Cobalt in the hard metal production industry - exposure via inhalation and skin and the inflammatory response in human keratinocytes

I dedicate this thesis to my parents and to my children

Örebro Studies in Medicine 220

MARIA KLASSON

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Abstract

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Cobalt is a strong sensitizer and can cause contact allergy upon both direct contact or from airborne exposure on the skin. In the skin, keratinocytes are the first cells to come in contact with the metal and will react and respond to the danger by triggering an alarm system resulting in an inflammatory response in the skin. Keratinocytes have been shown to produce IL-1β, which is one of the most potent inflammatory agents in our body and is associated with a variety of diseases.

The aims of this thesis was to investigate cobalt air concentrations for different particle fractions for possible use as proxies for other article measures and to examine if cobalt skin and inhalable air exposure contributes to uptake. Also, to investigate the effect of cobalt on cultured human keratinocyte cell viability, pro-inflammatory cytokine/chemokine release and NLRP3 inflammasome activation using cells cultured at low or high calcium (the latter yielding a more differentiated cell type).

Air exposure to cobalt was found in all departments and for all work tasks in the hard metal production facility and exposures were in general below the Swedish OEL for inhalable cobalt. The highest exposure levels were found in the powder production department and for laboratory and furnace work. Good correlations for the mass based measures enable us to use the findings for future references. When personal inhalable air levels of cobalt, cobalt skin levels skin and biological monitoring of cobalt in blood were analysed, the skin exposure was determined to be import as a route of uptake. Skin exposure to cobalt in the hard metal industry, could further affect the total uptake in the same order of magnitude as air exposure. *In vitro* investigations of cobalt using the human keratinocyte cell line HaCaT, showed that $CoCl_2$ triggered an alarm system in cells where the proinflammatory cytokines/chemokines IL-6, CXCL8 and CCL2, known to be involved in skin inflammation, were secreted in a time- and dose-dependent manner. Comparing HaCaT cells of high- and low differentiation stages indicated that the effect of cobalt chloride on cell toxicity occurs throughout the living epidermis. $CoCl_2$ exposure also resulted in secretion of the pro-inflammatory cytokines IL-1β and IL-18, and caspase-1, which indicates activation of the NLRP3 inflammasome in the cells. $CoCl_2$ regulates the inflammasome both as primer and as an activator. Our mRNA results indicates a negative feedback mechanism in the inflamamsome due to the exposure. The inflammatory response in general is more dose than time dependent, which be important for understanding the mechanisms of allergic sensitization.

Keywords: cobalt, cobalt chloride, skin, HaCaT, in vitro, cell viability, pro-inflammatory cytokines, NLRP3 inflammasome.

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I. **Maria Klasson**, Ing-Liss Bryngelsson, Carin Pettersson, Bente Husby, Helena Arvidsson, Håkan Westberg. **Occupational exposure to cobalt and tungsten in the Swedish hard metal industry – air concentrations of particle mass, number and surface area.** *Annals of Occupational Hygiene 2016, Jul;60(6):684-99*.

II. **Maria Klasson**, Magnus Lindberg, Ing-Liss Bryngelsson, Helena Arvidsson, Carin Pettersson, Bente Husby, Håkan Westberg. **Biological monitoring of dermal and air exposure to cobalt at a Swedish hard metal production plant: Does dermal exposure contribute to uptake?** *Contact Dermatitis, 2017 Oct;77(4):201-207*

III. **Maria Klasson**, Magnus Lindberg, Eva Särndahl, Håkan Westberg, Ing-Liss Bryngelsson, Kaya Tuerxun, Alexander Persson. **Dose- and time-dependent changes in viability and IL-6, CXCL8 and CCL2 production by HaCaT-cells exposed to cobalt. Effects of high and low calcium growth conditions.** (Manuscript)

IV. **Maria Klasson**, Magnus Lindberg, Ing-Liss Bryngelsson, Håkan Westberg, Kaya Tuxerxun, Alexander Persson, Eva Särndahl. **Dermal exposure to cobalt – possible effects of cobalt exposure on inflammasome, cytokine and mRNA response studied *in vitro* in keratinocytes.** (Manuscript)

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LIST OF ABBREVIATIONS

ASC	Apoptosis-associated speck like protein containing a CARD
B cells	B lymphocyte, a type of white blood cell
BEI	Biological Exposure Index
CARD	Caspase activation and recruitment domain
CASP-1	Caspase 1/ interleukin 1 converting enzyme
$CoCl_2$	Cobalt Chloride
CVD	Chemical vapor deposition
CXCL8	Chemokine (C-X-C motif) ligand 8 (same as IL-8)
DAMP	Danger/damage associated molecular pattern
Data RAM	Data-logging Real-time Aerosol Monitor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EC50	Half maximal effective concentration
HaCaT	Cultured human keratinocytes
HNO_3	Nitric acid
ICP-MS	Inductively coupled plasma mass spectrometry
IgE	Immunoglobulin E, a type of antibody
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-18	Interleukin 18
NLR	(NOD) like receptors
$NLRP_3$	NOD like receptor protein 3
NOD	Nucleotide oligomerization domain
OEL	Occupational Exposure Limit
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PM	Particulate matter
PRR	Pattern Recognition Receptor
PVD	Physical vapor deposition
PYD	Pyrin domain
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
T cells	T lymphocyte, a type of white blood cell
TLR	Toll Like Receptor
TWA	Time-Weighted Average
WC	Tungsten Carbide

BACKGROUND

Prologue

Cobalt is a metal used in many applications. It is found in, for example aircraft engine parts, tools, dental and other implants, jewelry, leather, batteries, and as a coloring agent. However, the exposure to cobalt endangers a risk to our health and problems resulting from exposure is found in the general population and in a variety of work environments. Both air exposure from use of the cobalt containing products and direct contact may lead to adverse health effects.

Occupational exposure to cobalt is well known to occur in the hard metal manufacturing or from the use of hard metal production in engineering. Hard metal is an alloy based on tungsten carbide in which cobalt is used as a binder, and the addition of cobalt allows increased resistance to wear, as it increases hardness and toughness in tools in which the alloy is used. Despite the desired qualities of cobalt, exposure to the metal has been associated with a range of adverse health effects, including cancer and respiratory, lung, heart and neurological problems [1-5]. Air exposure in the hard metal industry has been well documented and in 2011, the occupational exposure limit (OEL) was decreased and changed from cobalt in the total dust fraction to the inhalable fraction. Also, there is increased interest in other particle measures and fractions because of the link between particle exposure size fractions and the different adverse health effects. The field of investigation regarding how particle fractions relate to one another has not been fully investigated, nor has the possibility of using different particle fractions as proxies been thoroughly investigated.

The uptake of cobalt occurs primarily through inhalation but also through the skin, and the relationship between skin uptake and blood and urine levels has been established [6, 7]. Cobalt ions are shown to penetrate the skin upon contact [8] and are toxic at the cellular level [9]. Keratinocytes, the dominant cell type in the skin, can produce several pro-inflammatory cytokines and chemokines known to contribute to the development of skin sensitization.

When I started work on this project there was no knowledge about the importance of skin uptake in relation to uptake via the respiratory tract during cobalt exposure, and we discussed the probability that particle exposure could be a significant source of uptake through the skin and not just uptake through inhalation. The relationship between exposure, cell toxicity

and inflammatory response, and disease development was, and still is poorly understood.

Regarding the inflammatory mechanisms elicited by body foreign substances, many mechanisms need to be elucidated. However, we do know that the inflammasome, which is a large intracellular multiprotein complex, plays a central role in the innate immune system and can detect and respond to several kinds of "danger" associated molecular patterns [10], including cobalt [11, 12]. It can also be activated by particles, such as asbestos, quartz and various types of nanoparticles [13]. Activation of the inflammasome via a receptor (NLRP) leads to the production and release of pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18, which occurs via the enzyme caspase-1 (the executing part of the inflammasome). IL-1β is one of the body's most powerful inflammatory agents by regulating the production of other cytokines [10, 14]. Inflammatory cytokines have been shown to play a key role in cardiovascular disease [15] and a variety of auto inflammatory diseases [16]. It is possible that upon exposure to cobalt, skin cells will react with an inflammatory response depending of the amount of exposure, but also that the duration of the exposure is of importance to the response.

When this PhD work started, no one had investigated if and how those parameters could affect the mechanisms known to be involved in skin sensitization to cobalt. Skin cells are known to be important cells with immune capacity, and are involved in innate as well as adaptive immune responses, but are often oversighted when talking about occupational skin exposure. Current OELs are based on adverse health effects linked to the exposure, but the underlying mechanisms of those health effects have not been taken into consideration. Cobalt skin exposure can lead to skin allergies since cobalt is one of the most common sensitizing metals [17-19] and approximately 4.5% of patch tested patients with eczema were allergic to cobalt [20].

Origin and use of cobalt

Cobalt is a silver grey metallic element with metallic properties and has the chemical symbol Co in the periodic table of elements. It can only be found in the Earth's crust in chemical combined form. Cobalt is a trace element and is required in small amounts in mammals as a part of vitamin B12, cobalamin.

The name cobalt is believed to originate from German miners around 16th century who were working in an unusually pesky ore which they believed to be silver. When the miners melted the ore, it did not melt properly and produced a toxic fume. The miners could not explanation why the silver disappeared and why the toxic fumes made them feel sick, other than that the ore was bewitched. The miners called the ore *kobold* after the devilish spirit from German folklore [21].

Cobalt compounds have been used for thousands of years as a blue coloring agent in ceramic glass, porcelain, pottery, tile and enamel. It was the Swedish chemist Georg Brandt who first isolated cobalt from the ore in 1735. However, it was not until the 20th century that, however, cobalt started being used for industrial purposes. In 1907, a scientist in the USA, E. Haynes, patented Stellite-type alloys that were highly resistant to corrosion and wear at high temperatures [22]. Cobalt was first added to tungsten carbide in 1923 to produce hard metals [23].

Cobalt is often used in alloys since it will provide cobalt-based alloys with many desired properties, such as resistance to wear, corrosion and heat. Cobalt-based alloys are used in hard metal tools, in the aerospace, jewelry, prosthetics, coins, stellite blades and computer shells for example [24-28]. In recent years, the demand of cobalt has increased due to the diverse range of industrial applications and the global production of cobalt has increased. Cobalt is also extensively used in rechargeable batteries [29], and is an important source of gamma rays used in cancer treatment [30]. Other sources of exposure to cobalt in our daily lives include cosmetics, detergents and leather tanning [31-33].

