/cm^2 and 62% (8/13) to 12.8 μg/cm^2 of nickel exposure. These values were chosen from a meta-analysis, where it was estimated that 95% of nickel-allergic individuals would have ACD to 370 μg/cm^2 and 50% to 12.8 μg/cm^2 of nickel exposure. Our result is slightly higher for the 12.8 μg/cm^2 exposure, which may be explained by the induction of local memory. Notably, it was also estimated that 1% would show ACD to a nickel exposure of 0.067 μg/cm^2, where we comparably report 15% having ACD to re-exposure of 0.05 μg/cm^2, potentially highlighting the importance of local memory in low dose-exposure.

Additionally, we found a subclinical immune activation. Exposure to 0.2 μg/cm^2 without manifestation of clinical ACD caused an immune activation, which was notably similar to the immune response of the higher dose exposures, which caused clinical ACD. Our findings suggest that the elicitation phase of ACD may involve a continuous immune response, with the accumulation of inflammatory signals eventually leading to tissue damage and clinical manifestation of ACD rather than a fixed threshold of exposure. This is a noteworthy observation, as it suggests that repeated or

prolonged exposure to low doses of nickel could lead to an accumulation of inflammatory signals and subsequent development of ACD, increasing the risk of low-dose exposures.

Pruritus, is also symptom of a contact allergic reaction. It is, however, often overlooked as contact allergies are mainly diagnosed by their visual clinical appearance. As a subjective symptom, pruritus is difficult to measure and is often disregarded in experimental studies. In the context of subclinical inflammation, it is possible that symptoms of pruritus could appear before, and independently of, elicited dermatitis. The presence of pruritus in individuals with contact allergies could indicate an effect of the subclinical immune activation, even in the absence of visible dermatitis. There have been anecdotal reports of individuals experiencing itching from wearing cheap jewelry and subsequently avoiding these products. Though no studies exist to support that this could be an allergic reaction, the high prevalence of nickel allergy in the general population and the common use of nickel in cheap jewelry could potentially explain many "unsafe" exposures.

We did not observe any clinical reactions to the nickel exposure in the healthy group, and a negligible immune response was observed at the transcriptomic level. Interestingly, the healthy participant group showed a significant upregulation of Ly96 for all exposures. Ly96 is involved in the activation of TLR4, which is essential in initiating the sensitization phase of nickel ions.[25,26,166] Despite the healthy controls not showing an allergic response to nickel, it is notable that all exposure groups showed a significant upregulation of a crucial component in the nickel sensitization phase.

Several studies have sought to identify biomarkers or unique immune phenotypes to distinguish different types of contact dermatitis. In nickel allergy, studies have reported IL5 and IL8, among others, though no consensus exist and further studies are needed.[41,167,168] We did not study the immune response of diagnostic exposures level of nickel, so we do not propose potential biomarkers for nickel dermatitis. Nonetheless, we show that a potential diagnostic use of biomarker for nickel dermatitis would likely also be an effective at a subclinical level and be able to discern nickel-allergic exposures at very low levels.

5. Nickel regulation and body piercings

The lower regulatory value of 0.2 μg/cm^2, was established to extend the protection of the regulation. The decision to lower the limit in piercing post assemblies was recommended by the European Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) based on a European Committee contracted investigation on "*the risk of sensitization of humans to nickel by piercing post assemblies.*"[78,130] While the report states there is insufficient information to make a complete risk assessment, it demonstrates evidence for an approximate doubling of the release rate of nickel ions from stainless steel to blood plasma compared to artificial sweat as used in the EN 1811. During the epithelialization of a body piercing, nickel ions are mainly released to blood plasma. Thus, the release limit was recommended to be halved to 0.25 μg/cm^2/week. Due to the limit of quantification in standard laboratories using EN 1811 and to achieve a high level of consumer protection, the final recommended migration limit was set to 0.2 μg/cm^2/week for all piercing post assemblies, which

was acknowledged by the CSTEE and amended in the EU nickel directive in 2005.[78,91,130] The regulatory migration limit for piercing post assemblies is hence a calculated approximation of the safety of nickel-releasing piercings. To date, there are limited clinical studies on exposure to nickel-releasing body piercings and symptoms of nickel allergy, and the results are inconclusive. One study showed that a grade of high-quality stainless steel commonly used in piercing post assemblies, with nickel release within the limits of the regulation, did not elicit ACD in any of the 25 nickel-allergic individuals tested, despite indications of nickel having been released and deposited onto the skin. However, it is noted that two of the 25 subjects developed erythema and itching after insertion of the piercing.[85] In contrast, two studies tested different stainless steel piercing alloys, in which 3/3 of nickel-allergic individuals[84] and 4/10 individuals[83] elicited ACD to piercing post assemblies with nickel release within the current regulatory limits.

Additionally, we found in Manuscript I that most nickel release was from the post component, which is in direct contact with the traumatized skin canal and may allow for direct release to the blood plasma during epithelization after piercing. Moreover, several earrings with measured nickel release had nickel release from several components with apparent skin contact. As the earlobe is relatively small, this aggregated exposure could be significant and increase the risk of nickel allergy. It is worth noting, however, that a larger area of exposed skin may elicit a slightly stronger response compared to a smaller area.[66]

Although there are general recommendations for piercers to use “hypo”-allergenic piercings such as glass, titanium, or plastic after the initial piercing perforation,[131] there are no legislative specifications for this except for the nickel regulation. We show in Manuscript IV that doses within the regulatory limits may in fact elicit nickel ACD in some sensitized individuals and produces a significant immune response also in the absence of clinical reaction. Hence, it is highly likely that a direct nickel release to the blood plasma during epithelization would pose a significantly increased risk – as well as likely also a risk for sensitization.

7. CONCLUSION

The purpose of the current studies was to evaluate the safety of nickel-releasing piercing post assemblies available on the market with regard to contact allergy and to determine whether low doses of nickel could trigger a subclinical immunological response before the onset of visible allergic contact dermatitis. Additionally, the aim was to validate the DMG spot test as a screening tool for detecting nickel release from metallic items. The findings have important implications for the regulation of nickel and other metals in consumer items and can be used in efforts to reduce the risk of contact dermatitis and allergic reactions among susceptible individuals.

Nickel release from earrings in the market still poses a significant problem, with at least 28.3% of tested earrings from the Danish market releasing nickel. Among these, 14.8% surpassed regulatory limits, suggesting the need for better enforcement of the current regulations. While EN 1811 is currently used to assess legislative conformity, it is time-consuming and expensive, which can pose a challenge for small manufacturers or retailers and lead to a reduction in testing. The DMG spot test could help legislative testing as a potential alternative to EN 1811 due to its high specificity and low false positive rate. Although the DMG spot test is less sensitive than EN 1811, it could still be valuable in identifying critical items with high nickel release. To facilitate rapid and affordable market surveillance for enforcers while limiting unnecessary costs, using the DMG spot test as a preliminary screening tool before EN 1811 testing could be considered as a potential amendment to the legislation.

The current regulatory limits for nickel exposure may not provide sufficient protection for individuals with nickel allergy. Exposure to the regulatory limits of nickel release (0.5 and 0.2 $\mu g/cm^2$/week) can trigger a clinical reaction in some individuals and cause significant immune activation at the transcriptomic level. We highlight the importance of local memory in increasing the risk of elicited dermatitis, which may be more prominent at lower doses. The identification of subclinical immune activation suggests that repeated or prolonged exposure to low doses of nickel could result in the accumulation of inflammatory signals and the subsequent development of allergic contact dermatitis.

In summary, we emphasize the need for better regulation and enforcement of nickel release in consumer items to increase protection against nickel allergy. The DMG spot test has the potential to serve as a screening tool, but further research is necessary to fully assess its usefulness and limitations. Furthermore, the current regulatory limits may not be sufficient when a previous induction of local memory is considered.

8. FUTURE RESEARCH PERSPECTIVES

Comprehensive and regular market surveillance is necessary to ensure that consumer products comply with regulations. Manufacturers and retailers should be made aware of the nickel regulation, and informative measures should be taken to increase compliance. Future studies could explore the feasibility and effectiveness of using the DMG spot test as a preliminary screening tool before EN 1811 testing to identify high-risk products and minimize unnecessary testing costs. Additionally, the DMG spot test may be optimized to increase sensitivity by eliminating potentially interferent binding of other metal ions.

Further studies are needed to identify the causative exposure of nickel allergy. Piercing has been found to be a major risk factor, and studies should elaborate on the specific risk of this exposure route. It is unknown whether the risk factor of the piercing exposure is only during the reepithelization or if the skin barrier remains compromised and allows further penetration than prior to perforation. It would be interesting to further investigate the actual skin barrier properties of a healed piercing.

Studies are needed to further elucidate the immunological aspect of the sensitization phase of nickel allergy and to fully understand the immune response of a nickel exposure. This may serve to identify potential biomarkers and allow for early diagnostics of nickel allergy or nickel exposure, even at low doses. Further studies should validate the finding of an immune response independently from clinical presentation and elaborate on the kinetics of low-dose exposures. Additionally, this effect may not be unique to nickel and could be studied in other contact allergens. Furthermore, as we found an upregulation of an essentially component in innate immune response to nickel in healthy participants, further studies are needed to elucidate the immunological aspect of the sensitization phase of nickel allergy, as the regulatory limit may not even be protective for healthy individuals.

While the chemical properties of nickel make it widely used, it may be replaced in several common alloys while retaining the same functionality. The socioeconomic cost of nickel contact allergy may justify complete replacement of nickel from some alloys in consumer products. Finally, many items still contain a substantial amount of nickel, chromium, and/or cobalt, despite not showing release. Further studies should investigate the protective effect of the metal alloy surface finish and how this may or may not diminish over time depending on the type of surface seal, as older items may be a significant overlooked source of exposure.

9. MANUSCRIPTS

1. Manuscript I: Nickel release from metallic earrings: A survey of the Danish market and validation of the nickel spot test

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ORIGINAL ARTICLE

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Nickel release from metallic earrings: A survey of the Danish market and validation of the nickel spot test

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Abstract

Background: Exposure to nickel releasing ear piercing jewellery may explain the persistently high prevalence of nickel allergy in Europe. While nickel release from earrings is regulated, field studies show that the regulation is not always respected. More knowledge is needed regarding the risk of piercing exposure including suitable screening methods.

Objective: To examine the proportion of earrings on the Danish market that release more nickel than allowed, and to validate the use of the dimethylglyoxime (DMG) test as a screening tool.

Methods: A total of 304 earrings were purchased and tested with the DMG test and X-ray fluorescence spectrometry. The level of nickel release was quantified in a selected subsample of 100 earrings by the European reference test EN 1811. The DMG spot test was validated against EN 1811 at different thresholds.

Results: Excessive nickel release according to the European regulation was found in 45 (14.8%) tested earrings. The sensitivity of the DMG test decreased with reduced levels of nickel release (sensitivity of 45.2% at ≥0.2 μg/cm^2/week vs 61.1% at >0.5 μg/cm^2/week).

Conclusion: Excessive nickel release is common in earrings on the Danish market. Because of low sensitivity, the DMG test has limited use in screening of earrings for research but may still be used clinically.

KEYWORDS
contact allergy, DMG, EN 1811, exposure analysis, jewellery, nickel, spot test, XRF

1 | INTRODUCTION

Nickel is the most prevalent cause of contact allergy worldwide despite regulatory efforts to reduce the incidence.[1-3] Around 14.5% of the European adult general population is sensitized to nickel.[4] Women are more commonly affected than men: 22.2% compared with 5.2%. This gender difference is associated with women's greater propensity to have ear and body piercings.[5-9]

Earrings and piercing jewellery are largely composed of metals. The exposure differs from that on intact skin, as the skin barrier initially is bypassed after the piercing procedure, and thus nickel and other metals may be released directly into the blood plasma.[10] Furthermore, after epithelialization of the pierced canal, the skin contact may be regarded as occluded, often during a prolonged time. This distinctive exposure has been recognized by the European Commission, which is why nickel release from piercing post assemblies is restricted to 0.2 μg/cm^2/week, lower than from items with contact with intact skin (0.5 μg/cm^2/week).[11-13] The lower limit only applies to the post assembly of the earring, meaning the parts inserted in and coming in direct contact with the pierced skin canal. The European standard

reference test EN 1811 yields a quantitative measurement of nickel released from an object under standardized conditions and is used to control for compliance with the regulatory limits.[14]

It has been discussed whether the continued high prevalence of nickel allergy in women is due to the regulation not being respected or whether the limits in the regulation are too high.[8,15-17] Several European studies of consumer items have shown nickel release exceeding the regulatory limits.[18-27] Only a few studies have focused on exposure from earrings and there is no recent survey of the Danish market. Most market surveys have used the dimethylglyoxime (DMG) spot test to rapidly screen for nickel release,[28] although its sensitivity is known to be modest. In some market studies, X-ray fluorescence (XRF) spectrometry has been used to rapidly determine the elemental composition of a metallic object.[29-31]

This study evaluated the elemental composition and nickel release from a random sample of earrings available on the Danish market. In addition, the DMG spot test was validated against EN 1811.

2 | METHODS

2.1 | Materials

From March to May 2020, a total of 304 earrings were bought from 35 different stores. Only unique earrings with a metal perforating post were bought. Earrings marked "nickel free" were not included. Of the 35 different stores, 15 were fashion stores (n = 86), 7 accessory stores (n = 40), 4 variety stores (n = 44), 2 supermarkets (n = 52), 2 beauty retailers (n = 8), 1 jeweller (n = 5), and 4 online accessory retailers (n = 69). All stores including the online retailers were situated in Denmark.

Approximately €3300 were spent, ranging from €2.5 to €50 (mean €12.9) per earring.

The posts from all 304 samples were analysed with the XRF spectrometer and the DMG spot test. Many samples lacked a lock and/or decorative part, or the part consisted of nonmetallic materials, usually plastic or glass. Hence, XRF analysis and DMG spot testing were only performed when the lock (n = 37), the decorative part with skin contact (n = 18), and/or decorative part without skin contact (dangle charm; n = 33) visibly consisted of metal.

2.2 | XRF spectroscopy

A handheld XRF device (X-MET8000 Series; Uedem, Germany) was used to measure the elemental composition of the earrings on up to three different components.

The XRF spectrometer determines the elemental composition by capturing the characteristic fluorescent radiation emitted when excited with high-energy X-ray beams.[29-31]

Each measurement was taken on the factory default settings for alloy measurements with 10 seconds' exposure time recommended by the manufacturer. Every sample measurement was done in triplets where the mean is presented as the result.