Cobalt in the hard metal production industry

Hard metal, also referred to as cemented carbide, is an alloy based on carbide cemented into a composite and with a metal acting as a binder. Cobalt or nickel are commonly used as binder matrixes in hard metals. The major compounds could be TiC (titanium carbide), TiN (titanium nitride), Ti(C)N (titanium carbide-nitride), and TaC (tantalum carbide) used as aggregates.

At the plant in which the cobalt measurements of this thesis were conducted, the hard metal produced comprises 70- 95% tungsten and 5- 30% cobalt.

Production of hard metal

The production of hard metal tools involves series of carefully controlled steps (shown in figure 1) to enable the final product to obtain specific properties and structure. The first step is the formation of tungsten carbide from tungsten oxide and elementary carbon through carburization to form tungsten carbide (WC) powder. Tungsten carbide and cobalt powders are then mixed, followed by granulation of the mixture. After granulation, the material is pressed into compacts, pre-sintered (heated to 500 °C) in an atmosphere of hydrogen or water to remove lubricant and then accurately machined into the desired shapes. Pieces such as cutting tool inserts are then sintered under vacuum at 1400- 1500 °C, depending on the amount of binder metal and the desired microstructure. During the final sintering, the binder melts and carbide particles are drawn together and will further compact and get the hardness near diamond. The products are sand blasted and covered with a protective layer. The last step is quality inspection of the finished product before storage and shipment. Exposure to cobalt occur at all steps of the production process. The amount of exposure for the specific production step can vary, due to the nature of the work.

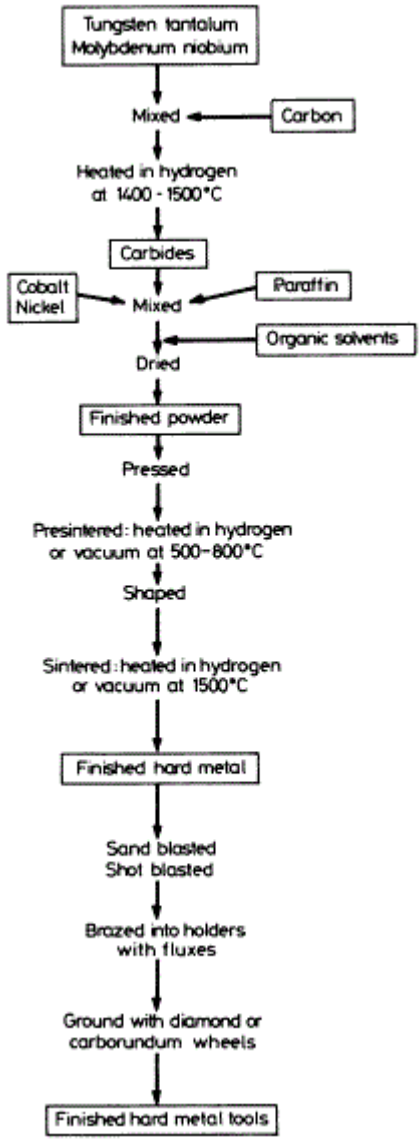

Figure 1. Process steps in the manufacturing of hard metal. Reproduced from Respiratory diseases in hard metal workers: an occupational hygiene study in a factory, Kusaka Y, Yokoyama K, Sera Y, Yamamoto S, Sone S, Kyono H, Shirakawa T, Goto S., Br J Ind Med. 1986;43(7):474-485, with permission from the BMJ Publishing Group Ltd.

Cobalt levels in air

Many individuals are exposed to dust and particles in their workplace. Conducting air measurements and the corresponding magnitude can help evaluate whether there is a health risk. Most previous occupational studies in the hard metal production industry have focused on cobalt in air. Occupational exposure measurements in the Japanese hard metal industry involving 275 participating workers in one study and 282 workers in another study, found cobalt air concentrations varying between 1 $\mu g/m^3$ and $6.39x10^3$ $\mu g/m^3$ [4, 34]. All steps in the manufacturing process were covered in the measurements, and the highest levels were found in the powder preparation area, followed by pressing, shaping and grinding departments. As a result of the study, local ventilation systems were installed in the Japanese factories for the work areas that had the highest exposure levels. In three French factories including 425 workers, the powder department generated the highest cobalt air levels [35]. The arithmetic means (AMs) presented were 45, 117, and 272 $\mu g/m^3$. A German study also reported cobalt air levels to be the highest in the powder department as ambient air levels ranged between 0.30 and 116 $\mu g/m^3$ [36]. Stefaniak et al. measured the total dust level in the hard metal production process and found levels between 128 and 4031 $\mu g/m^3$ [37] with the highest levels found in the powder mixing work area. Upon identification of the particles, they were found to be almost exclusively cobalt particles.

Measuring air levels is important since these assessments can be used to compare the level of exposure to the current exposure limits in order to prevent adverse health effects among workers. Industries can also monitor air quality in order to eliminate emissions and implement preventive measures to eliminate exposure.

Cobalt levels on skin (and surfaces)

A study measuring dermal exposure to cobalt in skin wipe samples from 41 hard metal workers across three different hard metal production facilities showed that the highest concentration was among the workers in the powder handling facility and also in areas of the metal separation and forming/machining facility [38]. The levels of cobalt found on the hands in the powder handling facility was 388 µg, for forming/machining workers 126 µg and for metal separation workers 88 µg. Administration employees had low to moderate cobalt levels on their hands. When cobalt on surfaces was measured, the highest level was found in the powder handling facility (690 $\mu g/100\ cm^2$). Another study conducted at companies manufacturing or

maintaining stellite blades found cobalt levels on skin measured with consecutive hand washing ranged between 0.4- 6.7 mg [7]. In an industry using cobalt containing hard metals for the development and manufacturing of gas turbines and space propulsion components, measurements were conducted on 37 workers in departments in which they handled cobalt [39]. The range of cobalt on all skin surfaces measured (forehead and areas on the hand) was 0.0013- 4.5 μg cm^2/h. The highest levels were found among workers who were sharpening hard metal tools.

Health effects and occupational cobalt exposure

In many workplaces, substances are emitted that can be harmful to the health. This include work shops, construction sites, foundries. However, airborne particle exposure in cities is also a worrying health issue. Exposures can be to dust and smoke, but also fumes from solutions and gases. Unfortunately, in the hard metal industry, exposure to particulate cobalt has been shown to potentially cause multiple adverse health effects. Exposure to the use and production of cobalt-containing alloys has implicated cobalt as the causing agent. The two main target organs are the skin and the respiratory tract, but other organs could also be affected.

Adverse health effects

Occupational exposure to cobalt is known to cause respiratory effects, including asthma [40-42], rhinitis, sinusitis, bronchitis [2, 35, 43], hard metal lung disease and dose-related decreased lung function over time in exposed workers [2, 42-45]. For people working in the hard metal production industry, an increased risk of developing lung cancer has been noted [5, 46, 47]. Concluded on the basis at the literature review, the International Agency for Research on Cancer has classified cobalt metal with tungsten as being probably carcinogenic to humans (Group 2A) [25].

Cardiomyopathy and myocardial disease have also been connected to cobalt exposure [48, 49], and in a cohort study of hard metal workers exposed to cobalt, an increased risk of ischemic heart disease was seen [3]. There have also been reports of cobalt causing systemic complications, such as audio and optic nerve symptoms and cognitive impairments as well as heart failure in patients with cobalt containing hip implants [1, 50-52].

Effects on the skin

Of particular interest in this thesis is the effect of cobalt on skin. Cobalt is an important sensitizer, which is known from clinical experience and from

animal testing [18, 53]. Cobalt is one of the most frequent and commonly encountered contact allergens in the work environment, and simultaneous exposure to several allergens is common in hard metal production and from using hard metal tools since a mixture of different metals is used in the alloy. Cobalt sensitization often coincides with nickel in the clinical patch testing of eczema patients [54], and nickel has been shown to act as an adjuvant during cobalt sensitization [55]. It has been suggested that there is a cross reaction or a concomitant reaction between cobalt and nickel [56]. However, more recent studies suggest that simultaneous reactivity to nickel-cobalt would be the result of multiple exposure and sensitization [57, 58].

Cobalt can cause allergic contact dermatitis upon exposure [17, 19, 59], and in some Northern European countries, 1- 3% of the population is allergic to cobalt [60]. In Sweden, approximately 4.5% of patch-tested patients with eczema were allergic to cobalt [20]. Cobalt allergy and hand eczema in hard metal workers has been shown to occur [53]. A case of hard metal lung disease concurrent with contact dermatitis suggests that allergies to the metal play a role in the onset of the disease [61].

Cobalt uptake and monitoring

The skin has not been given appropriate level of attention despite it being a target organ for exposure of many harmful substances. Exposure to cobalt can cause inflammation of the skin [17, 18] and uptake via the skin also contribute to the total uptake [6, 7, 62]. Although skin cells have not been considered to be active immune cells, they have been shown to have an inflammatory capacity [63] and keratinocytes can react and respond to a variety of substances. In order to understand the full effect of occupational exposures, it is important to have knowledge about the exposure routes and the actual uptake, as well as how to monitor the exposure.

Cobalt deposition on the skin

Exposure to cobalt can occur from both direct contact with the metal and from airborne exposure [17, 19]. Cobalt has been shown to be deposited on the skin from short repetitive contact with metals containing cobalt. Midander et al. conducted a study in which five volunteers handled cobalt-containing hard metal discs (6% and 15% cobalt) with their hands, and the cobalt levels were analyzed after 30 minutes [64]. The discs were shown to release cobalt at concentrations that could elicit an allergic response in the skin of cobalt sensitized patients. In the same study, several different cobalt-containing hard metal discs were placed in artificial sweat for a period of

two minutes to one week. Cobalt was released from all the discs and the amount of cobalt that was released increased with increased time in the sweat. Cobalt was shown to have already been released after two minutes in the artificial sweat and the levels corresponded to the lowest patch test concentration in the serial dilution tests on the patients [64]. Knowledge about release and deposition is of interest to understanding allergy risk when handling cobalt-containing materials.

Occupational exposure measurements of cobalt in an industry manufacturing hard metal components found that the cobalt levels of the workers was higher on the hands than on the forehead [39]. These results indicate that the cobalt found on the skin was mostly accounted for by direct contact rather than deposition on the skin from airborne particles.

Uptake, distribution and excretion

The uptake of cobalt in the hard metal production industry has previously been shown to mainly occur via inhalation of cobalt particles, whereas uptake via the skin has not been received sufficient attention. However, cobalt uptake via the skin has been shown to be important as an entry route [6, 7, 62, 65]. When an analysis of cobalt levels in air, blood and urine was conducted in a study from a hard metal factory, no relationship was found between the air concentration and the corresponding urine concentration [62]. Thus, it was suggested that cobalt uptake would be accounted for by uptake via the skin [62]. In a study in which four healthy volunteers were exposed to fresh mixed powder containing cobalt (approx. 5- 15%) and WC (95-85%) by holding their hand in a box containing the mixture for 90 minutes, urinary cobalt levels increased and remained at high levels for 48- 60 hours [6]. This finding clearly showed that cobalt had been absorbed through the skin [6]. Skin uptake of cobalt was also noted in a study of cobalt exposure in the wet sharpening of hard metal and stellite blades. The excretion of cobalt in urine was elevated in five volunteers after skin exposure to a grinding coolant solution used in the industry, showing that cobalt was absorbed through the skin [7]. Skin uptake of cobalt was also seen in a study of 76 workers in a hard metal production industry when air levels, skin levels and cobalt in urine was investigated in a Swedish hard metal industry [65]. It is possible that some of the uptake can also be accounted for by gastrointestinal absorption from unintentional transfer from the hands and clothing to the mouth [66] and most likely, exposure takes place simultaneously through the respiratory, dermal and gastrointestinal routes.

After exposure to cobalt and administration in the body, the cobalt concentration in blood and serum is initially high. The levels decreases rapidly and gradually due to uptake in tissues, mainly the liver and kidneys [67]. The elimination of cobalt mainly occur via urinary excretion but can also occur to a lesser extent via fecal elimination [68, 69]. In the first excretion phase most of the cobalt is eliminated within a few days. The second phase is slower and last several weeks. Retention in the tissues can last for several years, and around 10% has a longer biological half-life and remains in the body for several years or even permanently [70-73].

Absorption through the skin

Skin or dermal absorption is a term that describe the transport of chemicals from the outer surface of the skin to the systemic circulation, comprising penetration, permeation and resorption [74]. To investigate absorption through the skin, *in vitro* diffusion cell systems can be used. An example of such a system is called a Franz diffusion cell, and it consists of a piece of human or animal skin attached between two compartments. A donor phase solution passes through the skin to the receptor phase solution. In studies using the Franz cell system, experiments showed that when cobalt powder was dissolved in artificial sweat, cobalt ions permeated through the skin to a higher degree than when the powder was dissolved in a saline solution [8, 75]. Skin absorption of cobalt is closely related to the capacity of the sweat to oxidize cobalt. Cobalt oxidizes in the presence of sweat and the ions are absorbed through the skin in the form of ions [7, 8, 76].

Several factors affect dermal absorption. The absorption rate is based on the concentration of the substance on the skin and the permeability of the skin, but also that the exposed area and duration of exposure effect the total amount absorbed [77]. Other factors are thickness of the skin, skin temperature, and the degree of skin hydration. Skin condition can have a significant impact on the penetration and permeation of the skin. It has been shown that cobalt powder penetrate damaged skin more easily than intact skin [8, 78]. One study found that cobalt-allergic individuals with irritated skin react to lower concentrations of cobalt than healthy individuals with intact skin [79].

Air exposure measurements

Air exposure assessments including measurements can be used to compare the levels of chemicals in a work setting with occupational exposure levels

(OELs), or to investigate the exposure-response relationships in epidemiological studies. There are several criteria to be taken into account when choosing a sampling strategy, including the choice of sampling and analytical methods and evaluation of the determined levels [80] (e.g. which method and equipment to use in order to obtain valid measurement data for compliance with the regulations and also standards to follow for sampling and analysis). Current Swedish regulations for cobalt concentrations in the air apply to inhalable air [81] and the term inhalable air refers to what can be breathed in through the nose and mouth. These particles correspond to particles with an aerodynamic diameter of < 50- 100 μm. The respiratory tract comprises three regions: the extra thoracic region (including the nose and throat), the thoracic region, and the alveolar region. Particles that enter the extra thoracic region constitute the inhalable fraction; those particles that reach into the thoracic region constitute the thoracic fraction; and particles deposited in the alveolar region constitute the respirable fraction (Table 1) [82]. The separation characteristics of our sampling devices correspond to the deposition in the human airways.

Table 1. Definitions and particle sizes for inhalable, thoracic and respirable air fractions.

Particle fraction	**Definition**	**Particle size 50% cut off**
Inhalable	Particle that can be inhaled through the nose and mouth.	< 50- 100 μM
Thoracic	Particles that pass the larynx.	< 10 μM
Respirable	Particles that penetrate beyond the terminal bronchioles into the gas exchange region of the lungs.	< 4 μM

In the studies of this thesis, personal sampling of cobalt in inhalable dust and in total dust was conducted in order to compare old and new sampling methods (Papers I). Total dust does not correspond to a specific particle fraction, but to the method used for sampling, and corresponds to inhalable dust for particle sizes up to 18 μm. In 2011, the occupational exposure limit for cobalt was changed to apply from cobalt in total dust to cobalt in inhalable dust. Thus, both exposure limits were included for comparison with previous performed studies. Real time monitoring of dust was also per-

formed, using an instrument that measures particles with a diameter of between 0.1 to 10 μm in the range 0.001- 400 mg/m^3 with optimal sensitivity to the respirable fraction of dust (< 5 μm).

In this thesis, stationary sampling of inhalable dust, total dust and real time monitoring was also performed. In addition, particulate matter 2.5 (PM 2.5) and 10 (PM10) were measured during stationary sampling. These measures are often used in the environmental monitoring of air pollution and refer to particles with a diameter of < 2.5 μm (PM 2.5) and < 10 μm (PM10). PM10 and PM2.5 are widely discussed in environmental analyses, corresponding to the thoracic and respirable fractions, respectively [82].

Particle surface area air concentrations were determined for particles in the 10 to 1000 nm size range and particle number concentrations were measured based on particles in the 20 nm to 1000 nm size range.

Dermal exposure measurements

In order to evaluate the risk of effects on the skin, a range of methods for assessment of skin exposure to chemicals has been developed. The assessments can be divided into three different categories: removal and recovery techniques, surrogate skin techniques and visualization techniques. Most of the methods used are removal techniques that include different washing and wiping techniques. Removal techniques samples the mass of material on an individual's skin at a particular point in time [83, 84]. Washing techniques often involve washing of the hand and it makes it difficult to determine the dose per unit area of the skin. Removal using wipes or tape stripping has the advantage that the area can be specified [85]. For the tape stripping method, a tape is placed on the skin and when it is taken off, chemicals absorbed in the skin are removed together with a few skin cell layers in the stratum corneum [86]. Removal with skin wipes is less invasive. In this thesis, a wiping method developed by Linden et al. has been used, in which cobalt metal is removed with an acid -wash [85].

Contaminants can also be recovered from gloves to estimate hand exposure or clothing for whole a body assessment of contamination [87]. In surrogate skin techniques, a patch is attached to the skin and the amount on the patch upon removal represent the amount of the contaminant exposed to the skin [88]. Visualization methods include the use of fluorescent tracers to measure skin contamination [84]. Most of the available methods measure the amount of contaminant on the skin and not what has already been absorbed through the skin.

There are also conceptual models for assessment of dermal uptake that assess the uptake of airborne particles resulting from the transport of mass between different compartments [89, 90]. In this thesis, no conceptual models has been used.

Biological monitoring

Biological monitoring is another method for assessing human exposure to chemicals, their metabolites or reaction products in various human specimens, including fluids, feces and hair. In the field of occupational health, biological monitoring is an important tool for assessing total exposure to assess the risk to workers. During risk assessment, using air exposures measurements only can give an underestimation of the exposure since skin and oral uptake is not accounted for in such measurements. Biological monitoring includes these routes of uptake and provides measurements of how much has entered the body.

Measurements of cobalt concentrations in samples from whole blood, plasma, serum, exhaled breath and urine have all been used as indicators of exposure [91-93]. Several studies has shown that both blood and urine are well suited to monitoring cobalt uptake since the concentration reflects the recent uptake from exposure [7, 62, 94].

Occupational exposure limits and reference values for biological exposures

Occupational exposure limits (OELs) are exposure levels for hazardous substances to ensure for a safe working life in the workplace. OELs can be either time weighed averages, air concentrations for an 8-hour shift, or ceiling limit values, averages for 5 or 15 -minute periods, depending on the critical effect of the substances. To set these limits, available research data are taken into account, particularly regarding carcinogenicity, mutagenicity, reproduction toxicity, and the acute effects of exposure. The value is then set to prevent occupational diseases or other adverse health effects in workers exposed to hazardous chemicals in the workplace, and is mainly based on inhalation. It assumes that the work force is health. However, there are also OELs that protect vulnerable groups, e.g. pregnant women. Swedish OELs are set to 10 mg/m^3 for inhalable dust and 0.02 mg/m^3 for cobalt in the inhalable fraction [81]. The American Conference of Governmental Hygienists has threshold limit values of 0.02 mg/m^3 set for cobalt [95].

Unlike inhalation exposure in the work environment, there are no exposure limits for dermal exposure. There are skin notations for a variety of

substance, assigned by the National Institute of Occupational Safety and Health, although currently not for cobalt. Bos et al. have suggested that a quantitative dermal occupational exposure limit should replace skin notation [96]. Unfortunately, there is lack of standardized measurement methodology, difficulty in calculating the absorption rate and limited information in the field of the adverse health effects that could result from dermal exposure [96].

Regarding the biological monitoring of cobalt, the biological exposure index (BEI) for cobalt in blood was excluded in the latest regulatory document by the American Conference of Governmental Hygienists, although when this thesis work started, and until 2020, the BEI for cobalt in blood was set to 17 nM [97, 98]. However, there are limits for biological monitoring, and Finland has a biomonitoring action limit (BAL) for cobalt in urine of 130 nM [99].

The skin

The skin is the body's largest organ, with a surface area of 1.5- 2 square meters and weighs approximately 4 kilograms in an average adult human. The skin has many functions; it protects us from microbes, fungus, pathogens, UV light and water loss, helps regulate body temperature contains nerve cells that permit the sensations of touch, heat and cold. The skin varies in thickness from 1.5 millimeters to 4 millimeters or more, depending on its location on the body. It has three layers, and the epidermis, composed of epithelial cells, is the outermost protective layer (Figure 2). The stratum corneum is the layer furthest out in the epidermis, and functions as the main diffusion barrier. Beneath the epidermis is the dermis, which contain connective tissue, hair follicles and sweat glands and is vascularized. The subcutaneous tissue is the deepest layer and comprises fat connective tissue. This thesis focuses on the epidermis, since cobalt particles will first make contact with cells in this layer upon skin exposure.