2.3 | DMG spot test

The DMG spot test was prepared by the hospital pharmacy in the Capital Region of Copenhagen. The test solutions were 1% DMG in ethanol and 10% ammonium hydroxide in water. DMG spot testing was done in the laboratory at the Department of Dermatology and Allergy, Gentofte Hospital.

Two drops of each solution were added to a cotton swab that was rubbed on the test area for 20 seconds. A positive result was indicated by a pink/red colouration of the swab, and a negative result was indicated by no change in colour of the swab. A doubtful test reaction, defined by a discolouration other than a reddish hue, was retested. If the reaction remained doubtful, the result was registered as negative.[18,28]

2.4 | EN 1811: Nickel release in artificial sweat

A subsample of 100 earrings was tested for metal release in artificial sweat, according to the current European standard reference test EN 1811:2011.[14] Earrings with a positive DMG spot test result and/or nickel content in the post (measured by XRF) were selected. Because of the limited numbers of test sample (n = 100), we excluded 36 samples with low nickel content (<10%) in the post.

Nickel release was measured by the ILAC-, UKAS-, and CPSC-accredited institution Eurofins | BLC Leather Technology Centre Ltd. (Northampton, UK) according to BS EN 1811:2011 +A1: 2015.[14] Prior to the test, samples were disassembled in up to five components and/or masked if necessary. Components were tested regardless of visible metallic constituents, with a more detailed subcategorization. The components were tested individually and later categorized into post, lock, decorative part, and dangle charm for data analysis. A total of 273 individual components were tested.

Each component was submerged in artificial sweat consisting of deionized water containing sodium chloride 0.5%, lactic acid 0.1%, and urea 0.1% with pH adjusted to 6.5. The test volume was about 1 mL of artificial sweat per cm^2 sample area. The samples were left at 30°C for 168 hours. The resulting nickel released in the artificial sweat was measured by inductively coupled plasma-mass spectrometry for nickel, chromium, and cobalt. Results from chromium and cobalt release will be reported separately. The lower limit of detection was 0.02 μg/mL. The measured release was compared with the surface area of the component, measured with digital callipers, and reported as $\mu g/cm^2$/week.

3 | RESULTS

3.1 | XRF spectrometry

Nickel was found in 39.8% (121/304) of the earrings; mainly in posts (n = 118). Nearly 19% of tested locks (7/37) contained nickel, whereas 51% of decorative parts contained nickel (26/51). Among the samples

containing nickel, the mean constitutional percentage of nickel was 10.9% in the post, 6% in the lock, and 11.2% in the decorative part.

An overview of the elemental composition of the earring parts is presented in Figure 1. Many components consisted of 100% of a single metal (eg, copper, silver, titanium, iron, nickel, or aluminium), resulting in a large elemental variance between the samples in all three categories.

The most common element was copper, found in 92% of posts (281/304), 100% of tested locks (37/37), and 98% of the tested decorative parts (50/51).

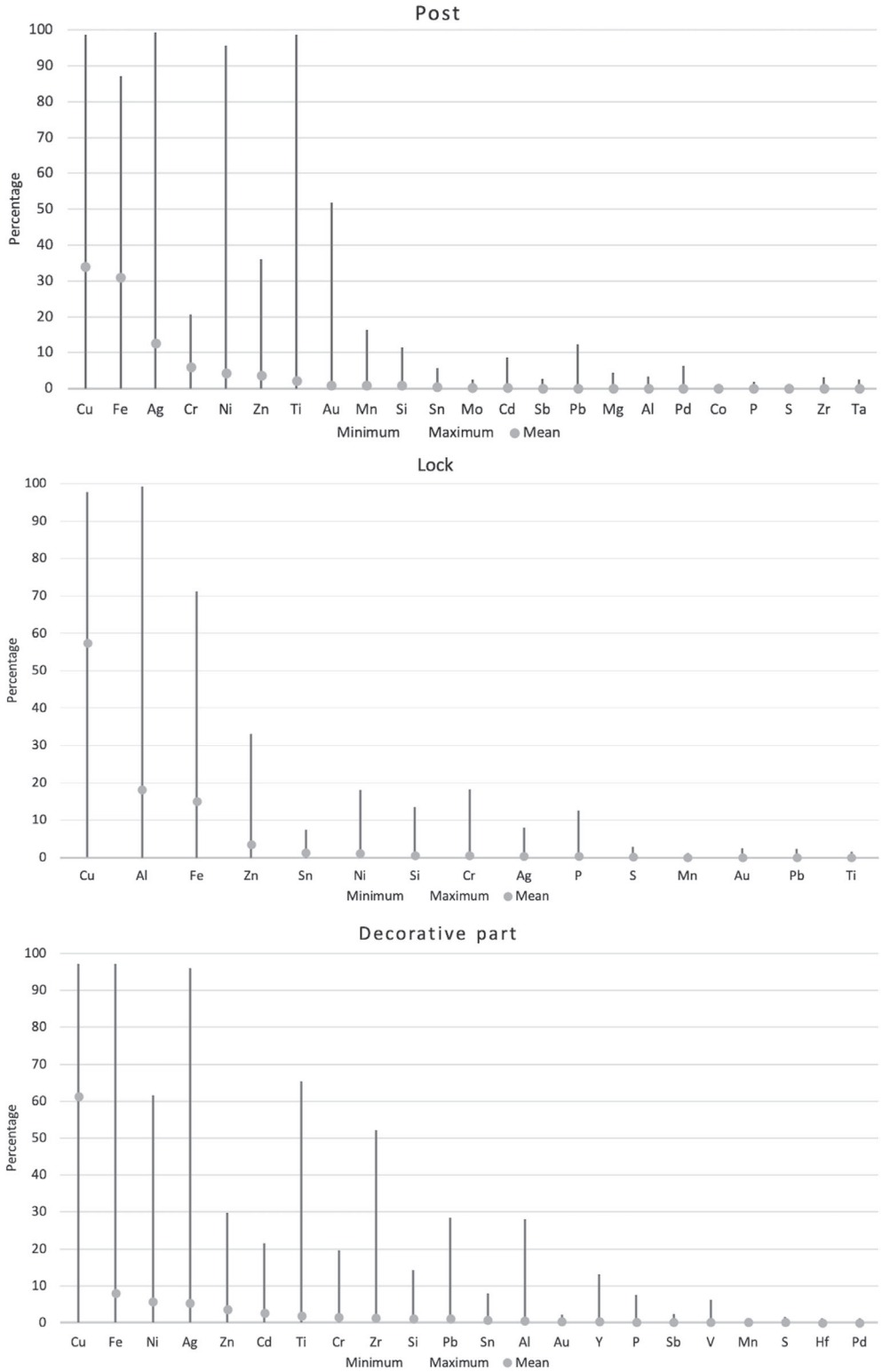

FIGURE 1 Mean, minimum, and maximum elemental compositions of post (n = 304), lock (n = 37), and decorative part (n = 51) of sampled earrings

TABLE 1 Earrings and earring components with nickel content (measured by XRF), positive DMG spot test result, and nickel release (measured by EN 1811)

	XRF Ni positive	Tested	DMG positive	Tested	EN 1811 positive	Tested (subsample)
Complete earring	**121 (39.8%)**	**304**	**28 (9.2%)**	**304**	**86 (86%)[a]**	**100**
Lock	7 (18.9%)	37	2 (5.4%)	37	37 (74%)[a]	50
Decorative part						
With skin contact	9 (50%)	18	7 (13.7%)	18	27 (42.9%)[a]	63
Dangle charm	17 (51.5%)	33	6 (11.8%)	33	33 (55%)[a]	60
Total components	151 (38.5%)	392	35 (8.9%)	392	171 (62.6%)[a]	273

Note: For dimethylglyoxime (DMG) and X-ray fluorescence (XRF) testing we broke the earring down to a total of 392 components and for EN 1811 the subsample of 100 was broken down to 273 components.
[a]Percentage of selected subsample; not representative for the market survey.

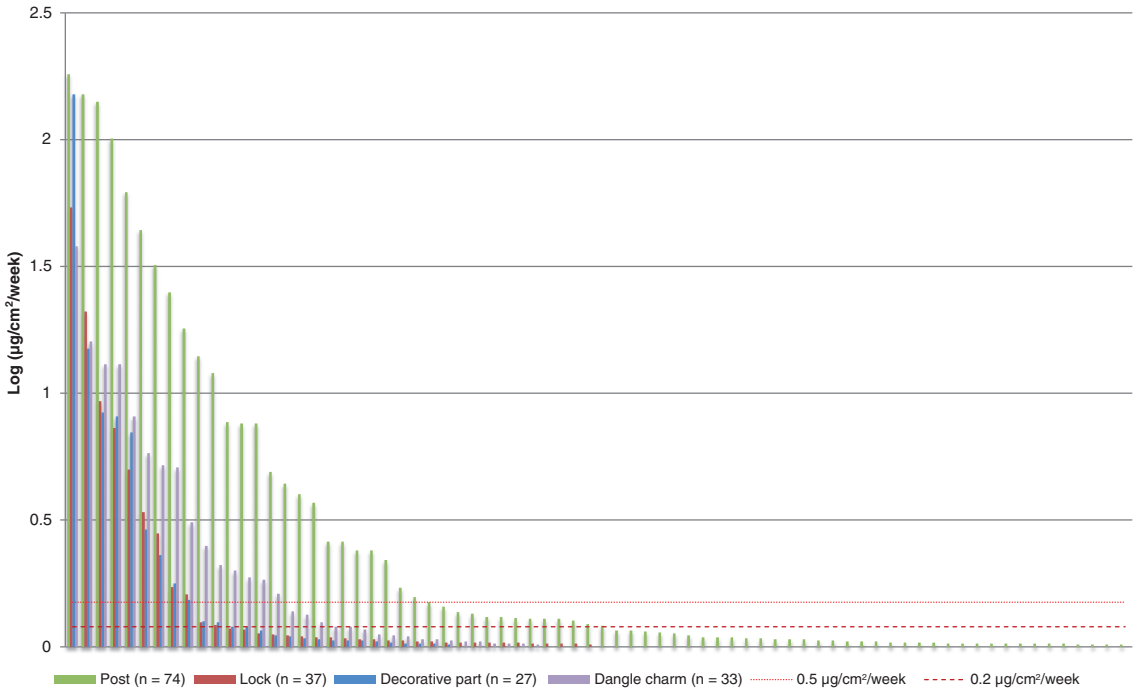

FIGURE 2 Nickel release from sample earring components measured by the EN 1811 on a base-10 logarithmic scale

TABLE 2 Nickel release from earrings tested with EN 1811 (n = 100)[a]

Result	Post	Lock	Decorative part	Dangle charm	Total
<LOD	26	13	36	27	102
0.02-0.2 µg/cm^2/week	36	26	14	13	89
0.2-0.5 µg/cm^2/week	**13**	**2**	4	5	24
Above 0.5 µg/cm^2/week	**25**	**9**	**9**	15	58
Total tested	100	50	63	60	273
Total positive	74 (**38**)	37 (**11**)	27 (**9**)	33	171

Note: Components with excessive nickel release are presented in bold. Limit of detection (LOD) = 0.02 µg/cm^2/week.
[a]Data presented based on tested components (n = 273) stratified according to the European regulatory limits[12,13]

TABLE 3 Sensitivity and specificity of DMG spot test at different thresholds for positive EN 1811 value

		EN 1811									
		>0.5			≥0.2			≥LOD			
Threshold (μg/cm²/week)		+	–	Total	+	–	Total	+	–	Total	
DMG	+	22	13	35	+	26	9	35	+	33	2	35
	–	14	130	144	–	31	113	144	–	81	63	144
Total		36	143	179		57	122	179		114	65	179
Sensitivity (95% CI)		61.1% (43.5%-76.9%)			45.2% (32.4%-59.3%)			29% (20.9%-38.2%)			
Specificity (95% CI)		90.9% (85%-95.1%)			92.6% (86.5%-96.6%)			97.8% (89.3%-99.6%)			

Note: Only components tested with the dimethylglyoxime (DMG) spot test and EN 1811 were included (n = 179). Data for the last two rows presented with the "exact" Clopper–Pearson 95% confidence intervals.[33] Limit of detection (LOD) = 0.02 μg/cm²/week.

TABLE 4 Sensitivity and specificity of XRF analysis as a predictor for nickel release verified by EN 1811

		EN 1811		
		+	–	Total
XRF	+	97	41	138
	–	17	24	41
Total		114	65	179
Sensitivity (95% CI)		85.1% (77.2%-91.1%)		
Specificity (95% CI)		36.9% (25.3%-49.8%)		

Note: Only components tested with X-ray fluorescence (XRF) analysis and EN 1811 were included (n = 179). Data in the last two rows are presented with the "exact" Clopper–Pearson 95% confidence intervals.[33]

3.2 | DMG spot test

Of all samples, 9.2% (28/304) were DMG positive in one or more components. About 6.6% (20/304), 5.4% (2/37), and 25.5% (13/51) of post components, locks, and decorative parts were found to be positive. The positive decorative parts were divided into seven parts with apparent skin contact and six without (Table 1).

3.3 | EN 1811

Of the 100 tested samples, 86 released nickel. Notably, the post showed the most frequent release among the tested components with 74 positive results (Figure 2). In general, results were skewed toward the limit of detection (0.02 μg/cm²/week) with a median of 0.22 μg/cm²/week and some high outliers with a maximum of 180 μg/cm²/week (Table S1).

Results were stratified for each component according to the limits set by the European regulation[12,13] (Table 2). Excessive nickel release, defined as over 0.2 μg/cm²/week in the post and/or lock component and/or over 0.5 μg/cm²/week in the decorative part with skin contact, was found in 45 earrings. The excessive nickel release was largely from the post component (n = 38) (Table 2 and Figure 2). Eleven locks and nine decorative parts showed excessive nickel release.

The component category "dangle charms" did not have any apparent prolonged skin contact during normal wear and thus is not covered by the European regulatory limits.

3.4 | Sensitivity and specificity of DMG spot test

To test the validity of the DMG spot test, the results were compared with the amount of nickel release (found by the EN 1811) for each component examined with both tests (n = 179). Sensitivity was calculated as $\frac{true\ positives}{true\ positives + false\ negatives}$ and specificity was calculated as $\frac{true\ negatives}{true\ negatives + false\ positives}$ in accordance with published methods.[28,32,33]

The DMG spot test had a sensitivity of 29% when all components with nickel release were regarded as true positives. If true positives were defined as more than 0.5 μg/cm²/week, the sensitivity was 61.1%. At a threshold of 0.2 or more μg/cm²/week, the sensitivity declined to 45.2% (Table 3).

The specificity for the DMG test was 97.8%, as nickel could not be quantified in two DMG spot test-positive components.

3.5 | Sensitivity and specificity of XRF analysis for nickel release

The XRF analysis measures elemental content and not the release of metal ions. However, a bivariate Spearman correlation analysis showed a moderate linear correlation between the nickel content and the nickel release (r = 0.592, n = 179, P < .001).

The sensitivity of the XRF analysis as a predictor for nickel release was 85.1% (95% CI 77.2%-91.1%) with a specificity of 36.9% (95% CI 25.3%-49.8%) of the total components (n = 179) tested with both tests (Table 4).

4 | DISCUSSION

4.1 | Nickel release from earrings

In this study, of the 304 earrings on the Danish market, nickel release was found from at least 28.3% (n = 86), and nickel release exceeded the limits of the regulation in at least 14.8% (n = 45) of products.

Previous market studies have shown that excessive nickel release from earrings is common throughout European countries. Studies using either EN 1811 or the DMG test have reported excessive nickel release from 10% to 18.4% of sampled earrings,[19,21,22] except for one Swedish study from 2010 in which the DMG spot test reported positivity as little as 3.7% (n = 107).[34] The most recent Danish survey from 2009 reported DMG spot test positive results in 14.7% of products among a total of 170 earrings tested.[18] In this study we report DMG spot test positive result in 9.2% of products (n = 304), demonstrating a decrease in positivity. In countries not covered by the European regulation, the percentage is much higher, ranging from 29.2% to 42.5%.[30,35,36]

The post component was the most common source of nickel release (24.3%, 74/304) and had additionally, by far, the highest levels of nickel release. A similar finding has previously been reported.[19] High release from the posts may be more critical than from other components of the earring, as the post is in direct contact with the blood plasma after piercing, bypassing the skin barrier. Further, metal ions in earrings may more easily be released to plasma than water.[37]Of the total earrings positive for nickel release (n = 86), in EN 1811, 20 (6.5%) had concurrent nickel release from the post, the lock, and the decorative part with apparent skin contact, resulting in an aggregated exposure. Because of the relatively small size of the earlobe, this aggregated exposure might be substantial, contributing to the risk of nickel allergy.

From a clinical point of view, the elicitation threshold for nickel exposure has been difficult to establish as it varies largely between individuals.[2] In a meta-analysis, Fischer et al[10] found that 10% of the sensitized population reacted to 1.04 μg Ni/cm^2 after occluded exposure on intact skin. In addition, the elicitation threshold for a penetrating exposure, such as the body piercing, may be lower than a single occluded exposure. Räsänen et al[37] found allergic reactions in sensitized individuals from the use of earrings releasing as little as 0.15 and 0.17 μgNi/cm^2/week. In 2011, and later clarified in 2015, the EN 1811 reference test was amended to introduce a measurement of uncertainty to make it easier to assess products for compliance. This amendment effectively increased the allowed level of nickel release to 0.35 μg/cm^2/week for post assemblies and 0.88 μg/cm^2/week for items intended for prolonged skin contact.[11,14] Together with the critical characteristics of a piercing exposure, this may question whether the European regulatory limits are sufficient in protecting the population from nickel allergy.

The XRF analysis on metal alloys is useful as a preliminary analysis to the EN 1811, as it positively identified 85.1% of nickel-releasing components and has some predictive value regarding the level of nickel release (Spearman r = 0.592, n = 179, P < .001). However, the low specificity (36.1%) does not make it suitable for analysing nickel release. The surprisingly high rate of false negatives (n = 17/41, Table 4) is likely because the XRF analyses a small delimited area, whereas the EN 1811 analyses the complete surface area of the tested component, including joints, surface irregularities, or smaller parts. Our XRF results also demonstrated the presence of other allergenic metals, which theoretically could promote sensitization.

In this study, the EN 1811 results and the interpretation of the DMG spot test results should be considered conservative. Our subsampling for EN 1811 analysis did not include all nickel-containing piercing posts. In our interpretation of the DMG spot test results, excessive nickel release from the decorative part was expressed by the limit for prolonged skin contact (>0.5 μg/cm^2/week). We did not specifically test the area of the decorative part in contact with the pierced canal, where excessive nickel release should be defined as 0.2 or more μg/cm^2/week.

4.2 | DMG spot test validation for screening piercing post assemblies

In our testing, the DMG spot test identified 28 earrings that released nickel, as opposed to 86 identified by the EN 1811.

We found that the DMG spot test had a sensitivity of 61.1% and specificity of 90.9% on objects that released more than 0.5 μg nickel/cm^2/week, close to that previously reported.[28] The sensitivity declined with decreasing levels of available nickel, noticeably below 0.5 μg/cm^2/week.

The DMG spot test has previously been used to assess nickel release from earrings,[21,22,30,34,35] which has a lower regulatory limit (0.2 μg/cm^2/week) than the *estimated* detection limit (0.5 μg/cm^2).[28] At a threshold of 0.2 or more μg/cm^2/week, the sensitivity of the DMG spot was 45.2%, making it less useful to correctly assess piercing post assemblies for excessive nickel release and thus the EN 1811 reference test should be preferred.

Because of the small test area of earring components, the volume of DMG test solution used in this study is arguably large. It could prove beneficial for the sensitivity of the DMG test to only use one drop of each solution when testing earrings, and even mix the solutions beforehand to ensure correct proportions of test solutions.

It should be emphasized that the DMG spot test is a qualitative screening tool for nickel release, with an estimated detection limit, and is not designed to screen for excessive nickel release. Thus, the false positives that emerge when setting a quantitative threshold (Table 3) cannot truly be regarded as false positive as these are still positive for nickel release. The decline in specificity is hence a construct and for the purpose of the DMG spot test as a screening tool, the specificity is reported to be 97.8%.

The two true false-positive results, with no nickel release, could be explained by the small test area of earring components, making it difficult to accurately and separately test a single component without touching other parts of the earring. In the EN 1811 test, this is avoided by dismantling and/or masking irrelevant components, which could also be applied for the DMG spot test of earrings to improve testing accuracy.

5 | CONCLUSION

We found that at least 14.8% of a random sample of 304 earrings released nickel in levels that may elicit allergic nickel dermatitis. The sensitivity of the DMG spot test declined at levels of nickel release less than 0.5 μg/cm^2/week. The quantitative EN 1811 reference test should therefore be used to correctly assess nickel release from

piercing post assemblies where the limit is only 0.2 μg/cm^2/week. The DMG spot tests may still be used to detect higher levels of nickel in clinical settings due to its rapidness and high specificity. A safe level of nickel release in earrings should be identified to better protect consumers from nickel allergy.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Michael Wennervaldt: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; validation; writing-original draft; writing-review & editing. **Malin Ahlstrøm:** Formal analysis; funding acquisition; methodology; supervision; validation; writing-review & editing. **Torkil Menné:** Conceptualization; methodology; project administration; supervision; validation; writing-review & editing. **Jacob Thyssen:** Conceptualization; methodology; supervision; validation; writing-review & editing. **Jeanne Duus Johansen:** Conceptualization; formal analysis; funding acquisition; methodology; project administration; supervision; validation; writing-original draft; writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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ORIGINAL ARTICLE

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Chromium and cobalt release from metallic earrings from the Danish market

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Abstract

Background: Chromium and cobalt are important skin sensitizers. It has, however, been difficult to identify causative exposures. Studies on nickel allergy have demonstrated piercing as critical for both sensitization and elicitation. It may be speculated that the same applies for chromium and cobalt.

Objective: To examine the content and release of chromium and cobalt from earrings randomly purchased in Denmark.

Methods: Three hundred four earrings were examined with x-ray fluorescence (XRF) spectrometry. Earrings with measured content of chromium or cobalt were spot tested with diphenylcarbazide spot test (n = 166) or Nitroso-R spot-test (n = 99), respectively. Chromium and cobalt release were quantified in a selected subsample (n = 100) with the artificial sweat test (EN 1811).

Results: Chromium was present in 54.6% (166/304) of earrings and cobalt was present in 72.0% (219/304),– measured by XRF. All chromium spot tests for chromium VI were negative. The cobalt spot test was positive for one component. Chromium release was found from 59/100 (median concentration = −0.06 μg/cm^2/week) and cobalt release from 29/100 (median concentration = −0.06 μg/cm^2/week) of earrings in tested subsample.

Conclusion: Earrings for piercing release chromium and cobalt and may on a case basis be a source of chromium and cobalt allergy.

KEYWORDS
chromium, cobalt, contact allergy, diphenylcarbazide, jewelry, Nitroso-R, spot test

1 | INTRODUCTION

Chromium and cobalt are important skin sensitizers. Contact allergy to chromium and cobalt are relatively common affecting, respectively, 0.8%-1.8% and 2.2%-2.7% of the general population.[1,2] They are even more frequently reported in patients with dermatitis, with 3.7% positive for chromium and 5.3% positive for cobalt of patients who were patch tested with the European baseline series.[3]

Causative exposures for chromium allergy have been described primarily as exposure to leather objects and previously cement-work.[4] For cobalt allergy it has been difficult to identify relevant exposures.[3,5] However, both chromium and cobalt are present in many common metal alloys and recent studies have shown release of these metal-ions from the metallic parts in various consumer items posing a potential allergy risk.[6-12] Only a few of these studies have included body piercings.

Two colorimetric spot tests have been developed based on the same principle as the widely used dimethylglyoxime (DMG) spot test for screening of nickel release.[13] Diphenylcarbazide (DPC) can detect hexavalent chromium (Cr[VI]) release,[7] and disodium-1-nitroso-2-naphthol-3,6-disulfonate (Nitroso-R salt) cobalt release.[14,15] Both of

the tests have successfully been used to show release from consumer items.[6,7,12,16] However, their sensitivity and specificity have been questioned.[17-19]

The purpose of this study was to evaluate chromium and cobalt content and release from earrings available on the Danish market and to compare the outcome of the DPC and Nitroso-R spot test to the quantitative measurement of metal released in artificial sweat according to EN 1811.[20]

Additionally, the measured levels of release will be discussed in context of what is currently know about induction and elicitation levels for chromium and cobalt, in an attempt to elucidate the risk.

2 | METHODS

The same earrings from a previously published study on nickel release[21] were tested for chromium and cobalt content and release. Of the earrings bought as an identical pair, we sought to subject the same earring to the various test. Tests were done in the following order: x-ray fluorescence (XRF), spot-test, artificial sweat test.

2.1 | Materials

Three hundred four earrings were bought from March to May 2020 from a wide variety of different stores in Denmark. Only unique earrings with a metal perforating post were bought. Approximately 3300 EUR were spent, ranging from 2.5 to 50 EUR (mean = 12.9) per earring. For further details see published article.[21]

2.2 | XRF spectroscopy

The elemental composition of the earrings was measured by a handheld XRF device (X-MET8000 Series, Uedem, Germany). The post from all 304 samples was analyzed, and measurements were only done when the lock (n = 37), the decorative part with skin contact (n = 18), and/or decorative part without skin contact (dangle charm) (n = 33) visibly consisted of metal.

Each measurement was taken on the factory default settings for alloy measurements with 10 seconds exposure time as per manufacturer's recommendation. Every sample measurement was done in triplets where the mean is presented as the result in weight percentage (wt%).

The detection limit of the XRF varies depending on the alloy tested and the element recorded. For stainless steel grades, the detection limit for chromium is 25 ppm (0.0025%) and 130 ppm (0.0130%) for cobalt with an average error of, respectively, 0.24% and 0.11%.

2.3 | Spot tests: DPC and NITROSO-R

The elemental composition measured by XRF was used to determine objects for spot testing.

All samples with chromium content in one or more components were tested with the DPC spot test (n = 166). Because very low levels of cobalt were detected in the majority of the samples, all samples with cobalt content above 0.1% (n = 79) in one or more components were subjected to Nitroso-R spot test. Furthermore, a random selection of samples that contained 0.002%-0.1% cobalt in one component was spot tested (n = 20) (Figure 1).

Several days passed between each type of spot-testing to allow re-oxidation of surface metal ions. If a test was positive, the sample was washed with de-ionized water and dried with a paper tissue to avoid cross-discoloration between the tests.[22]

Both spot tests were prepared and performed in our laboratory at the Department of Dermatology and Allergy according to previously published methods.[7,14,15]

For the DPC spot test for Cr(VI), 0.4 g of 1,5-DPC (CAS no. 140-22-7) (Merck KGaA, Darmstadt, Germany) was dissolved in a mixture of 20 mL of acetone (Merck KGaA, Darmstadt, Germany) and 20 mL of 96% ethanol. Then 20 mL 75% H_3PO_4 (Merck KGaA, Darmstadt, Germany) and 20 mL of de-ionized water were added. A cotton swab was soaked in the solution and rubbed on the test area for 30 seconds, and reading was performed after 2 minutes. A positive result was indicated by purple/red coloration of the swap and a negative result was indicated by no change in color.

For the Nitroso-R spot test for cobalt, 0.008 g disodium-1-nitroso-2-naphthol-3,6-disulfonate (Nitroso R salt) (CAS no. 32588-53-7) (BocSciences, Hamburg, Germany), 0.04 g oxalic acid (Merck KGaA, Darmstadt, Germany), and 2 g sodium acetate (Merck KGaA, Darmstadt, Germany) were dissolved in 40 mL de-ionized water. A cotton swab was dipped in the solution and rubbed against the test area for 30 seconds. A positive result was indicated by yellow/red coloration of the swap and a negative result was indicated by no change in color.

All doubtful tests, defined by a discoloration other than a hue of the positive color indication, were retested. If the reaction remained doubtful the result was registered as negative.

2.4 | Metal release in artificial sweat

A subsample of 100 earrings was subjected to metal release testing in artificial sweat according to the current European standard reference test EN1811 for control of compliance with the EU REACH nickel regulation.[20,23,24] The subsample was chosen with a primary analytical focus on nickel and defined mainly by the nickel content of earrings.[21] Included in this sample were, 75 of 166 (45%) earrings with chromium content as measured by the XRF and 85 of 219 (39%) earrings with cobalt content; several earrings had content of both metals (Figure 1). The test was performed by Eurofins | BLC Leather Technology Centre Ltd. (Kings Park Road, Moulton Park, Northampton, NN3 6JD, UK) according to BS EN 1811:2011 + A1: 2015,[20] with additional measurements of chromium and cobalt release by inductively coupled plasma mass spectrometry (ICP-MS). The resulting release was divided with the measured surface area of the component and reported as $\mu g/cm^2$/week. The lower limit of detection (LOD) was 0.02 $\mu g/cm^2$/week.