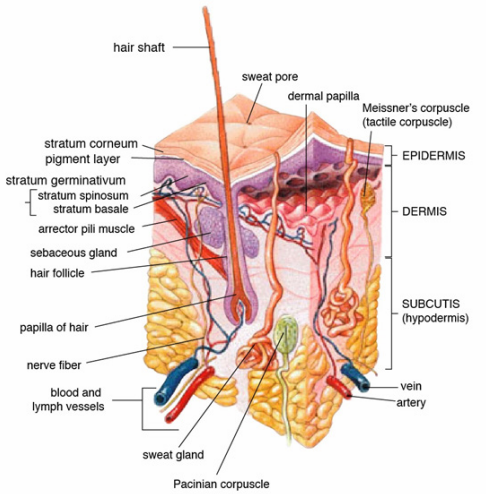

Figure 2. The skin. Reprinted with permission from Wikimedia Commons.

Epidermis

The epidermis is a keratinized stratified squamous epithelium comprising four or five layers, around 0.2 mm thick. The epidermis comprises four different cell types, keratinocytes, melanocytes, Merkel cells and Langerhans cells. The keratinocytes comprise approximately 95% of the cells in the epidermis. Keratinocytes produces the fibrous protein keratin that gives the epidermis its protective properties. Keratin inside the cells attaches to proteins complexes called desmosomes on the cell surface. Desmosomes are a type of tight junction that tightly connect the keratinocytes to each other. They mediate cell-to-cell adhesion and communication [100].

The bottom layer of the epidermis (Figure 3), the stratum basale (stratum germinativum) comprises a single row of cells that undergo continuous mitosis. These basal keratinocytes differentiate and migrate upward, toward the surface of the skin. There they will eventually form the stratum corneum, the outermost horny layer of the epidermis, and also the main protective barrier of the skin. During this upward migration the cells lose their ability to proliferate, and cells in the stratum spinosum, stratum granulosum and stratum corneum are non-proliferating keratinocytes. It is this gradual differentiation of the keratinocytes that is utilized in our model, in

which calcium is used as an inducer for differentiation. The less differentiated cells represent cells in the basal layers of the epidermis and the more differentiated cells in our model represent cells further out in the epidermis.

The process by which keratinocytes migrate upward is called "terminal differentiation" and the keratinocytes gradually lose their nuclei and become flat, dead cells filled with keratin fibers, called corneocytes. The membrane of the corneocytes, or the cornified envelope, is thickened and reinforced by strong covalent bonds between the proteins in the membrane, also called SS-bonds. This intercellular space in the stratum corneum contains lipids derived from the lamellar bodies. These are ceramides, sterols and free fatty acids [101]. Those lipids provide the stratum corneum its diffusion barrier function and help regulate extensive water loss. Since the cornified envelope on the keratinocytes in the stratum corneum is relatively impermeable to most compounds, the major route of penetration has been identified as the tortuous/ intercellular pathway between the corneocytes [102].

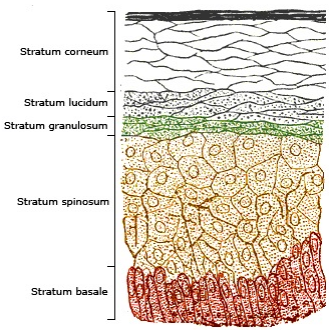

Figure 3. Epidermis. Reprinted with permission from Wikimedia Commons. Modified from the original Grey's diagram here: http://en.wikipedia.org/wiki/Image:Gray941.png

Cobalt cell toxicity and models

Cobalt cell toxicity is well documented, including the uptake, distribution in the body, metabolism, and excretion of the metal. After the entry of cobalt ions into the skin, the ions will activate different signaling pathways causing damage to the cells, and can even lead to cell death. The magnitude of the damage depends on the exposure dose and time [103]. To study the

effects of cobalt exposure in the skin and to understand the mechanisms, *in vitro* skin models using the cell line HaCaT are often used. HaCaT is an immortalized human skin cell line, and HaCaT cells have been shown to be similar to normal human keratinocytes.

HaCaT cell line and the use as models

To study chemicals and other toxic substances *in vitro,* skin models are commonly used. Models can be with donated skin, artificial skin or biopsies.

HaCaT is a spontaneously immortalized human keratinocyte cell line. It is derived through culturing of human adult keratinocytes in long-term, high temperature and low calcium growth conditions. The name HaCaT originates from Ha = human adult, Ca = calcium and T = temperature. The HaCaT cell line is capable of both proliferating and differentiating normally [104] when compared to neonatal epidermal keratinocytes [105]. HaCaT cells have been shown to exhibit essential markers for differentiation, such as the different keratins produced by the cells and the specific marker representing a specific phase in the keratinization, or terminal differentiation, of the cell. This quality has been shown to be maintained throughout high passage levels, and also without the cell line forming tumorigenic phenotype, and with a stable genetic balance [104, 106, 107]. HaCaT cells are also capable of forming organized and typically differentiated epidermis-like tissue *in vitro* when cultured with dermal fibroblasts [108]. Due to the specified properties of HaCaT, the cell line is often used as a model to study skin irritants and drugs. However, when interpreting data from *in vitro* models in which HaCaT has been, used it is important to interpret the results with care since differences, such as variation in some gene transcription profiles [109], signaling pathways [110], and stress response, have been shown to exist between normal keratinocytes and the cell line [111].

Cobalt uptake and distribution in cells

Absorption of cobalt in the skin is known to occur [6, 8] and the metal is also retained in the tissues [72]. The passage of cobalt through the skin is not completely clear, but in the stratum corneum cobalt will enter the extracellular phase (fat). As the metal ions continue on their way, they will enter the cells, and can therefore interact with cell components and cause damage to the cell.

Cobalt is found inside the cell, both in the cell nucleus and in the cytosol [103, 112-115]. HaCaT cells treated at the sub toxic level of 40 µM $CoCl_2$

show that the cobalt intracellular content was below the detection limit. However, at 400 µM, cobalt is mainly found within the nucleus and the cytosol of the cell [112]. In another study, cobalt content was also measured in HaCaT cell pellets after 24 hours of incubation of $CoCl_2$, and more than 85% of the available cobalt had accumulated in the cytosolic fraction of the cells [103]. Bresson et al. saw similar a distribution at 400 µM cobalt, 77% in the cytosol, 22% in the nuclear fraction and 1% in the membrane [113]. HaCaT cells were also shown to be capable of concentrating the metals present in the media 12.5 times for 1 mM $CoCl_2$ [116].

The presence of cobalt in the cell nucleus indicates that cobalt can possible interact directly with nuclear proteins and genomic DNA. Exposure to cobalt has shown changes in gene expression in several cell types, including lung epithelial cells [114, 115], and cobalt may possibly cause DNA damage and interfere with DNA repair mechanisms [112, 117, 118].

Cell viability and toxicity of cobalt

To investigate toxicity, several models were utilized including a straight forward approach in which cells *in vitro* were exposed to cobalt chloride. Cobalt is toxic to cells and several studies have discovered the negative impact on cell viability of $CoCl_2$ on HaCaT cells using *in vitro* models [119-122]. Cell viability is cell's ability to metabolize, i.e. to be viable. In HaCaT cells treated with a $CoCl_2$ concentration of 400- 600 µM for 24 hours, it was found that the cell viability in the exposed cells significantly decreased in a dose-dependent manner [119]. Another study also showed a dose dependent decrease in cell viability in HaCaT cells exposed to 300 to 800 µM $CoCl_2$ for 24 hours [120].

In an earlier study performed on HaCaT cells, exposure to 1 mM $CoCl_2$ for four hours was found to be harmful to the cells, with a 53.1% reduction in cell viability with small cytosolic effects already detected at 0.1 µM. The EC50 value for cobalt chloride was estimated to be 475 µM [116]. Other studies have also determined that a $CoCl_2$ concentration of 500 µM result in a 52% decrease in cell viability compared to the control [121] and also to be the LD50 dose [122].

It is suggested that $CoCl_2$-induced cytotoxicity in HaCaT cells is associated with the overproduction of reactive oxygen species (ROS). ROS are chemically reactive molecules that contain oxygen and easily reacts with other molecules in a cell. An intracellular increased level of ROS causes oxidative stress in the cell, which results in ROS-mediated damage and ROS

also regulates cellular signaling pathways that contribute to disease [123, 124].

The intracellular ROS content in HaCaT cells is concentration –dependent with enhanced production after exposure to 400- 600 μM $CoCl_2$ for only two hours [119]. Another study also detected noticeable increase in ROS levels in HaCaT exposed to 500 μM $CoCl_2$ for two hours [120]. Levels as low as 100 μM $CoCl_2$ led to a significant increase in ROS levels [121]. ROS can be produced during biochemical reactions in the cell's mitochondria, which would further explain the impact on metabolic activity that cobalt exerts upon exposure.

The inflammatory response

An inflammatory response occurs when tissues are damaged by foreign agents such as viruses or bacteria, toxins, trauma, heat, stress or exposure to irritant chemicals. It is a defense system for the body to prevent the spread of the damaging agents to nearby tissues, eliminate foreign agents and debris and restore the tissue to a healthy condition. The inflammatory response can last from a few hours up to a few days referring to acute inflammation, or for an extended period reflecting the biological aspects of a chronic inflammation. When the inflammatory response is initiated, the immune system mobilizes, and a series of seemingly complex reactions take place in which the adaptive and innate immune system working together to defend the body.

The immune system

Our immune system comprises two subsystems, the innate immune system and the adaptive immune system. The innate immune system is the first to respond to pathogens, and has previously been thought not to retain a memory and to be non-specific, i.e. anything that is identified as foreign or non-self could cause an innate immune response. However, innate immune cells have recently been found to acquire trained immunity which means they are able to adapt to previous encounters with pathogens through epigenetic, transcriptional and functional reprogramming [125]. The innate immune system provides immediate host defense since the components are constitutively present in our body [126] and it is therefore often referred to as our first line of defense [127, 128]. The major function of the innate immune system include recognition of foreign agents and recruitment of specialized cells to the site of infection via production of cytokines and chemokines, and activation of the adaptive immune system via antigen

presentation [129]. The cells involved in the immune system have specialized functions including phagocytosis of foreign agents such as particles, viruses, bacteria and dead cells and also direct killing of pathogens. Innate immune cells include white blood cells such as basophils, dendritic cells, eosinophils, Langerhans cells, mast cells, monocytes and macrophages, neutrophils and NK cells, although keratinocytes have also been shown to be important cells with immune capacity [129]. As the innate immune system is mediated by receptors, which detect threats leading to the induction of inflammatory responses and innate host defense, sensing by the receptors also activates the adaptive immune system [130], also referred to as innate control of adaptive immunity [131].

The adaptive immune system, also called the acquired immune system, will engage the foreign agent with high specificity to a particular foreign agent but is relatively slow to develop at first exposure. Adaptive immunity involves a tightly regulated interplay between antigen-presenting cells and T and B lymphocytes [132]. When an antigen is presented to the B lymphocytes, the cells produce an antibody that will contain a unique antigen-binding site capable of binding to that specific antigen. T lymphocytes react directly against a foreign antigen that is presented to them on the surface of a host cell [133]. Large antigens can be recognized as they are while small molecules, haptens, must first bind to a protein to be recognized [134]. Allergies are T- cell-driven reactions [135-137] and dendritic cells (DCs) migrate to the draining lymph node for antigen presentation, leading to the generation of allergen-specific T helper type 2 (Th2) cells, which in turn, help B cells to produce antibodies as well as developing into memory B cells providing long-lived immunological memory. Memory B cells can mature and differentiate into plasma cells that produce large amounts of allergen-specific IgE antibodies that bind to the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils, thus leading to the IgE sensitization of individuals against allergens. Mast cells and basophils are present in the dermis and can release compounds upon activation that are responsible for the symptoms of the immediate phase and acute inflammation [138].

Inflammatory cytokines and chemokines in keratinocytes

The skin has long been considered to be a barrier to the outer environment of the body. However, the skin is now considered to be an essential part of the first line of defense. The majority of cells that makes up the skin are keratinocytes and current knowledge shows that keratinocytes are capable of detecting and reacting to foreign substances, and produce a variety of

inflammatory signaling [139]. Human keratinocytes can produce a wide range of cytokines as a response to different stimuli or threats. Cytokines are small soluble proteins that are produced a broad range of cells, including immune cells. Among the best studied cytokines produced by inflammatory cells are interleukin-1β (IL-1β), IL-6, IL-12, tumor necrosis factor (TNF) and the chemokine CXCL8 (interleukin-8, IL-8). Cytokines play an important role in mediating innate immune responses by acting as pro-inflammatory or anti-inflammatory mediators, and some cytokines, for example IL-6 may have both properties depending on the circumstances in which they are produced. Cytokines can act on the cells that secrete them, nearby cells as well as distant cells. Cytokines initiate their biological action by interacting with cells that express receptors specific to the cytokine. Cells in the immune system uses the secreted cytokines for communication and the pro-inflammatory cytokines recruit cells to the site of inflammation and induce cells to proliferate and differentiate. Cytokines with chemotactic activities are also called chemokines [140, 141]. Chemokines play an important role in innate immunity as they control cell migration and cell positioning throughout development, homeostasis, as well as inflammation [142].

Cultured keratinocytes constitutively produce or can be induced to produce a large variety of cytokines and chemokines including IL-6, IL-8, IL-1α, IL-1β and IL-18 [143, 144]. *In vitro* models using HaCaT cells has showed that upon exposure to 500 μM $CoCl_2$, HaCaT cells both upregulate the mRNA and secrete the pro-inflammatory cytokines IL-6, IL-1β and TNF [122]. Yang et al. also detected increased levels of IL-6, IL-8 secreted from HaCaT cells at 500 μM $CoCl_2$ exposure [119] and another *in vitro* study found increased IL-6, IL-8 and IL-1β levels after 500 μM $CoCl_2$ [120]. The ability of the HaCaT cells to produce several pro-inflammatory cytokines show that keratinocytes have an inflammatory capacity and most likely those cytokines produces take part in the inflammatory process in the skin, i.e. skin allergy.

Molecular mechanisms of metal allergy in the skin

Contact dermatitis is a result of exposure to allergens or irritants that have triggered an immune reaction in the skin. The allergen or irritant induces a "danger signal" in the skin by activating receptors that trigger the production of inflammatory cytokines. Contact dermatitis is a type of hypersensitivity reaction and involves a priming of naïve T cells, called the sensitization phase, or induction stage, and activation of primed T cells upon re-exposure to the antigen, the elicitation phase [145, 146].

The molecular mechanism of metal allergy in the skin is complex and involves both innate and adaptive immune responses. In the sensitization phase, the presence of an antigen (the metal) is not enough to generate an immune response. This phase also requires the activation of the innate immune system [146-148]. Upon penetration, the metal attaches to the major histocompatibility complex (MHC) expressed on Langerhans cells (LCs) and dendritic cells (DCs). However, metal ions are considered to be incomplete antigens and must therefore first bind to proteins or peptides to be able to bind to the MHC [147]. The MHCs on LCs and DCs are upregulated and subsequently become activated by cytokines secreted from the surrounding keratinocytes. These cytokines are produced by innate immune responses to danger signals via activation of toll like receptor (TLRs) 4 receptor and activation of the inflammasome.

The activated LCs and DCs are so called antigen presenting cells and will migrate to the draining lymph nodes where they present the allergen (the metal) to the naïve T cells which results in proliferation, activation and differentiation of the T cells. The primed T cells migrate out of the draining lymph node and back to the skin, which is referred to as the sensitization phase. In the following elicitation phase, the subsequent application of the same hapten leads to uptake by cells, which is presented to the recirculating hapten-specific T cells. The activated T cells produce inflammatory cytokines and chemokines at the site of exposure that promote an allergic reaction, leading to the development of characteristic skin lesions [149]. At several points during the sensitization phase, cytokines produced by the keratinocytes affect the maturation and migration of the DCs to the lymph nodes [150-152]. Upon re-exposure to the same danger signal (metal) and uptake of cells, presentation to the primed T cells will occur. The activated T cells produce inflammatory cytokines and chemokines at the site of exposure that promote an allergic reaction.

Figure 4 shows an illustration of the molecular mechanisms in nickel allergy. Cobalt share similar mechanisms as nickel given the similar physical properties of cobalt and nickel, including two electrons in its outer shell, and the fact that cobalt bind to and activate TLR4 [153, 154]. Cobalt binding to the TLR4 receptor, elicits immunogenic stimulation of the cell, resulting in the production of IL-6, CXCL8/IL-8, and pro-IL-1β. Cobalt has also been shown to activate the NLRP3 inflammasome in keratinocytes, leading to the production of IL-1β [119, 120, 122]. IL-1β is one of the body's most powerful inflammatory agents as it regulates the production of other cytokines [10, 14] and induces the onset of a range of diseases. Keratinocytes

play an important role in the innate phase of immune responses as cytokines released by keratinocytes are known to regulate maturation, recruitment and migration of cells involved in the inflammatory response in the skin.

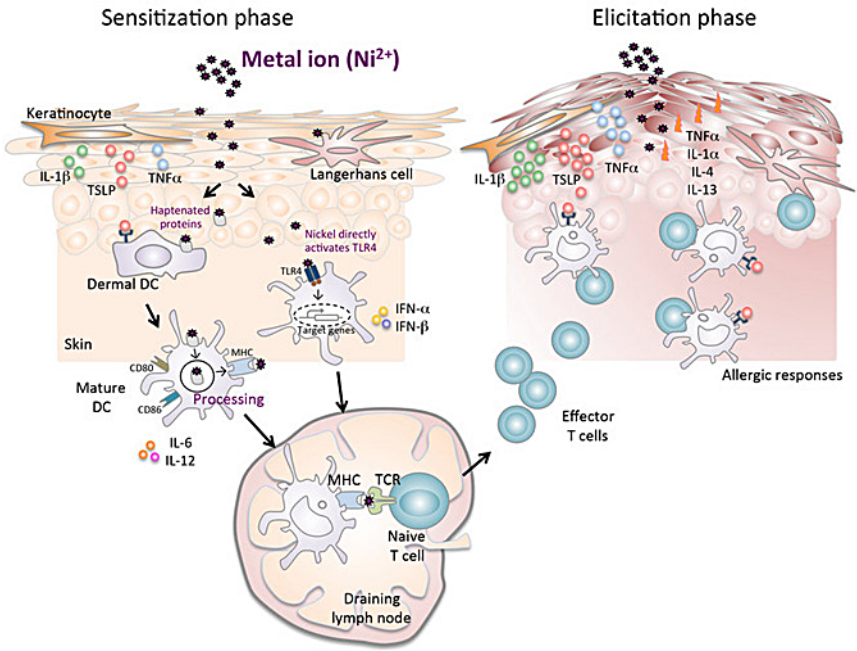

Figure 4. Molecular mechanism of metal allergy. Reprinted from Molecular Mechanisms of Nickel Allergy, Saito, M., Arakaki, R., Yamada, A., Tsunematsu, T., Kudo, Y., & Ishimaru, N, International Journal of Molecular Sciences, 2016;17(2):202.

Detection by receptors and triggering of the innate immune system

In order to mount a sufficient innate immune response capable of killing and/or eliminating a threat, cells need to be able to sense and recognize the threat. The recognition is conducted by special receptors that are collectively called pattern recognition receptors (PRRs). PPRs recognize various molecules derived from microorganisms, so-called pathogen-associated molecular patterns (PAMPs), for example lipopolysaccharide (LPS) [126]. PRRs can also recognize damage-associated molecular patterns (DAMPs), which are derived from host cells, dead or dying cells and in response to signals such as hypoxia. DAMPs are often created in environments of trauma or tissue damage, and are sometimes referred to as a contributing factor to

sterile inflammation [155, 156]. However, DAMPs are also exogenous substances such as silica, asbestos, as well as chemicals such as nickel and cobalt [120, 157-159]. In this thesis, the focus is on two receptors that have been showen to be involved in detection and in the inflammatory response due to exposure to cobalt ions i.e. the TLRs and the nucleotide oligomerization domain (NOD) – like receptors (NLRs).

A major group of pattern recognition receptors that plays an important role in the innate immune recognition are TLRs located on the cell surface that trigger host cells gene expression in response to pathogens. Humans have at least ten TLRs and many of them uses the transcription factor nuclear factor kappa-light-chain-enhancer (NF-κB) signaling pathway to trigger gene expression. NF-κB is a protein complex in the mammalian cell that mediates the transcription of genes to produce pro-inflammatory cytokines and chemokines. Examples of those cytokines and chemokines are IL-6, IL-8/CXCL 8 and the pro form of IL-1β [160, 161]. This indicates that the NF-κB signaling pathway is crucial for the activation of the inflammasome [162] and the production of cytokines and chemokines known to contribute to inflammation in the skin. Another group of receptors are NLRs, which are cytoplasmatic receptors. Due to their location, they detect signals of intracellular invaders [163]. Based on their N-terminal domain, NLRs are divided into different groups. Upon recognition and binding of the receptor, a speck like multi protein called the inflammasone is assembled, resulting in the production of the pro-inflammatory cytokines IL-1β and IL-18 [164].