The chromium analysis did not distinguish between states of chromium (III and VI) and thus represents total chromium.

3 | RESULTS

3.1 | XRF spectroscopy

Chromium was present in 54.6% (n = 166) of the 304 earrings in one or more components. Regarding tested components 53.3% posts (162/304, content median = 11.8%), 24.3% locks (9/37, content median = 0.04%), and 51% decorative parts (26/51, content median = 0.4%) had chromium content.

Cobalt was present in 72% (n = 219) of the 304 earrings in one or more components.

Regarding tested components 65.5% posts (199/304, content median = 0.07%), 75.7% locks (28/37, content median = 0.06%), and 84.3% decorative parts (43/51, content median = 0.02%) had cobalt content.

3.2 | DPC and NITROSO-R spot tests

All 166 earrings that contained chromium were negative with the DPC spot test.

Of the 99 tested earrings, one lock component of an earring was found positive for cobalt release with the Nitroso-R spot test. Notably, only one lock of an identical earring pair was positive (Figures 1 and 2).

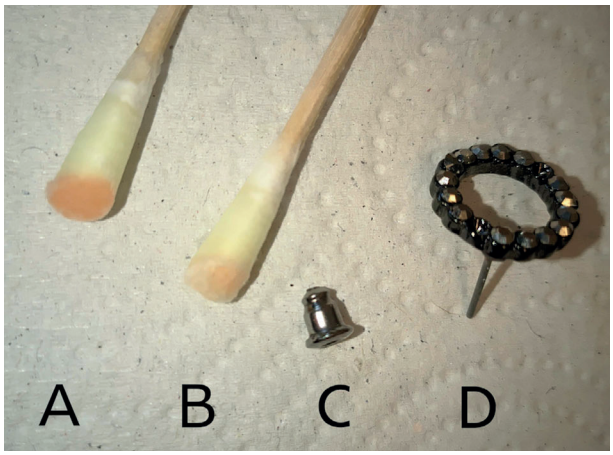

FIGURE 2 Positive Nitroso-R spot test of the lock component of an earring. A, Positive control. B, Positive test. C, Positive lock component. D, The earring post and the decorative part tested negative

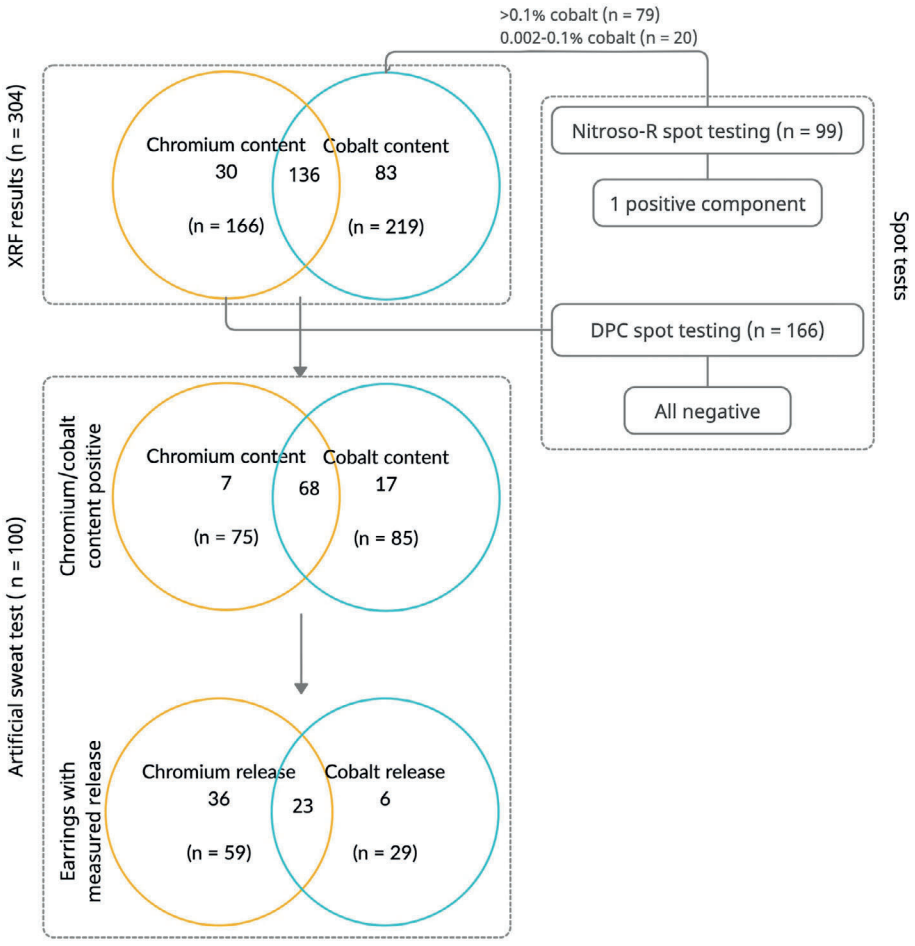

FIGURE 1 Summary of study methods and results. Top circles: Earrings positive for chromium and/or cobalt *content* as measured by the x-ray fluorescence (XRF) analysis of all 304 earrings. All chromium-positive earrings were diphenylcarbazide (DPC) spot tested. A subsample of cobalt positive earrings was Nitroso-R spot tested. Middle circles: Distribution of chromium and/or cobalt *content* positive earrings in a subsample of 100 earrings chosen for artificial sweat testing (eight earrings did not have any measured chromium or cobalt content). Bottom circles: Earrings positive for chromium and/or cobalt *release* as measured by the artificial sweat test

	N	Median	Mean	Minimum	Maximum
Post	43	0.06	10.88	0.02	110
95% CI		0.04-0.12	4.33-18.33		
Lock	15	0.06	1.07	0.02	14.00
95% CI		0.03-0.27	0.07-2.96		
Decorative part	10	0.49	6.58	0.02	51.00
95% CI		0.03-4.50	0.58-16.77		
Dangle charm	15	0.07	0.12	0.02	0.88
95% CI		0.04-0.11	0.06-0.24		
Total positive components	83				

TABLE 1 Chromium release from earring components measured by the artificial sweat test

Note: Values are reported in μg/cm^2/week. A total of 59 earrings were positive in one or more components. Lower limit of detection = 0.02 μg/cm^2/week.

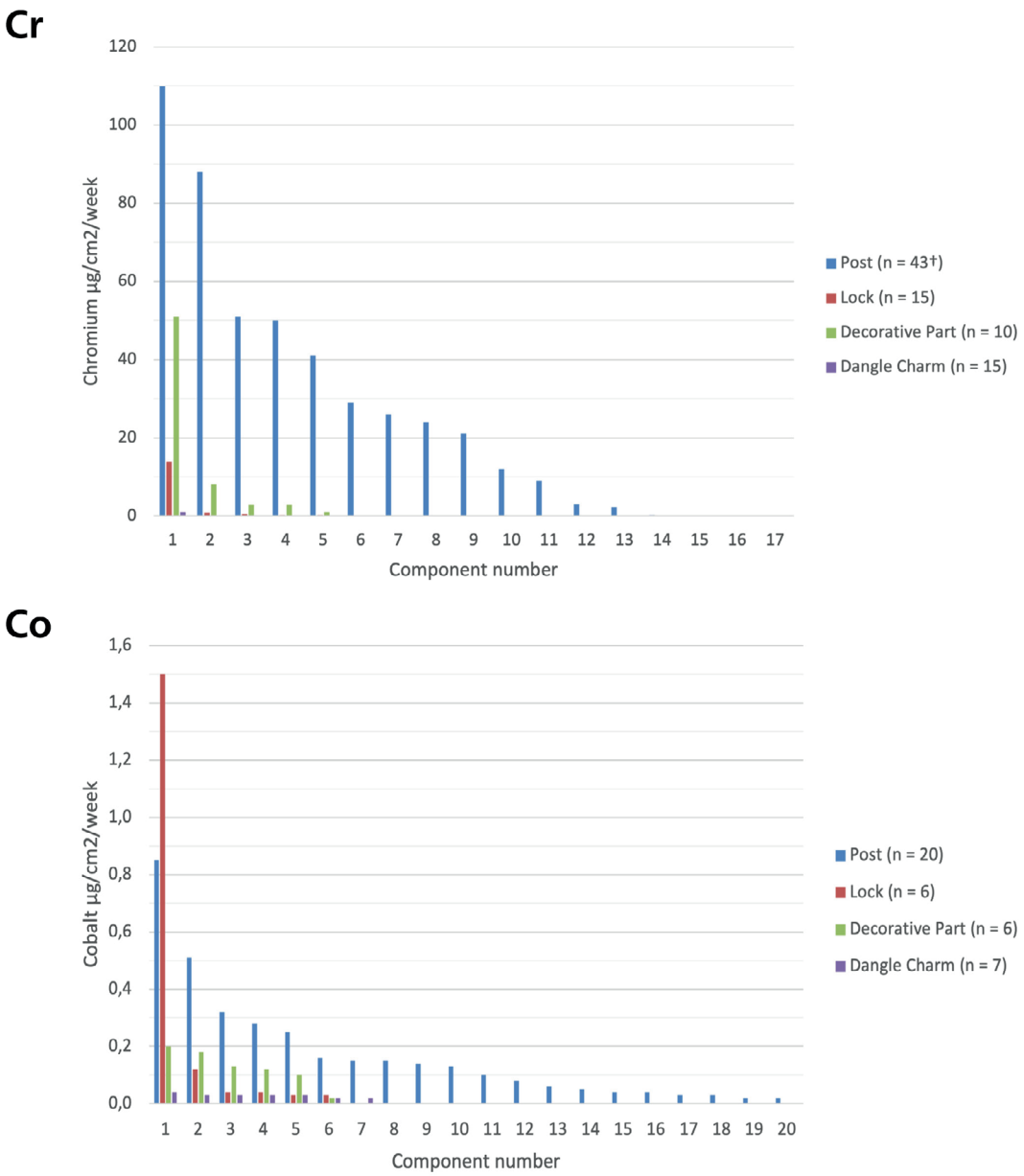

FIGURE 3 Chromium and cobalt release from earring components measured by artificial sweat test. Each bar represents one component. (†Post components with chromium release <0.1 μg/cm^2/week are not shown)

TABLE 2 Cobalt release from earring components as measured by the artificial sweat test

	N	Median	Mean	Minimum	Maximum
Post	20	0.12	0.17	0.02	0.85
95% CI		0.05-0.16	0.09-0.27		
Lock	6	0.04	0.29	0.02	1.50
95% CI		0.03-0.81	0.04-0.78		
Decorative part	6	0.13	0.13	0.02	0.20
95% CI		0.06-0.19	0.08-0.17		
Dangle charm	7	0.03	0.03	0.02	0.04
95% CI		0.02-0.03	0.02-0.03		
Total positive components	39				

Note: Values are reported in μg/cm^2/week. A total of 29 earrings were positive in one or more components. Lower limit of detection = 0.02 μg/cm^2/week.

3.3 | Chromium and cobalt release in artificial sweat

Chromium was released from 59 of the tested earrings (59/100); release from more than two components were found from 20 earrings. A total of 83 components were positive with a median of 0.06 μg/cm^2/week (0.04-0.11, 95% confidence interval [CI]) with some high outliers. The majority of positive components were posts (n = 43), which also had the highest release value (110 μg/cm^2/week) (Table 1, Figure 3).

Cobalt release was found from 29 (29/100) earrings in one or more components, of which 10 had several positive components. A total of 39 components were positive, with a median of 0.06 μg/cm^2/week (0.04-0.13, 95% CI). The majority of positive components were posts (n = 20); however, a single lock was a high outlier with a value of 1.5 μg/cm^2/week (Table 2, Figure 3).

A Spearman bivariate correlation analysis showed a moderate correlation between released chromium and cobalt (r = 0.48, $P < .01$). There was no significant correlation between either of the two metals and nickel released from the same samples.

4 | DISCUSSION

4.1 | Chromium and cobalt release

In this study, we found that both chromium and cobalt were widely present and often released from earrings on the Danish market.

There have been few published studies assessing the release of chromium or cobalt from earrings and piercing jewelry,[8,9,11,18,25-27] of which only four (including a follow-up) have performed quantitative measurements.[8,11,25,27]

With several high outliers, we found a median release of 0.06 μg chromium/cm^2/week (n = 59/100) and 0.06 μg cobalt/cm^2/week (n = 29/100). In comparison, a Turkish study in 2020 found a *mean* release of 2.04 μg chromium/cm^2/week (standard deviation [SD] = 4.68) and a *mean* release of 0.20 μg cobalt/cm^2/week (SD = 2.73) from a sample of 223 cheap earrings.[11] The authors did not note how many samples were positive for release.

Our findings of cobalt release are slightly higher than reported in a similar German survey in 2008 (median = 0.013 μg/cm^2/week).[8] A 2014 follow-up of the survey notes a decline in the levels of cobalt released, however, with no decrease in the number of cobalt releasing earrings.[25] In addition, Hamann et al found cobalt release from earrings bought in the United States in levels ranging 0.02-0.05 ug/cm2/week (n = 35/96).[27]

Of interest, we report a modest correlation in the release of chromium and cobalt but not with the release of nickel, a finding also described in a similar study.[11] This is likely due to the alloy composition; however, we are not able to further elaborate with the current data.

Cobalt is frequently seen as a contaminant in various nickel-containing alloys, as cobalt occurs naturally in nickel ores and exists as a trace element in nickel ore extract.[28] Nickel was also a common constituent[21] and might explain some of the very low levels of cobalt content measured by the XRF.