Inflammasomes

Inflammasomes are a group of intercellular multi-protein complexes that mediate the activation of potent inflammatory mediators. The term inflammasome is derived from the word *inflammation* and the suffix "*some*", and is used to define molecular complexes [165]. It was also chosen to highlight the similarities in function with apotosome, a protein that mediates apotosis [166].

Inflammasomes are essential parts of the innate immune response against foreign pathogens or threats. Their activation starts by the sensing of PAMPs and DAMPs, which results in inflammasome assembly, leading to the release of pro-inflammatory cytokines that trigger a downstream cascade of inflammatory responses [167, 168]. The essential components of inflammasomes are a sensor protein, i.e. the NLR, the adaptor protein ASC and the inflammatory protease caspase-1. Binding of a pathogen to the sensor protein leads to assembly and formation of the speck-like protein that

the inflammasome constitutes. When the inflammasome is activated, caspase-1 will cleave the precursor forms of IL-1β and IL-18 to their active and immunocompetent forms. Four different receptors have been shown to form inflammasomes, and three of them belong to the NLR [169]. Of special interest in this thesis is the NLRP3 inflammasome because of its ability to be activated by a surprisingly diverse set of stimuli, including cobalt ions.

The NLRP3 inflammasome

The NLRP3 inflammasome, previously called the NALP3 inflammasome, is the most studied inflammasome and is activated by a wide range of different stimuli, i.e. crystals, toxins, viral RNA, malarial hemozoin and metals, including cobalt [11, 12, 170-175].

The NLRP3 inflammasome constitute the sensor molecule NLRP3, the adaptor protein ASC and pro-caspase-1 (Figure 5). The NLRP3 protein comprises a pyrin domain (PYD) and the ASC protein accommodate PYD and CARD domains. ASC binds to other pyrin-containing proteins via its own pyrin domain, and recruits caspases via CARD/CARD interaction [176-178]. The activation of caspase-1 is via its interaction with ASC. Upon activation, the NLRP3 receptor dimerizes and binds ASC via pyrin domain interactions. ASC subsequently interacts with caspase-1 via their respective CARD domains. Caspase-1 undergoes auto-proteolytic cleavage (the breaking of peptide bonds between amino acids in proteins) to become in its active form. In turn, caspase -1 can cleave the precursor forms of IL-1β and IL-18 to their biologically active forms, leading to pro-inflammatory responses [179-183].

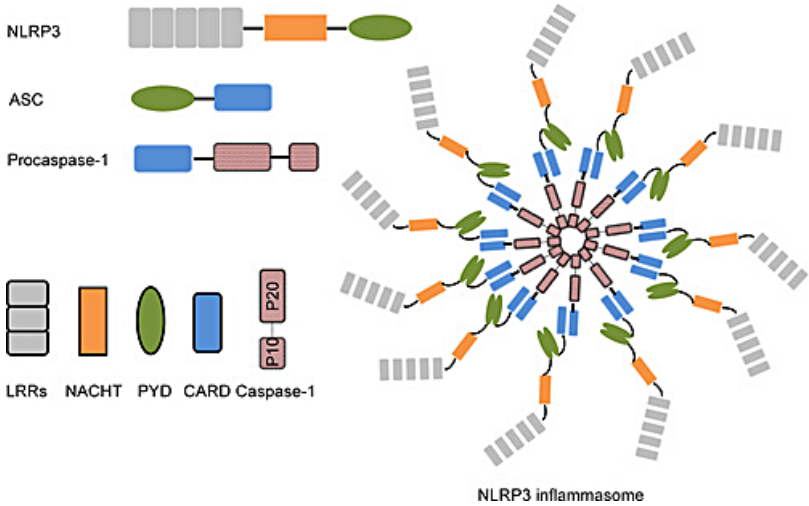

Figure 5. NLRP3 inflammasome structure. Reprinted from Ma, C., Liu, S., Zhang, S., Xu, T., Yu, X., Gao, Y., Zhai, C., Li, C., Lei, C., Fan, S., Chen, Y., Tian, H., Wang, Q., Cheng, F., Wang, X. "Evidence and perspective for the role of the NLRP3 inflammasome signaling pathway in ischemic stroke and its therapeutic potential (Review)". International Journal of Molecular Medicine 42.6 (2018): 2979-2990

Activation of the NLRP3 inflammasome requires two distinct signals for the multi protein to assemble (Figure 6). The first signal typically involves ligation of a TLR that initiates (via NF-κB) -the expression of pro-IL-1β and NLRP3. This initial step is often called the "priming" signal or "signal 1" [162]. The second signal, also known as the activating signal, is induced by a wide variety of substances that are sensed by the NLRP3 protein. Examples of those signals are microbial cell wall components, nucleic acids, pore-forming toxins and, environmental crystalline agents such as silica and endogenous molecules, including ATP and uric acid crystals [157, 184-188].

Because of the significant structural differences between the inducers of activation (i.e. particles, pathogens, metals), the NLRP3 inflammasome is not thought to bind directly to its activators or to be dependent on a single pathway for activation [189, 190]. Rather than being a traditional ligand-receptor interaction, PAMP/DAMP are proposed to be sensed via distress signals capable of mediating the activation of NLRP3. Including changes in cell volume, rupture of lysosomes, production of reactive oxygen species

(ROS), K^+ efflux and Ca^{2+} signaling have all been proposed as distress signals capable of mediating the activation of NLRP3. [186, 191-195].

Keratinocytes require stimulation of both TLR and NLR receptors for the secretion of mature IL-1β and IL-18 [196]. Watanabe et al. suggests that primary human keratinocytes contain the necessary elements to form an inflammasome. They found activated fragments of caspase-1, IL-1β and IL-18 in supernatants six hours after stimulation of the cells [197].

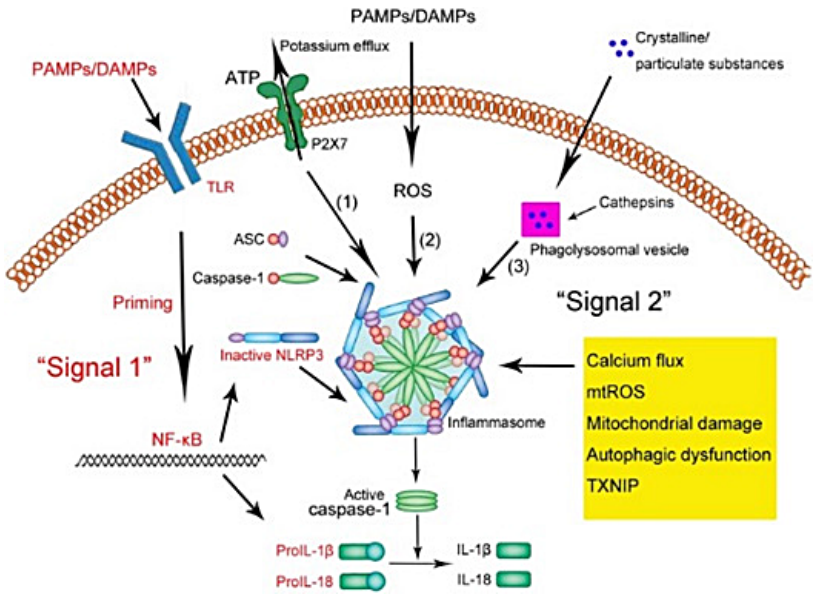

Figure 6. Schematic illustration of the NLRP3 inflammasome activation. Reproduced Shao B-Z, Xu Z-Q, Han B-Z, Su D-F and Liu C (2015) NLRP3 inflammasome and its inhibitors: a review. Front. Pharmacol. 6:262. doi: 10.3389/fphar.2015.00262, Copyright © 2015 Shao, Xu, Han, Su and Liu [198].

Activation of the inflammasome by cobalt

The NLRP3 inflammasome is activated by a range of different PAMPs and DAMPs and it is logical that cobalt would too because of the immunological effects linked to cobalt. In investigations on the effect of cobalt ions released from wear and corrosion of alloys used in prosthetics, there are evidence of inflammasome activation due to cobalt exposure [11, 12]. When a human monocyte cell line was exposed to cobalt ions, IL-1β secretion peaked at 0.1

mM $CoCl_2$. The model also revealed the abolishment of IL-1β as a caspase-1 inhibitor was added, supporting that the activation is dependent on the active form of caspase-1 in the cells. Further, the cells showed diminished IL-1β production in the presence of a nicotinamide adenine dinucleotide phosphate (NADPH) inhibitor, suggesting that inflammasome activation is dependent on NADPH generated ROS in the cells [11]. Metal ions may cause a dysfunction of the mitochondria, which in turn are responsible for most ROS production [189]. The production of IL-1β in human monocytes was also shown to be increased with increasing concentration of $CoCl_2$ and cobalt particles [12]. However, inhibition of the inflammasome has also been reported in mouse brain mixed glial cells, and mouse bone marrow-derived macrophages after $CoCl_2$ treatment [199].

Cobalt ions have also been shown to activate the human toll like receptor, TLR4, in several cell types including human microvascular endothelial cells and signals provided by NF-kB activators are necessary but not sufficient for NLRP3 activation [162]. Upon exposure to cobalt ions, the endothelial cells secretion of IL-6 and IL-8 increased [154, 160, 200]. One model showed that the secretion could be prevented by a TLR4 antagonist proving that activation of TLR4 was responsible for the inflammatory response [201].

Since it has been suggested that the inflammasome is activated upon exposure to cobalt and IL-1β and IL-18 are produced upon exposure to $CoCl_2$, this indicates that the inflammasome is activated upon exposure to cobalt and should be of interest in understanding the underlying mechanisms of skin inflammation. To our knowledge, this thesis is the first to study mechanisms involved in skin inflammation caused by cobalt exposure to keratinocytes with focus on the inflammasome.

AIMS

The overall aim of this study was to gain an increased understanding of the present cobalt air concentration levels of different particle size fractions in the hard metal industry, and the importance of dermal exposure compared to inhalation for the total uptake. We also investigated the toxicity and inflammatory response from cobalt in the skin by establishing *in vitro* models using cultured human keratinocytes. These experiments could help to understand the inflammatory processes behind dermal allergic sensitization.