4.2 | Induction and elicitation risk

The sensitization reference dose of chromium for safe human exposure has been calculated to 0.01 μg/cm^2, an approximation based on data from animal studies.[29] More recent investigations based on patch testing estimated the minimum threshold to elicit dermatitis in 10% (MET_{10}) of sensitized individuals to 0.18 μg/cm^2 for Cr(III) and 0.03 μg/cm^2 for Cr(VI).[30]

Our results of chromium release do not distinguish between oxidation states. The conversion between Cr(III) and Cr(VI) is dynamic and depends on a range of environmental factors, such as humidity, pH, and temperature.[31] From stainless steel, chromium is released primarily as Cr(III) due to the presence of iron as a reductive agent, but other alloys may be more prone to oxidation and the formation of Cr(VI).[32] However, once chromium is in contact with or has penetrated the skin barrier, it may be that interactions with immune cells and proteins such as reactive oxygen species may facilitate the conversion to the more potent Cr(VI).[31,33]

In our study, all chromium positive components (59/100) surpassed 0.02 μg/cm^2/week (as this was the LOD of the EN 1811);

however, the exact deposition of chromium on the skin under real-life use of earrings is not known.

Cobalt is a strong skin sensitizer, and the dose to elicit dermatitis in 10% (ED_{10}) of sensitized individuals has been estimated to 0.06-1.95 μg/cm^2.[34] In our study, 29% of the subsampled earrings released cobalt with a median of 0.06 μg/cm^2/week, making it likely that several earrings may release critical levels of cobalt.

There are no specific studies or approximations on the induction levels for cobalt sensitization, but it is established as a stronger skin sensitizer than chromium or nickel,[35,36] and safe levels must thus be assumed lower (current European legislative limits nickel in piercing post assemblies to 0.02 μg/cm^2/week[23,24]).

The MET_{10} and ED_{10} have been derived from dose-response patch testing on intact skin. From studies of nickel, it is indicated that the elicitation threshold at piercing exposure is lower than at an occluded exposure such as the patch testing.[37] Furthermore the piercing exposure has been associated with a higher risk of allergy to nickel.[38,39] This stresses the potential risk of elicitation from chromium and cobalt released from earrings. Release of chromium and cobalt in our study was seen primarily from the post component, which is in direct contact with the pierced skin canal.

Warshaw et al analyzed the correlation between body piercings and chromium and cobalt allergy and found no association with cobalt allergy alone and a negative correlation with chromium allergy.[38] It should be noted, however, that the negative correlation was not significant when adjusting for gender, as few pierced males (4.4%) were included in the study, whereas the male group simultaneously had a higher prevalence of chromium contact allergy.

Nevertheless, along with leather, jewelry has been recorded as the most common source of exposure to cobalt in dermatitis patients.[5] Our results indicate that on a case basis both chromium and cobalt release from earrings may be of clinical significance.

4.3 | Spot tests

In our spot testing, all samples were negative in the DPC test for chromium release and only one sample (0.3%) was positive in the Nitroso-R test for cobalt.

The DPC spot test only detects Cr(VI) with a detection limit estimated to 0.25 ppm Cr(VI).[7] We were not able to differentiate between the oxidation states in the artificial sweat test. It is likely that the measured levels of chromium were primarily of Cr(III) and thus not detectable by the DPC spot test; however, the sensitivity and possibility of false negatives of the DPC spot test have not been fully validated to draw this conclusion.[7]

Studies have shown that primarily Cr(III) is released from specific types of biomedical applied steel; however, it is strongly dependent on the environment and alloy production.[31,33,40,41]

Bregnbak et al[18] did a similar study of DPC spot testing 848 items of jewelry, of which 707 were body piercings. All 848 items tested negative. There was no investigation of chromium release by any other methods.

The detection limit of Nitroso-R is estimated to 8.3 ppm cobalt.[14] The estimated ED_{10} range of 0.06-1.95 μg/cm^2 corresponds to 30.8-259 ppm.[34] The lock component that was Nitroso-R spot-test positive released 1.5 μg cobalt/cm^2/week, which was the component with the highest measured cobalt release. Cobalt release of LOD-0.85 μg/cm^2/week were measured for other components. This indicates the usefulness of the Nitroso-R spot test for screening objects for cobalt release of certain clinical relevance.

In 2010, Thyssen et al[9] performed a Danish cobalt screening survey of 354 jewelry and hair clasps, of which 170 were earrings; 3 earrings (1.8%) released cobalt, found by Nitroso-R spot test. Comparably, we found one (0.3%) Nitroso-R positive earring.

5 | CONCLUSION

In conclusion, earrings for piercing may on a case basis be an overlooked source of chromium or cobalt allergy. The cobalt spot test Nitroso-R seems to be able to detect exposures with some clinical relevance. A spot test for, or including, Cr(III) would be of value.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Michael Wennervaldt: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; visualization; writing-original draft; writing-review & editing. **Malin Ahlstrøm:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; supervision; validation; writing-review & editing. **Torkil Menné:** Conceptualization; investigation; methodology; supervision; validation; writing-review & editing. **Morten Haulrig:** Formal analysis; investigation; methodology; writing-review & editing. **Farzad Alinaghi:** Formal analysis; investigation; validation; writing-review & editing. **Jacob Thyssen:** Conceptualization; formal analysis; methodology; project administration; supervision; validation; writing-review & editing. **Jeanne Duus Johansen:** Conceptualization; formal analysis; funding acquisition; methodology; project administration; supervision; validation; writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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3. Manuscript III: Copper release from metals may mask positive nickel spot test results

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Copper release from metals may mask positive nickel spot test results

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KEYWORDS: copper, diagnostics, dimethylglyoxime, DMG, metal release, nickel, nickel allergy

The dimethylglyoxime (DMG) spot test is widely used to screen for nickel release that may cause allergic nickel contact dermatitis in allergic individuals. It is a colorimetric test based on the chelation of DMG molecules to free nickel ions which form the complex $Ni(DMG)_2$ that is bright pink-red.[1,2] The test has high specificity, but modest sensitivity, which has caused criticism for the test's usability, especially when used on metals that have low nickel release.[3,4]

DMG molecules do not have specific affinity for nickel ions, and can also chelate with copper ions.[5,6] This has sometimes been overlooked in the utilization of the test for nickel screening purposes, though it is described in the European Committee of Standardization's report CR 12471 concerning the use of the DMG spot test for nickel release.[7] In this study, we demonstrate a masking effect of copper ions that may result in an incorrect reading of DMG spot test to nickel.

METHODS

To test the colour change of DMG in reaction to various concentrations of nickel and copper, dilution series of $NiCl_2$ and $CuSO_4$, respectively, were made. A stock solution of 10% $NiCl_2$ (wt/v) was made by dissolving 1 g $NiCl_2$ (Merck KGaA, Darmstadt, Germany; ≥98%, CAS no. 7718-54-9) in 10 mL distilled water, from which a serial dilution of 1%, 0.2%, and 0.1% was made. Similarly, 1 g $CuSO_4$ (Merck KGaA; 98%, CAS no. 7758-99-8) was dissolved in

10 mL distilled water (10%) and further diluted to 5%, 1%, 0.1%, and 0% (for 0%, only distilled water was used). The concentrations of nickel were chosen based on the estimated detection limit of the DMG spot test,[2] which corresponds to 0.05% $NiCl_2$ in our set up. Higher concentrations of $CuSO_4$ were used to test the extent of the effect.

A DMG test solution was prepared by mixing 0.5 mL of 1% DMG solution in ethanol with 0.5 mL of 10% ammonia hydroxide in a 1.5-mL Eppendorf tube. Both solutions were prepared by the hospital pharmacy in the Capital Region of Copenhagen.

From both the $NiCl_2$ and the $CuSO_4$ dilutions series, 0.5 mL of each concentration was mixed in a 1.5-mL Eppendorf tube. The tube was thoroughly shaken in hand for 20 seconds and 10 μL was added to an 8-mm filter paper, along with 10 μL DMG test solution.

From a previous data set of earrings,[3] one earring component had a simultaneous copper and nickel release (43 and 0.87 $\mu g/cm_2$/week; unpublished data), which is close to the REACH item 27 of Annex XVII limit value for nickel release,[8] and close to the detection limit of the DMG test. The earring was DMG spot tested by adding two drops of DMG test solution to a cotton stick and rubbing it against the object for 30 seconds.

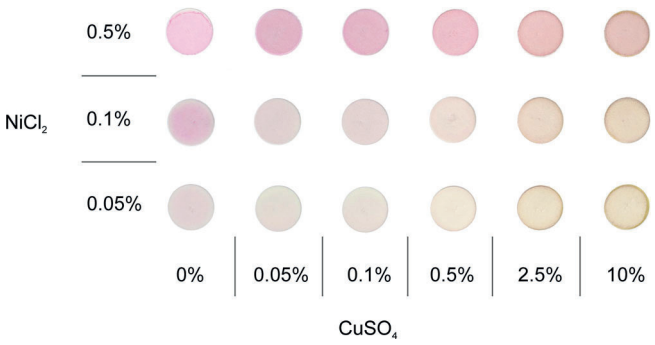

FIGURE 1 DMG spot test reactions at different concentrations of $NiCl_2$ and $CuSO_4$. The positive DMG spot test reaction (pink-red) was proportionally discoloured brown-yellow by increasing concentrations of $CuSO_4$. These results are from an experimental setup and serve as proof of concept and are not directly applicable to a regular DMG spot test. DMG, dimethylglyoxime

FIGURE 2 An inconclusive DMG spot test of an earring with known nickel (0.87 $\mu g/cm^2$/week) and copper release (43 $\mu g/cm^2$/week), due to a masking effect caused by brown discolouration of the DMG test reacting with released copper. DMG, dimethylglyoxime

RESULTS

The pink-red colouration of the DMG spot test as a result of $NiCl_2$ was proportionally discoloured brown-yellow by increasing concentrations of $CuSO_4$ (Figure 1). The discolouration depended on the nickel and copper concentrations; at 0.05% $NiCl_2$, a slight addition of copper (0.05% $CuSO_4$) diminished the red colouration. While the red colouration in 0.1% $NiCl_2$ was hardly distinguishable in the presence of 0.5% $CuSO_4$. At 0.5% $NiCl_2$, the red colouration remains distinguishable but fades with a higher concentration of $CuSO_4$.

The DMG-spot-tested earring that showed no red colouration was registered as inconclusive (Figure 2).

DISCUSSION

The presence of copper ions can effectively mask a potentially positive result of a DMG spot test (Figure 1). The masking effect occurs in part by brown discolouration, which makes a red colouration indistinguishable, and in part by the compete binding to the available DMG molecules. The sensitivity of the DMG spot test is partly dose dependent,[3] and similarly, the masking effect of copper was found to be more prominent at higher levels of copper ions and lower levels of nickel ions.

The DMG spot test has an estimated detection limit of 0.5 $\mu g/cm^2$/week.[1,2] From our results this is partly confirmed, as a weak reaction was demonstrated at 0.05% $NiCl_2$, which is comparable to 0.5 $\mu g/cm^2$ of available nickel in our set up (when adjusting for available DMG). However, at this level, a slight addition of copper (0.05% $CuSO_4$) begins to mask the result.

The tested earring with nickel release of 0.87 $\mu g/cm_2$/week was expected to become DMG spot test positive, but this result was possibly masked due to the brown discolouration caused by a DMG-copper reaction (Figure 2). We did not have other samples with nickel release over 0.5 $\mu g/cm^2$/week to further elaborate on the limit of copper release to give a masking effect.

Copper is widely used in many metal alloys, also in combination with nickel. While copper-nickel alloys are mainly used in the industry, we identified the co-constituting presence of nickel and copper in one or more components of several earrings (117/304, 38.5%) of a previously characterized sample of earrings (unpublished data).[3] Many earrings were not of an identifiable alloy. Copper-nickel alloys are commonly used in European coinage and have a high level of nickel release.[9] Euro coins have been found to be positive in DMG spot testing,[10] which further suggests that a masking by copper is only relevant for items with low levels of nickel release.

The DMG copper reaction itself is brown-yellow and is recognizable at high levels of copper. It has been custom to label a DMG spot test of another colour than red as either negative, inconclusive, or

doubtful. Our results emphasize the use of the inconclusive or doubtful category, as the object still might have excessive nickel release.

The DMG molecule can also chelate with other 2+ charged metal ions, such as palladium and cobalt, which might further mask a positive DMG result, depending on the concentration of available ions. In cheap earrings, cobalt is a frequent constituent and often released, while palladium is rarely seen.[11,12]

While the experimental setup and the real-life use of the DMG spot test are not directly comparable, due to the difference in DMG concentration, these results serve as a proof of concept and may explain some false-negative results in DMG spot testing and its resulting mediocre sensitivity. Because of its rapidness and low cost, the DMG spot test is widely used in various market studies and by consumers at home. The DMG spot test additionally been shown to improve diagnostic practice by visualizing nickel accumulation from multiple exposure on the hands.[13,14] We stress the importance of only registering objects as negative if there is no colouration when DMG spot testing for excessive nickel release, as a discolouration other than red could be masking a potentially positive result.

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AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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4. Manuscript IV: Subclinical immune responses to nickel in sensitized individuals - A dose-response study

Wennervaldt M, Vaher H, Ahlström MG, Bischofberger N, Menné T, Thyssen JP, Johansen JD, Bonefeld CM. (Manuscript)

Original Article

Subclinical immune responses to nickel in sensitized individuals - A dose-response study

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Conflicts of interest
The authors declare no conflicts of interest.

1. ABSTRACT

Background: Nickel is the leading cause of contact allergy in Europe, with 14.5% of the adult population sensitized. Despite regulations limiting nickel release from consumer items, the incidence and prevalence of nickel allergy remain high.

Objective: In this study, we investigate the clinical and subclinical immune response to low-dose nickel exposure on pre-exposed skin to further understand the safety of current regulatory limits.

Method: 13 Nickel allergic and 13 healthy controls were patch tested twice with a 3-4 weeks interval. In the first test the diagnostic concentration (2000 µg/cm^2) was used. In the second test, the skin areas were re-tested with 0.2, 0.5, 12.8, and 370 µg/cm^2 nickel sulfate. After 48 hours patch reactions were read, and biopsies were collected. The transcriptomic immune profile was analyzed with Nanostring nCounter and qPCR.

Results: Two nickel-allergic participants (15%) had clinical reactions to the regulatory doses (0.2/0.5 µg/cm^2) upon re-exposure to nickel. We found an immune activation in all skin areas upon re-exposure to nickel which was predominantly mediated by up-regulation of cytokines and chemokines. 81 genes were found to be up-regulated in all skin areas re-exposed to nickel independent from the clinical response. Interestingly, 101 genes were found to be differentially expressed in skin areas exposed to 0.2 µg/cm^2 even when no clinical response was seen.

Conclusion: As the first, this study demonstrates that immune activation can be induced in skin with local memory to nickel upon challenge with nickel doses within the regulatory limits. Thus, suggesting that the regulatory limits in the European nickel regulation may not provide sufficient protection against low-dose exposures.

2. INTRODUCTION

Approximately 14.5% of the European adult general population are sensitized to nickel,[1] and the prevalence remains high among female adolescents.[2,3] Nickel allergy has a significant negative impact on the quality of life of individuals afflicted and is a substantial burden in terms of socioeconomic and occupational costs.[4,5] The ubiquitous use of nickel in industrialized societies makes avoidance difficult. In 2001, a regulation was implemented in Europe limiting nickel released from consumer items with prolonged skin contact: earrings limited to 0.5 µg/cm^2/week and body piercings limited to 0.2 µg/cm^2/week.[6,7] The purposes of this regulation were to prospectively lower the incidence of nickel allergy and to protect the current nickel-sensitized population against eliciting exposures.

Many metallic items covered by the nickel regulation such as watches, buttons, jewelry, and ear- and body piercings are site-localized exposures that often are in repeated, close contact with the skin for extended periods of timeRecent studies have shown that nickel is deposited and accumulated in the stratum corneum, and that short, repeated skin exposure to nickel, can cause allergic contact dermatitis.[8–10]

The immune response to contact allergens consists of two phases: sensitization and elicitation. Upon nickel exposure on the skin, nickel ions activate skin resident cells inducing the production of a cascade of pro-inflammatory and regulatory cytokines and chemokines.[11–14] Activated antigen-presenting cells migrate to the draining lymph nodes and present nickel-modified self-proteins to nickel-specific naïve T cells. This leads to T cells activation and differentiation with some of the T cells becoming memory T cells. Studies have found that during sensitization and elicitation of allergic contact dermatitis, there is an induction of local skin resident memory T (T_{RM}) cells.[15] The T_{RM} cells persist in the skin and induce a faster and enhanced allergic response upon re-exposure to the same skin area, consequently lowering the threshold for elicitation of allergic contact dermatitis.[16,17]

Newer studies have shown an allergen-specific correlation between allergic nickel contact dermatitis and several cytokines and chemokines.[17–20] However, the immunological profile is not completely elucidated, and few clinical studies exist. These studies have mainly investigated high-dose exposure to nickel, causing a clinical reaction. It is not known if and how the immune system responds to low-dose exposure to nickel nor if a subclinical response can be detected.

Despite many years of enforced regulation of nickel release from consumer items in Europe,[12,21] nickel is still the leading cause of contact allergy. There are several possible explanations for the persistently high incidence and prevalence of nickel allergy in younger generations. One such explanation could be

that the regulations may not be respected or may not offer sufficient protection. In this study, we have investigated the clinical and subclinical immune response of low-dose nickel exposures on skin having a local memory response to nickel, to further elucidate the safety of the regulatory limit values from both a clinical and immunological perspective.

3. METHODS

3.1. STUDY POPULATION

We recruited 13 nickel-allergic adult participants and 13 healthy volunteers as controls. The inclusion of nickel-allergic participants was based on a positive patch test between 2017-2020 of at least 2+ according to ESCD criteria[22] to nickel sulfate 5% at a patch test performed in the Department of Dermatology and Allergy, Herlev Gentofte hospital. Exclusion criteria for both groups were pregnancy or breastfeeding, use of topical corticosteroids on the lower back, systemic immunomodulatory treatment within the past 2 weeks, and prolonged exposure to ultraviolet radiation such as solarium or sunbathing within 3 weeks. The nickel allergic group consisted of 12 females and 1 male with a median age of 47.2 years (range 22-66), while the control group consisted of 9 females and 4 males with a median age of 30.0 years (range 21-62). There was no significant difference in age between the two groups. All participants gave informed written consent to the study and to the publication of their results including photos of skin reactions. The study was approved by the ethical committee of the Capital Region of Denmark (H-19080328) and the Danish Data Protection Agency and registered in ClinicalTrials.gov (NCT04438330).

3.2. STUDY DESIGN

The study was a double-blinded clinical trial divided into two patch test periods with 3-4 weeks of rest between patch test periods (Figure 1, A). The first patch test (Patch test A) consisted of six chambers: four with the diagnostic concentration of nickel sulfate 5% (2000 μg/cm^2) in petrolatum (pet.), an empty control, and a vehicle (pet.) control. It was placed on the participant's lower back on day 0 and the exact location was photographed and noted by proximal and distal measurements to the spine, hip, scapula, and nearby birthmarks. On day 2, the border of the patch test was marked with a skin marker and the patch was removed. Reactions were scored according to an experimental scale (0-8);[16,23] an extension of the official ESCD criteria,[22] where *+?* was equal to 1-3, *+* equal to 4-6, *++* equal to 7, and *+++* equal to 8 (supplementary table 1). On day 4 and day 7 reactions were read again. The allergic reactions were left to heal over three to four weeks and the participant was instructed to frequently redraw the marked area until the end of the study. Patch test B consisted of an experimental dose range of nickel sulfate dissolved in demineralized water (aq.) to obtain a more uniform solution at low Ni concentrations (Figure 1, B). On day 0 of Patch test B, the exact area was identified by the participant's continued skin marking, photograph, and measurements, and the patch test was placed. The patch was removed on day 2 and potential reactions were scored. Punch biopsies of 4 mm were also taken from each exposed area on day 2. The biopsies intended for transcriptomic

analysis were stored in RNA*later* (Invitrogen, Thermo Fisher Scientific) for 24h at 4°C and then at -20°C until analysis. Biopsies intended for qPCR were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

All patch testing was done at the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev-Gentofte, Denmark, according to standard procedure in 8 mm Finn chambers with 20 mg sample per chamber. All samples were prepared in-house with nickel sulfate ($NiSO_4 \cdot 6H_2O$, Merck KGaA, Darmstadt, Germany, ≥98%, CAS no. 10101-97-0) dissolved in either petrolatum (Patch test A) or demineralized water (Patch test B) and color-coded. All readings were done by MW and assisting nurse; both participants and assessor were blinded.

3.3. MULTIPLEX TRANSCRIPTOMIC ANALYSIS

All six collected skin biopsies from five nickel allergic participants and five controls were subjected to multiplex transcriptomic analysis by nanostring nCounter on the Human Immunology V2 panel consisting of 594 target genes at BioXpedia A/S, who also performed the preliminary RNA extraction, purification, and quality control. Basic statistics of the results including differential expression analysis were also made by BioXpedia A/S.

3.4. QPCR

All six collected skin biopsies from eight nickel-allergic participants and eight controls were homogenized with Precylles 24 homogenizer (Bertin Instruments). Total RNA was extracted and purified with an RNeasy mini kit and RNase-Free DNase Set (Both from Qiagen) according to the manufacturer's instructions. RNA concentration and quality were measured with Nanodrop 2000 (Thermo Fisher Scientific). qPCR was performed using 100 ng of RNA for each sample using the TaqMan™ Fast Virus 1-Step Multiplex Master Mix (Thermo Fisher Scientific) and the following TaqMan™ Gene Expression Assays (*CCRL2* Hs00243702_s1; *CXCL8* Hs00174103_m1; *EEF1A2* Hs00951278_m1; *IL1B* Hs01555410_m1; *IL18RAP* Hs00187256_m1) (Thermo Fisher Scientific) and measured on Lightcycler 480 (Roche Diagnostics).

3.5. STATISTICS AND DATA ANALYSIS

nCounter transcriptomic results were normalized using nSolver 4.0 (Nanostring) and the geNorm algorithm.[24] Results were grouped by exposure and differential expression analyses and volcano plots were made by BioXpedia in R version 4.0.2 and plotted with the R-package ggplot2.[25,26] Normality in the groups was tested with Shapiro-Wilk test decisive for subsequent analysis with independent

sample t-test or Wilcoxon rank sum test. Results are presented as non-adjusted p-values with a significance level below 0.05. Heatmaps and Venn diagrams were made in R version 4.2.1 with the R-packages pheatmap and eulerr.[26–28]

In qPCR the target gene expression was normalized human *EEF1A2* using ΔΔCt calculation and results are presented as relative exposure to the participant's own blank (empty) control. The results were considered significant at a P value less than 0.05, data are represented as the mean with SEM (standard error of the mean). Data visualization and statistical analyses were performed with GraphPad Prism 9 (GraphPad Software) using the unpaired Student t-test or Ordinary one-way ANOVA with Šídák´s multiple comparison test.

4. RESULTS

4.1. NICKEL DERMATITIS FROM REEXPOSURE TO LOW-DOSE OF NICKEL SULFATE

To investigate the potential of low-dose nickel exposure to elicit nickel allergic dermatitis in skin areas previously exposed to nickel, the skin reactions from the two patch tests were evaluated by comparison with control exposures by blinded assessors. The dose range was based upon a dose-response metanalysis[29] where 95% of nickel-allergic individuals were estimated to have a clinical patch reaction to 370 μg/cm^2 and 50% to 12.8 μg/cm^2, along with the regulatory concentrations[6] 0.5 μg/cm^2 and 0.2 μg/cm^2, a vehicle control of demineralized water and blank control. The clinical response was scored both after Patch test A and B using an experimental scale (Supplementary table 1). In Patch test A, all nickel allergic participants scored 4 (+) or more on day 2 or day 4 readings, performed with 5% (2000 μg/cm^2) nickel sulfate pet. confirming their allergy. After a rest period of 3 to 4 weeks, re-exposure was made to a dose range of nickel, determined as explained above. No clinical reactions were observed among the healthy participants. In total, 12 of 13 (92%) nickel-allergic individuals had a clinical reaction with a score of at least 3 to the highest dose of 370 μg/cm^2 (corresponding to 0.925% nickel sulphate). Among these, 10 (77%) reacted with scores of 5 or 7, which are equivalent to + or ++ per ESCD criteria. Eight of 13 (62%) nickel allergic individuals had a clinical reaction scored 1-3 to 12.8 μg/cm^2 (0.0320%), while two of 13 (15%) and one (8%) had a reaction scored 1-2 to 0.5 μg/cm^2 (0.0125%) and 0.2 μg/cm^2 (0.005%), respectively (Figure 1, D, E, and supplementary table 2). Though the allergic responses generally were of clinically mild degree (Figure 1, C), they were clearly distinguishable from control exposures. A dose 10,000 times lower than what is typically used for diagnostic testing was sufficient to trigger a clinically visible allergic response. This indicates that an immune response must be activated at the molecular level even at such low doses.

4.2. RE-EXPOSURE TO LOW LEVELS OF NICKEL INDUCES IMMUNE ACTIVATION

To examine the possibility of a subclinical immune response to nickel exposures, biopsies were collected from each exposure site and immune-related transcripts were analyzed by nanostring nCounter. Differential expression analysis comparing gene expression of immune-related genes in skin areas exposed to nickel compared to vehicle exposed skin were made. In nickel-allergic individuals, 208 (201 up-regulated, seven down-regualted) significantly differentially expressed genes (DEGs) were identified at skin areas exposure to 370 μg/cm^2 of nickel, 287 (all up-regulated) DEGs of 12.8 μg/cm^2 of nickel, and 101 (100 up-regulated, one down-regulated) DEGs of 0.2 μg/cm^2 of nickel (Figure 2, A,

B). The results from the 0.5 μg/cm^2 exposure group were excluded due to low mRNA content and high normalization factors. Interestingly a broad immune response was seen to 0.2 μg/cm^2, driven by all participants, despite none of these participants having a clinical reaction to this exposure (supplementary table 2). The response pattern was similar in all skin areas re-exposed to nickel, with the highest number for up-regulated genes found in skin biopsies collected for skin areas re-exposed to 12.8 μg/cm^2 of nickel (Figure 2, A, B). Interestingly, 81 DEGs, all upregulated, were found in all skin biopsies that had be re-exposed to nickel compared to the vehicle exposed skin arease indicating that a similar immune response are induced which seems to be independent of the clinical presentation (Figure 2, B).

Few DEGs was found in skin upon re-exposure to nickel in healthy participants. Nine (one up-regulated, eight down-regulated) significantly regulated DEGs were found in skin upon re-exposed to 370 μg/cm^2 of nickel, three (two up-regulated, one down-regulated) DEGs were found in skin areas re-exposed to 12.8 μg/cm^2 of nickel, and three (two up-regulated, one down-regulated) DEGs were found in skin areas re-exposed to 0.2 μg/cm^2 of nickel. One gene was significantly upregulated in all three exposures, namely LY96 (Figure 2, A, B). Comparing the DEGs found in skin re-exposed to nickel of the nickel allergic group with the DEGs found in the healthy control group the majority of DEGs are only regulated in the allergic individuals (Figure 2, C). Thus, showing that a specific subclinical immune activation is induced in skin of nickel allergic individuals upon re-exposure to nickel, also with low dosis of nickel, which is not seen in healthy controls.

4.3. UP-REGULATION OF CYTOKINES AND CHEMOKINES DRIVE THE IMMUNE RESPONSE TO NICKEL

To further investigate what drives the immune response in the allergic group, we subsetted the different transcriptomic markers after nanostring's gene annotations (nSolver). We found that the majority of differential expressed immune-related genes induced upon re-exposure of the skin to nickel in nickel allergic individuals where related to cytokine/chemokines responses. In skin re-exposed to 370 μg/cm^2 of nickel, 93 (90 upregulated, and three downregulated) DEGs related to cytokine/chemokine response were found (Figure 3, A). In accordance 135 DEGs related to cytokine/chemokines responses were found, all up-regulated, in skin upon re-exposure to 12.8 μg/cm^2 of nickel, and 46 DEGs (45 upregulated and one downregulated) related to cytokine/chemokines responses were found in skin upon re-exposure to 0.2μg/cm^2 of nickel (Figure 3, A). The immune response showed a similar phenotypic profile across the different nickel concentrations but varied in the intensity of fold change, where the strongest response was found in

skin areas re-exposed to 12.8 µg/cm^2 of nickel (Figure 3, A, B). Re-exposure of the skin to either of the three nickel dosis resulted in an up-regulation of 22 cytokines and 17 chemokines. Among these were *IL1B*, *IL8*, *IL18RAP*, and *CCRL2.* We sought to validate these findings through qPCR. It should be noted that there was a large individual variation among participants. However, the baseline variance was similar in both the allergic and healthy groups, as shown by their response to the vehicle (Aq.) control (Supplementary figure 1). The analyzed cytokines (IL1B, IL8, and IL18RAP), and chemokine (CCRL2) show a tendency of dose-dependent upregulation with the highest response to the highest exposure dose (370 µg/cm2) in the allergic group (Figure 3, C). Interestingly, there was no correlation between the measured response at the transcriptional level and the clinical score. This indicates that the expression of various cytokines and chemokines already are induced at a subclinical level independent from clinical presentation.