The specific aims of the thesis work were:

I. To determine exposure air concentration levels of inhalable cobalt and tungsten and other particle exposure measures for risk assessment and investigate the possibility of using these data as proxies for exposure measures.

II. To examine whether cobalt skin and inhalable air exposure contributes to uptake determined as cobalt in blood.

III. To compare effects of cobalt on the viability of cultured human keratinocytes (HaCaT) cultured at low or high calcium (the latter yielding a more differentiated cell type) and to determine the pro-inflammatory cytokine release.

IV. To investigate dose- and time-resolved conditions in which $CoCl_2$ affect HaCaT cells to exhibit changes in proinflammatory cytokine release and NLRP3 inflammasome activation as a model of events in the skin following cobalt exposure.

METHODS

The materials and methods used in the different studies are summarized in this section. For a more detailed description, readers are referred to the individual papers (Papers I through IV). Considerations regarding methods and materials are further discussed in this section.

Overview

In Papers I and II, measurements were performed at a hard metal production facility, where there is exposure to cobalt. Personal air measurements and stationary area measurements of cobalt, measurements of cobalt on skin and biological monitoring of cobalt in blood were performed. In study I, the focus was on personal and stationary area measurement to evaluate the airborne exposure to cobalt, and to investigate the possibility of using other exposure measures as proxies for personal exposure. In study II, the focus was to investigate the total uptake expressed as cobalt in blood or urine with air and dermal exposure levels.

Studies III and study IV were both conducted in a laboratory environment using a human keratinocyte cell line for *in vitro* experiments with cobalt. In study III, the toxicity and inflammatory response in the skin cells after exposure to cobalt was investigated. In study IV, the focus was on the inflammatory response, specifically on the activation of the NLRP3 inflammasome.

Study objects

The Swedish hard metal production company, at which the air sampling, skin sampling and biological monitoring were performed for studies I and II is one of the world leading companies in the production of hard metal cutting tools. The company was producing approximately one million carbide inserts per week at the time and employed around 1200 workers.

The workers who participated in the studies were stationed in departments in the facility in which exposure to cobalt was expected to be found. The following departments were selected for personal exposure and area measurements: powder production, pressing, maintenance (pressing), charging/de-charging, physical vapor deposition (PVD) and chemical vapor deposition (CVD) furnaces, periphery grinding (shape and grade), process laboratory, warehouse and inspection. Informed oral and written consent was obtained from each participant and 72 persons agreed to participate.

The cells used in all the *in vitro* experiments were the immortalized human keratinocyte cell line HaCaT. The cells were an appreciated gift from Drs. Mikael Ivarsson and Anita Koskela von Sydow, Örebro University.

Ethical considerations

Studies I and II were approved by the Ethics Committee of the Regional Ethical Review board in Uppsala, Sweden (Dnr 2007/260).
Studies III and IV were exclusively performed using the immortalized human keratinocyte cell line HaCaT. Thus no ethical approval was needed.

Papers I and II

Sample collection (Papers I and II)

Workers were exposed to cobalt during all the steps in the process of production of hard metal, from mixing of powder to inspecting the final products. Measurements were taken in all departments in which cobalt exposure occurs. The study design was carefully planned and the sample collection was performed over a period of two years. The personal sampling and the stationary air sampling were conducted during a whole eight-hour work shift. Blood sample used for further analysis were taken at the beginning of the shift, before the participants had started working, and after the work shift. Skin sampling was conducted once for each participant. The participants worked normally for two hours without washing their hands and the amount of cobalt exposure was measured using an acid wash technique. Personal air measurements, skin measurements and blood measurements were taken on the same day for the worker, which made it possible to investigate the uptake in blood by correlating air, skin and blood cobalt concentrations.

Personal air sampling (Papers I and II)

The personal measurement equipment comprised a sampling head with a filter inside, see figure 7. A tube is connected from the sampling head to a pump that draws air in to the sampling head and through the filter in which the dust is deposited. The position of the filter head is very important in order to obtain accurate measurements and should be located in the workers' "breathing zone". The breathing zone is within approximately 30 centimeters of a person's nose and mouth and the location ensures that the air

drawn trough the filter reflects the air that the worker is breathing. Depending on what dust fraction to be measured, different filter heads and filters with different pore size and material are used. The pump is set to operate at a specific airflow according to the instructions. The air sampling of inhalable dust was performed according to general methods for sampling and gravimetric analyses of respirable and inhalable dust [202]. Sampling of total dust were performed according to a modified version of the National Institute of Occupational Safety and Health Manual of Analytical Methods 0500 [203]. Determination of the amount of dust on each filter was made gravimetrically. For determination of cobalt in the dust, filters and dust were then dissolved with acid and analyzed using inductively coupled plasma mass spectrometry (ICP-MS) [204].

We chose to measure cobalt in the inhalable fraction and total dust fraction. The inhalable fraction refers to the classification of the particles depending on their size and target area in the respiratory system. The inhalable fraction of cobalt is the fraction used for comparison with the OELs, the upper limit of the acceptable concentration of a hazardous substance in a workplace that is set to prevent occupational diseases or other adverse health effects. Measuring cobalt in the total dust fraction is important since the transition from sampling cobalt in the total dust fraction was changed to the inhalable fraction in 2011 [81]. Measurements were conducted during a full eight-hour work shift.

Real time monitoring of dust concentrations was performed using and aerosol monitor (DataRAM). The instrument was placed in the breathing zone and registrations of dust concentrations were made every 10 seconds. Using real time monitoring enables the detection of peaks of exposure arising from certain work operations or work tasks. Exposure can be due to the nature of production processes, but often the amount of exposure a worker is subjected to often depends on personal behavior. Real time monitoring is a useful tool in monitoring such high peak exposure work tasks or processes and preventative measures can be used to minimize the exposure or a change in how the specific work tasks resulting in the peak can be implemented.

Figure 7. Personal air measurements.

Stationary air sampling (Paper I)

Stationary measurements were performed simultaneously as personal measurements. The sampling equipment was strategically placed in the same departments in which the workers were stationed. Stationary measurements of total and inhalable dust were performed in accordance with the standardized sampling methods used for personal exposure measurements. Another way to define airborne particles, often used in air pollution is PM 2.5 and PM 10, where PM stands for particulate matter. PM 2.5 is fine inhalable particles with a diameter of < 2.5 μm and PM 10, which are inhalable particles with a diameter < 10 μm, were also measured. Further, a diffusion particle sensor, A-trak, was used to measure the particle surface area ($\mu m^2/cm^3$) in the size range 10- 1000 nm. Also, the particle number concentration (p/cm^3) was measured with a P-trak in the size range 20- 1000 nm. The sampling time for all stationary measurements was eight hours.

Since air measurements included sampling several different particle size fractions, our correlation analysis that used these fractions provided us with the opportunity to compare our results with other research reports.

Skin exposure measurements (Paper II)

A range of methods has been developed for the assessment of skin exposure to chemicals. Removal techniques are the most commonly used techniques to sample chemicals that have been deposited on the skin. Such techniques can be washing, rinsing, wiping and tape stripping. The skin exposure meas-

urements in our study were conducted using an acid-wash technique developed by Linden et al. [85]. This technique is non-invasive and has been shown to have a high recovery. It is well suited to assessing the amount of cobalt that end up on the skin. The measurements in our study were all performed on the hands (Figure 8).

Participants first washed their hands thoroughly with soap and water, and then the areas of interests were washed with a 1% HNO_3 to further remove possible cobalt contamination on the skin. One finger was covered to avoid exposure and was used as a reference. The participants then worked as usual for two hours without washing their hands during this period. After two hours of exposure time, the skin areas on the worker's hand were washed with 1% HNO_3 drained patches and stored in plastic containers for later analysis.

Figure 8. Skin sampling of cobalt.

Biological monitoring of blood (Paper II)

Biological monitoring is a way of assessing exposure of a chemical by measuring the chemical itself or its breakdown products in biological samples. Blood and urine are well suited to the biological monitoring of cobalt since level shave shown to correlate well with air levels and reflects recent exposure of cobalt metal [62, 91, 94] with the blood reflecting acute/immediate exposure whereas cobalt in urine relate to average exposures spanning several days. In this study, blood samples were chosen as an indicator for cobalt exposure because they reflects acute exposure. Samples were collected on four occasions for each participant. Samples were taken before the start of the work shift and after the work shift. The difference between the same

day before and after shift blood concentration reflects the uptake of cobalt in the blood on that day and exposure for an eight-hour work shift. In our statistical analyses, these values represent the uptake of cobalt in blood from air and skin exposure to cobalt.

Chemical analysis (Papers I and II)

Determination of the deposited amount of dust on the filters was made gravimetrically. A standard analytical procedure for metal analysis on each filter and the skin exposure samples for each participant was performed using inductively coupled plasma mass spectrometry (ICP-MS) for cobalt. The ICP-MS is a type of mass spectrometry that uses inductively coupled plasma to split up the materials into individual atoms that lose an electron and become positively charged ions. The ions pass through a quadrupole in which they are separated according to their mass/charge and then the ions hit a special detector. It has the advantage of high sensitivity and specificity, and the ability to simultaneously analysis of quantitative and confirmatory analysis for many metals. The limit of detection (LOD) is defined as 3 SD for a concentration with signal-to-noise ratio of 3. The detection limits are < 40 μg for dust and, < 0.01 μg for cobalt [205]. The corresponding air concentrations are < 0.040 mg/m^3 for dust and, < 0.00001 mg/m^3 for cobalt for an 8- hour full work shift sample and a flow rate of 2 L/min. Measurements below LOD were assigned LOD/$\sqrt{2}$ before the final calculations [206]. All analyses were performed at the laboratory at the Department of Occupational and Environmental Medicine, Örebro University Hospital, Sweden. The laboratory is accredited for sampling and analyses of both dust and metals by the Swedish Board of Accreditation and Conformity Assessment (SWEDAC).

Papers III and IV

Culturing of the HaCaT cells (Papers III and IV)

Culture conditions vary widely for different cell types, but the artificial environment in which the cells are cultured is constituted by the same factors for all kinds of cells. The cells require a substrate or medium that supplies the essential nutrients, growth factors, hormones, gases and a regulated environment for temperature and pH. HaCaT cells are adherent cells, growing in monolayers, and must be cultured while attached to a solid or semi-solid substrate. The HaCaT cells used in studies III and IV were cultured under

controlled conditions at 37 °C and 5% CO_2. They were cultured in cell culturing flasks in Dulbecco's Modified Eagle's Medium growth medium (DMEM) containing 0.15 mM calcium and supplemented with fetal bovine serum (FBS), L-glutamine and gentamicin. The cells were passaged with trypsin when they reached an estimated 70% confluency. Prior to the experiments, cells were cultured in DMEM containing 1.8 mM calcium for seven days to induce differentiation or kept in medium containing 0.15 mM calcium for a low differentiated phenotype [207-209].