5. DISCUSSION

To the best of our knowledge, this is the first study to investigate the subclinical response to low-dose nickel exposure. Our results reveal immune activation in nickel-allergic individuals exposed to various doses of nickel on skin areas with local memory, irrespective of the presence of clinically elicited dermatitis. Notably, we detected subclinical inflammation at levels typically deemed safe, which also elicited a clinical allergic response in two individuals when exposed to skin with local memory to nickel.

The regulatory limiting values, 0.2 and 0.5 μg/cm^2/week, are generally considered safe for nickel allergic individuals, though few studies have assessed the risk of these low-level exposures. Our findings indicate that these low levels may elicit allergic nickel dermatitis: 15% (2/13) of the allergic participants had a reaction to re-exposure of 0.5 μg/cm^2 of nickel and 8% (1/13) to 0.2 ug/cm^2 in skin areas previously exposed to 2000 μg/cm^2 of nickel.

A previous study found a significant increase in local vesicle formation and blood flow after repeated daily exposure to 0.01% nickel chloride (comparable to 0.4 μg/cm^2), suggesting frequent low-dose environmental exposures to nickel could contribute to and maintain dermatitis and/or hand eczema in patients with nickel allergy.[30] Another study have found lowest threshold for patch testing was 0.5 μg/cm^2, while in a repeated open application test (ROAT), a dose of 0.035 μg/cm^2 twice daily over a week elicited a reaction in 4 out of 18 participants.[23] Furthermore, it has been shown that nickel accumulates in the skin during repeated exposures,[31] increasing the risk of elicited dermatitis.[32] Although our reported allergic reactions to low-dose exposures were mild, continued, or repeated exposure could potentially worsen dermatitis or prolong a current skin lesion.

The clinical scoring was performed double-blinded, with two independent assessors, and the reaction was visibly distinguishable from the control exposures. One participant had a minor reaction (score 2) to the 12.8 μg/cm^2 concentration and no reaction to 370 μg/cm^2. This participant had weak reactions (score 2-4) to the initial challenge of 2000 μg/cm^2. It is difficult to explain why there was no reaction to the 370 μg/cm^2 challenge. However, the pathogenesis of nickel dermatitis is complex, and the skin barrier is not uniform. Additionally, there is significant variability in the elicitation threshold to nickel exposure.[33]

We observed a significant increase in the expression of immune-related genes at a low dose of 0.2 μg/cm^2 in nickel-exposed skin areas without clinical signs of allergic dermatitis. This, suggesting an immune response to the presence of nickel ions. However, this response was not sufficient to trigger clinically relevant dermatitis. Interestingly, only 4 out of 101 DEGs at 0.2 μg/cm^2 were unique to this

dose, indicating a similar immune response independent of clinical presentation. This suggests that the threshold for eliciting an allergic response may not be fixed, but rather fluid, with inflammation signaling potentially accumulating before manifesting as dermatitis. In contrast, previous research reported a tendency towards clinical dependency in transcriptomic response to nickel in patients with 2+ or 3+ reactions compared to those with 1+ reactions, albeit at 5% nickel exposure.[34] Our study found a broader and more intense response at 12.8 µg/cm^2 than at 0.2 and 370 µg/cm^2. It may be a matter of kinetics, that the reaction develops with a different pace dependent on the size of stimulus. A study has found increased immune response to 96h of 5% nickel exposure compared to 48h,[19] though further studies on kinetics of low dose exposure in relation to immune activation are needed. Interestingly, the healthy participant group showed a significant upregulation of *LY96* for all exposures. LY96, also called MD2 (myeloid differentiation factor 2), is involved in the activation of TLR4,[13,14] the interface for an allergic response to nickel ions.[13] It has been described in cell studies that both TLR4 and LY96 are required for an inflammatory response to nickel ions and thus crucial to initiate the sensitization phase of nickel ions.[14] Despite the healthy controls does not show an allergic response to nickel, it is notable that we report upregulation of a crucial component of the nickel sensitization phase, even at low levels of nickel exposure. However, the sensitization phase is inherently more intricate,[35] and it has also been reported that nickel and cobalt ions can activate TLR4 independently from LY96,[36] although disputed.[37]

In response to increasing doses of nickel exposure, various cytokines were found to be upregulated. IL1B and IL18, both members of the IL1 family of cytokines, play a central role in regulating inflammatory and immune responses to contact allergens.[35,38–40] Our results are consistent line with previous reports that *IL1B* is upregulated in nickel dermatitis.[17,19,38] Notably, *IL1B* also appears to be upregulated at low exposures (0.5 µg/cm^2) in some participants, indicating a potential role in the early stages of the elicitation phase.

Like IL1B, IL18 is a pro-inflammatory cytokine that is upregulated in response to TLR4 activation.[19,38] While, we observed a significant upregulation of the IL18 acceptor-associated protein, *IL18RAP*, at 370 µg/cm^2 exposure, the involvement of IL18 in nickel dermatitis remains unclear. IL8 is used, among others, in *in vitro* assays to identify contact sensitization for the purpose of risk assessments.[18] Produced by dendritic cells and keratinocytes, IL8 and other cytokines promote neutrophil and T cell migration to the exposed skin area.[41] A previous *In vitro* study has found that *IL8* is upregulated in response to contact with metal allergens and downregulated for irritant exposures.[42] Additionally, a transcriptomic study in humans demonstrated upregulation of IL8 in skin biopsies from nickel contact dermatitis,[19] consistent with our findings.

In conclusion, our findings present evidence of immune activation in response to low-dose nickel exposure in skin with local memory even in the absence of a clinical reaction. We also demonstrate that exposure to the regulatory limits defined in the European nickel regulation may cause nickel dermatitis in skin areas having local memory to nickel. Additionally, we observed an upregulation of a key immunological component in nickel sensitization in non-sensitized participants. Hence, it could be discussed if these current regulatory limits pose sufficient protection for consumers against nickel allergy.

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7. FIGURE LEGENDS

Figure 1. Study design and clinical results A: Schematic of study design. B: Concentrations of induction patch test (A) and experimental patch test (B). C: Clinical reactions from experimental doses (patch test B) on pre-exposed skin. D: Number of allergic participants with clinical reactions to the different nickel dose challenges (n = 13). E: Dose-response curve of nickel concentrations and clinical reading score.

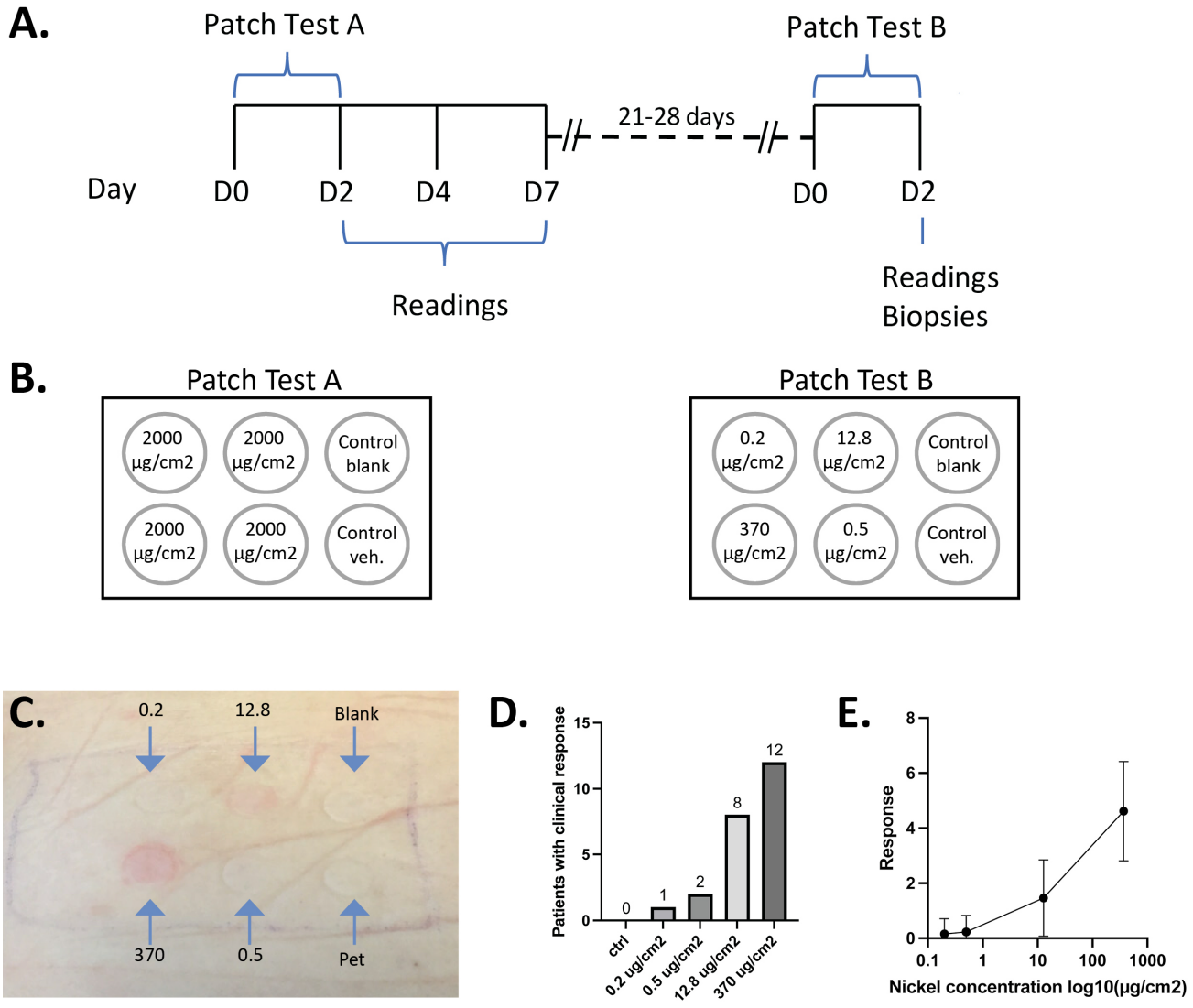


Figure 2. Nickel exposure induce immune activation upon re-exposure in allergic individuals. Transciptomic results from Nanostring nCounter analysis on the Human Immunology V2 panel from skin biopsies taken on day two of nickel re-exposure. A: Heatmaps of genes that are differentially expressed in one or more exposure for 0.2 μg/cm^2, 12.8 μg/cm^2, and 370 μg/cm^2 exposures compared with vehicle control (Aq.) for allergic and healthy groups. B: Venn diagrams showing the overlap of significant DEGs in different exposures in both allergic and healthy participants, along with the number of DEGs in each exposure group. C: Volcanoplots of DEGs in the allergic and healthy groups for exposures to 0.2, 12.8 and 370 μg/cm^2 of nickel.

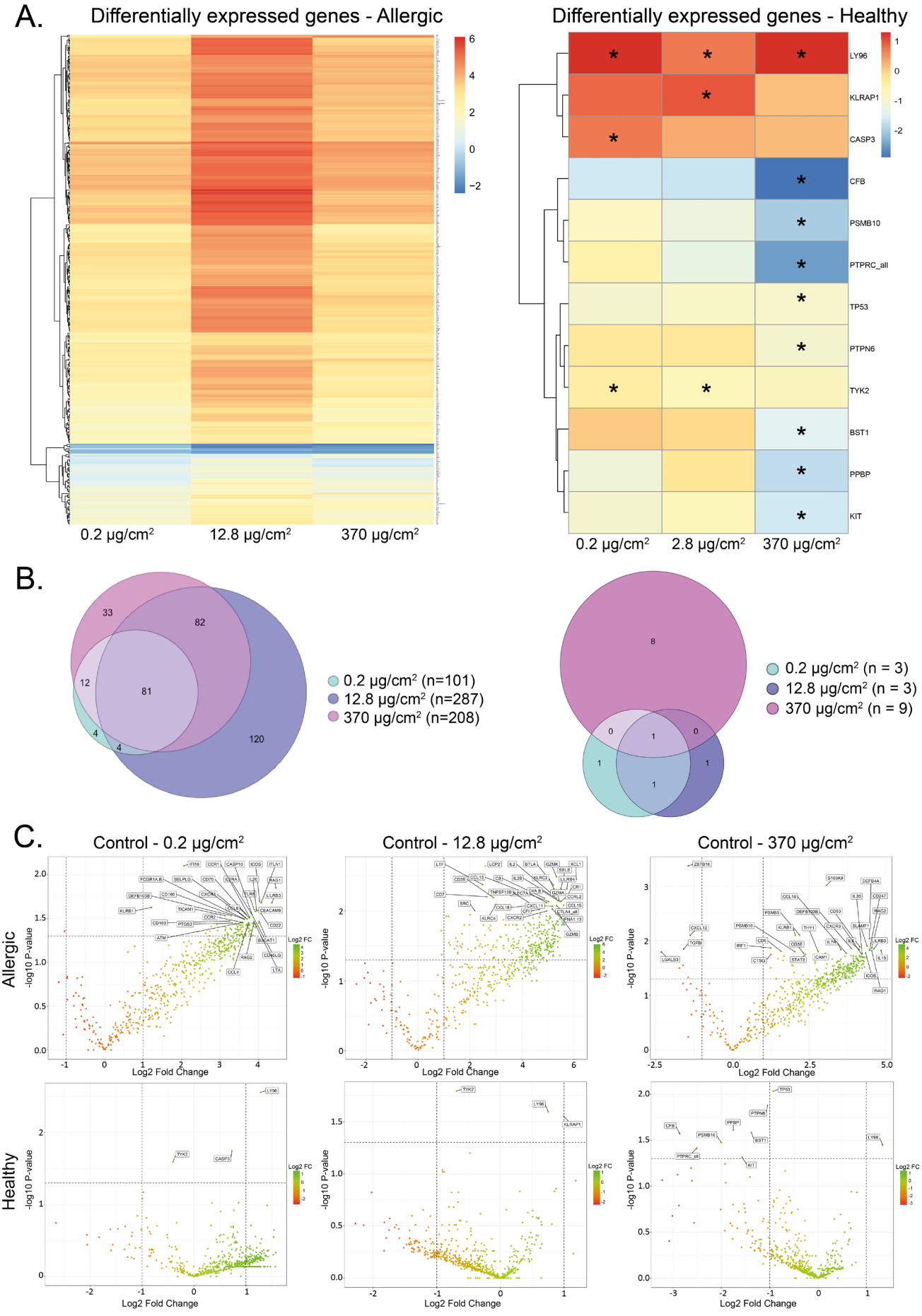
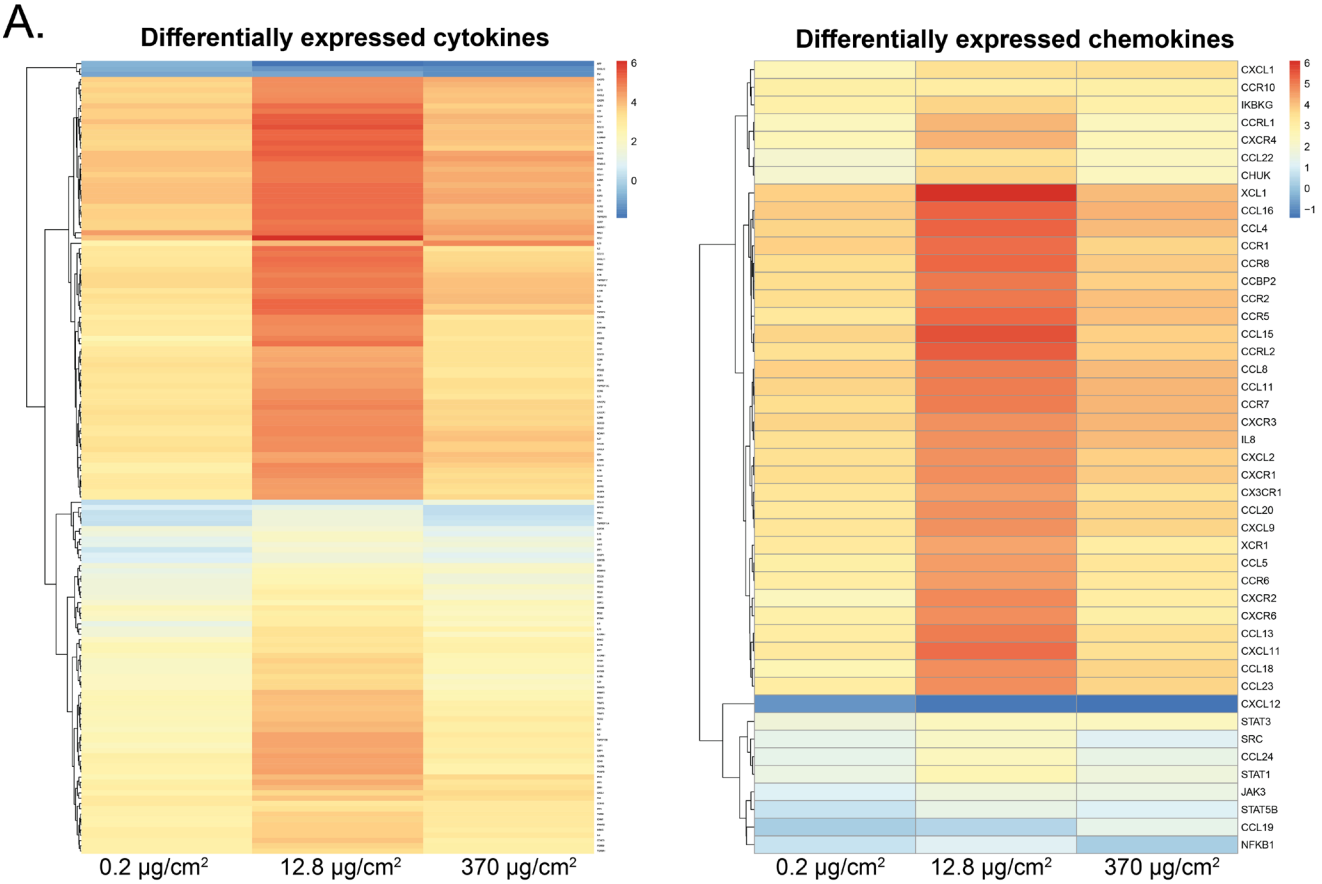
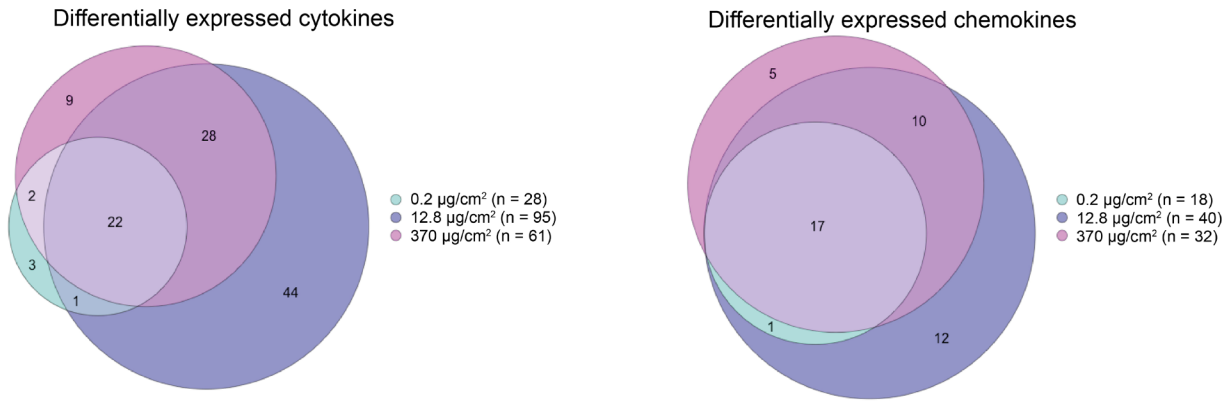

A.
Differentially expressed genes - Allergic
0.2 µg/cm²
12.8 µg/cm²
370 µg/cm²
Differentially expressed genes - Healthy
LY96
KLRAP1
CASP3
CFB
PSMB10
PTPRC_all
TP53
PTPN6
TYK2
BST1
PPBP
KIT
0.2 µg/cm²
2.8 µg/cm²
370 µg/cm²
B.
33
82
12
81
4
4
120
0.2 µg/cm² (n=101)
12.8 µg/cm² (n=287)
370 µg/cm² (n=208)
8
0
1
0
1
1
1
0.2 µg/cm² (n = 3)
12.8 µg/cm² (n = 3)
370 µg/cm² (n = 9)
C.
Control - 0.2 µg/cm²
Control - 12.8 µg/cm²
Control - 370 µg/cm²
Allergic
Healthy
-log10 P-value
Log2 Fold Change
Log2 FC

Figure 3: The immune response to nickel is driven by the up-regulation of cytokines and chemokines. Transcriptomic results are from Nanostring nCounter analysis on the Human Immunology V2 panel from skin biopsies taken on day two of nickel re-exposure. A: Heatmaps of cyto- and chemokines that are differentially expressed in one or more exposure for 0.2 μg/cm^2, 12.8 μg/cm^2, and 370 μg/cm^2 nickel exposures compared with vehicle control (Aq). B: The Venn diagram displays the number of significantly differentially expressed cyto- and chemokines in each exposure group, as well as the overlap between the exposures in both allergic and healthy participants. C: qPCR results of five selected immune-related targets in a different cohort. Results are presented as the participant's relative expression to their blank control exposure. One-way ANOVA with Šídák´s multiple comparison test, * $P < 0.05$, *** $P < 0.001$.

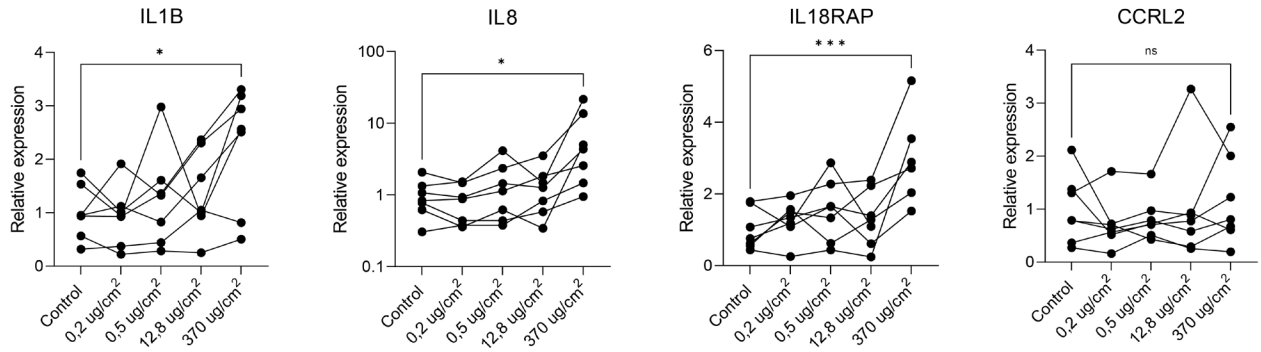
A.


Differentially expressed cytokines
Differentially expressed chemokines
0.2 µg/cm²
12.8 µg/cm²
370 µg/cm²
CXCL1
CCR10
IKBKG
CCRL1
CXCR4
CCL22
CHUK
XCL1
CCL16
CCL4
CCR1
CCR8
CCBP2
CCR2
CCR5
CCL15
CCRL2
CCL8
CCL11
CCR7
CXCR3
IL8
CXCL2
CXCR1
CX3CR1
CCL20
CXCL9
XCR1
CCL5
CCR6
CXCR2
CXCR6
CCL13
CXCL11
CCL18
CCL23
CXCL12
STAT3
SRC
CCL24
STAT1
JAK3
STAT5B
CCL19
NFKB1

B.


Differentially expressed cytokines
9
28
2
22
3
1
44
0.2 µg/cm² (n = 28)
12.8 µg/cm² (n = 95)
370 µg/cm² (n = 61)
Differentially expressed chemokines
5
10
17
1
12
0.2 µg/cm² (n = 18)
12.8 µg/cm² (n = 40)
370 µg/cm² (n = 32)

C.


IL1B
IL8
IL18RAP
CCRL2
Relative expression
Control
0,2 ug/cm²
0,5 ug/cm²
12,8 ug/cm²
370 ug/cm²
*
*
* * *
ns

8. SUPPLEMENTARY

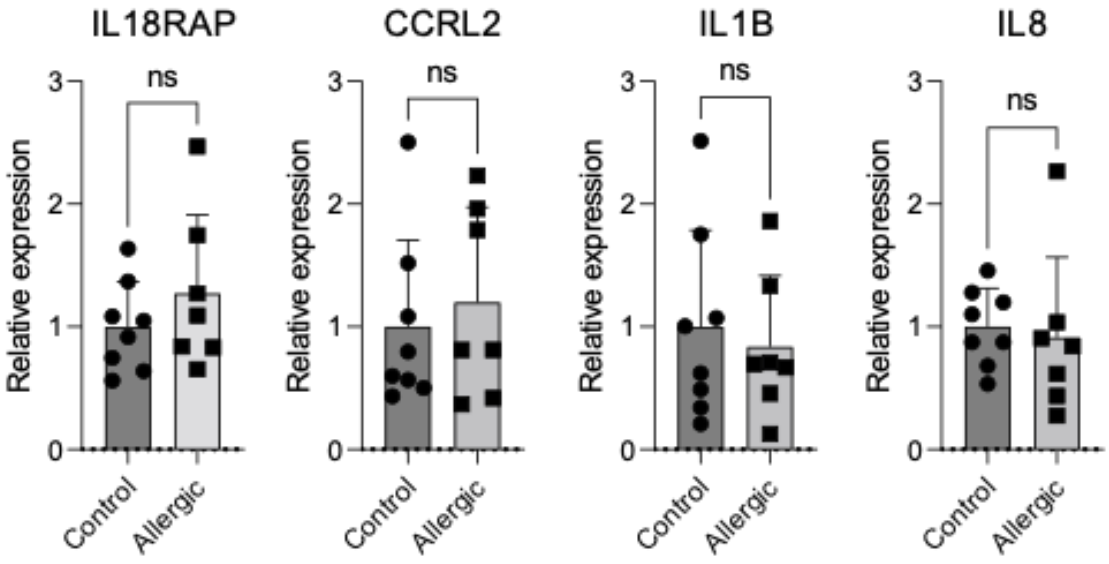
Supplementary table 1. Extended experimental scale for scoring severity of allergic contact dermatitis based on the ESCD criteria.

Score	*Morphology*	*ESCD equivalent*
-	No reaction	-
1	Few papules, no erythema, no infiltration	+?
2	Weak erythema, no infiltration, no papules	+?
3	Weak erythema, few papules, no homogenous infiltration	+?
4	Erythema, homogenous infiltration	+
5	Erythema, infiltration, few papules	+
6	Erythema, infiltration, papules	+
7	Erythema, infiltration, papules, few vesicles	++
8	Strong erythema, infiltration, vesicles, possible bulla	+++

Supplementary table 2: Patch test reaction at re-exposure (Patch test B) 3-4 weeks after patch testing with 5% nickel sulfate. ESCD equivalent to scorings: 1-3: +?; 4-6: +; 7: ++; 8: +++. Samples from participant no. 11 were excluded from qPCR due to poor RNA concentration.

ID	*370 µg/cm2*	*12.8 µg/cm2*	*0.5 µg/cm2*	*0.2 µg/cm2*	*Vehicle*	*Blank*	*Biopsy Analysis*
1	5	1	0	0	0	0	nCounter
2	3	0	0	0	0	0	nCounter
4	5	3	0	0	0	0	nCounter
5	7	3	0	0	0	0	nCounter
6	7	3	0	0	0	0	nCounter
3	3	0	0	0	0	0	qPCR
7	5	0	0	0	0	0	qPCR
8	5	3	0	0	0	0	qPCR
9	5	0	0	0	0	0	qPCR
10	5	0	2	2	0	0	qPCR
11	5	3	0	0	0	0	~~qPCR~~
26	5	1	1	0	0	0	qPCR
29	0	2	0	0	0	0	qPCR
Total reactions	**12**	**8**	**2**	**1**	0	0	

Supplementary figure 1: The nickel allergic and healthy group elicit a similar high baseline variance. Relative expression of IL18RAP, CCRL2, IL1B, IL8, and GZMA in vehicle control (Aq.) exposure in a patch test of the control group and the allergic group analyzed by qPCR. Data represent mean with SEM. Student's t-test.



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