Differentiation of the HaCaT cells (Papers III and IV)

In the skin, the keratinocytes are in different differentiation stages depending on whether they are basally located or further out towards the surface of the skin. In order to study cell response in these two stages of differentiation we used a model with calcium in different concentrations added to the growth medium. One of the most studied and used ways to trigger cell differentiation is to use extracellular calcium as an inducer. For an undifferentiated phenotype low calcium of 0.03 mM up to 0.1 mM is required and, to induce differentiation, high calcium of > 1 mM is required [207-209]. When using calcium as an inducer for differentiation the differentiation markers should start to appear 48 to 72 hours after the calcium switch. The HaCaT cell in studies III and IV were cultured under low calcium conditions to keep the cells as a low differentiated phenotype representing basally located cells in the epidermis. Differentiation in the cells was initiated by switching to high calcium culturing conditions in order to achieve a more differentiated phenotype representing cells located further out in the epidermis (Figure 9).

Other triggers of differentiation can include confluency of the cells and culturing temperature. The cells are sensitive to cell density and should not exceed 75- 80% confluence for maintaining the cells in a basal state. Higher cell density could lead to differentiation in the cells [207]. HaCaT cell line can also be reverted back to basal cells, and 72 hours after switching from high calcium to low calcium, the cells will already lose their tight cell junctions, be less compacted and show changes in their morphology [209].

One way of visually distinguishing basal HaCaT cells from more differentiated cells is the morphology. Cells in low calcium will be elongated and spindle shaped and lack the cell-to-cell tight junctions, while cells in high calcium will have a more cubical shape and be tighter packed [207].

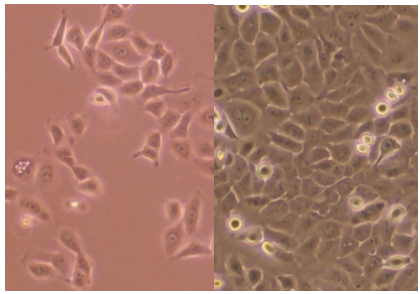

Figure 9. HaCaT cells cultured in a low (left) and high (right) calcium medium.

Cell viability assay (Paper III)

Cell viability is a measure of the proportion of live, healthy cells in a sample. However, it does not take into account the cells phase in the cell cycle. In study III, cell viability was measured using the Cell-Titer Blue viability assay and analyzed using a microplate reader. The assay provides a measure of the cells metabolic activity and is based on the ability of living cells to metabolize a redox dye (resazurin) substrate into a fluorescent product (resorufin). The viability assay provided intervals for time and cobalt concentration for cell toxicity to be used in the subsequent experiments investigating the inflammatory response.

Immunoassay analyzed using flow cytometer (Papers III and IV)

An immunoassay measure the presence of a molecule in a solution. Immunoassay tests are based on the use of an antibody that will specifically bind to the antigen of interest. The antibodies used in the immunoassay must have a high affinity for the antigen. In studies III and IV, the cytokine/chemokine production was measured using a bead-based immunoassay. A bead-based immunoassay is a method that uses two antibodies that bind to different sites of the antigen. This results in the capture of the antigen between the two antibodies. One of the antibodies is connected to a bead, and the other antibody carries a fluorochrome for quantification.

The immunoassay was analyzed using a flow cytometer. Since the assay contains two different sized beads and 13 different populations of capture antibodies, the assay allows for quantification for 13 cytokines in each sample. Flow cytometry is a laser-based technology used to analyze cells or particles. The principle behind flow cytometry is that the cells or particles or in our case, beads, are suspended in a fluid and lined up in a single row that

will pass thorough a flow chamber in which lasers excite the fluorescent dye. The emitted light from the dye is then quantified as fluorescent intensity and in the case of our immunoassay, each cytokine was detected on an isolated population of beads differentiated by size, allowing and inherent fluorescence able to distinguish be-tween cells or particles depending on size, quantification of multiple cytokines simultaneously.

Caspase-1 assay (Paper IV)

Measurements of caspase-1 activation were performed using a caspase-1 assay kit, FLICA 660. It is an *in vitro* method in which a fluorescent inhibitor probe enters the living cell and binds to active caspase-1. The fluorescent signal was read using a flow cytometer. This method allowed us to detect and quantify the populations of cobalt-exposed HaCaT cells and differentiate between cells with active caspase-1 and the cells that did not express active caspase-1.

Gene expression analysis using PCR (Papers III and IV)

Polymerase chain reaction, PCR, is a method used to amplify a sample of DNA in order to obtain enough copies in the sample for a quantitative analysis to be possible. Three major steps are involved in a PCR, and each step takes place at different temperatures. First, the double stranded DNA opens up to allow the second stage, in which a specific florescent probe (primer) binds to a single DNA strand and a polymerase attaches and start copying the strand. Last, the strands are coupled and a double stranded DNA molecule is put together again. This is one cycle. During this cycle, the double stranded DNA molecule is amplified into two double stranded DNA molecules, and the number of copies increases exponentially. Usually, 30- 40 cycles are made and the gene expression, i.e. the amount of available copies, is quantified due to the accumulated fluorescent signal from the gene specific probes.

When the starting material is RNA, it must first be transcribed into complementary DNA (cDNA). This process is called reverse transcription. An enzyme called reverse transcriptase generate the cDNA, which can then be used as a template for the qPCR reaction. This combination of reverse transcription and PCR allows for detection of low amounts of RNA in a sample.

Statistical analyses

Paper I

Personal air measurements and stationary air measurements were calculated as an 8-hour time weighted average concentration (8 hour TWA) for the full workday. For descriptive purposes, standard parameters (arithmetic mean (AM), standard deviation (SD), geometric mean (GM), geometric standard deviation (GSD) and range) were calculated.

Further, Spearman´s rank correlation based on personal and stationary area measurements comparison, including inhalable and total dust, particle number and particle surface area concentrations, was performed. Regression analysis was used to analyze relations between personal exposure measurements and stationary measurements.

Paper II

Concentrations for the thumb, index finger, middle finger, ring finger and palm were added up to represent cobalt skin exposure and concentrations have been converted to the average for one hour. Blood concentrations used in statistical analyses is the difference between before and after shift samples, and reflects the uptake of cobalt during a work shift.

Rank correlation and simple linear regression analysis were performed to study the relationship between skin, blood and inhalable air cobalt concentrations. In addition, linear multiple regression analysis was used to study the relationship between cobalt in blood, skin exposure and inhalable air exposure together. The effect on the uptake in blood when air and skin exposure concentrations changes are also presented.

Papers III and IV

In Paper III, cell viability is presented in the diagrams as fluorescence signal of the exposed cells compared to the unexposed control cells. Mann-Whitney was used to compare the dose-dependent effects over time.

In Papers III and IV, immunoassay data are presented in the diagrams with each point representing concentration data from one measurement. Data from gene expression analysis are presented in diagrams as the median value of the relative mRNA levels. High and low calcium growth conditions are shown.

A linear mixed model was developed to describe the relationship between the different exposure times, $CoCl_2$ concentrations and high and low calcium incubation of the cells. Linear mixed models are extensions of simple

linear models that predicts the dependent variables as a function of the independent variables. The mixed model takes into account both variations explained by the independent variable and variations not explained by the independent variables. The model is useful in settings with repeated measurements or measurements in cluster. It also has the advantage of dealing with missing values. The mixed linear model was used for both cytokines/chemokines and gene expression. In the linear mixed model, as one input variable was changed at a time and the other variables were kept constant, correlations for each of the exposure times, $CoCl_2$ concentrations, exposure groups and high- and low calcium could be revealed. The results of the mixed model analysis are presented as odds ratio which allows for quantitative evaluations of the differences for a certain strata compared to a reference group rather than answering whether there are differences or not alone. The equation of the mixed model is described in detail in Paper III and in paper IV.

In paper IV, the presentation of the caspase-1 activity is expressed in percentage as fluorescence signal of the activated caspase-1 in exposed cells compared to unexposed cells. A paired t-test was used to investigate the effects of cobalt exposure on caspase-1 activity. The gating strategy we used to determine the FLICA-stained cells (activated cells) is also shown in Paper IV. The mixed linear model was also used for caspase-1 activity in the HaCaT cells to describe the effects of $CoCl_2$ exposure time and concentration and the differentiation status of the cells.

RESULTS AND DISCUSSION

A summary of the main findings of Papers I-IV is described and discussed in this section. For a more detailed description, the reader is referred to the respective papers (Papers I-IV)

Cobalt air levels in the hard metal production industry (Paper I)

The aim of Paper I was to determine exposure air concentration levels of inhalable cobalt and tungsten and other particle exposure measures for risk assessment and investigate the possibility of using these data as proxies for exposure measures.

Personal exposure measurements

In our study, personal exposure measurements were performed on 72 workers in the hard metal production facility (table 2). Inhalable and total dust were measured. The levels of cobalt in the inhalable fraction ranged between 0.000028 and 0.056 mg/m^3 (AM 0.0030 mg/m^3) and 6% of the samples exceeded the Swedish OEL (0.02 mg/m^3). The levels of cobalt in total dust ranged from < 0.000011 to 0.028 mg/m^3. Previous studies have reported general air concentrations for cobalt ranging between 1 μg/m^3 and 6.39x10^3 μg/m^3 [34, 36] and compared to those, our levels seem very low. The low levels most likely reflects the continuous work with production techniques, for example eliminating exposure by enclosing the processes in which high exposure is generated, as well as improvement of ventilation. Decreased OELs leads to reduced exposure levels in the industries as well as an awareness of the health issues arising from exposure to cobalt. The highest cobalt air exposure levels were found for the work in the powder production department and for work in the PVD- furnace. The difference in cobalt air exposure levels between the departments lies in the different stages and in the production process and possibly to some extent the workers' personal behavior.

Real-time monitoring was also performed on 52 of the participants using a personal data logger (DataRAM). The measurements were conducted for a full eight hours work shift and the workers also kept a work diary where they wrote down time and duration of their work tasks throughout the day. The Data-RAM logs data every 10 seconds and therefore detects the exact work tasks that gives the high peak exposures linked to adverse health effects [210, 211]. When the peaks are identified, preventitative measures can be implemented lower emissions from these specific tasks. Direct monitoring of exposure is also useful from a pedagogical perspective and can help

the workers identifying whether a specific behavior is causing the high peak exposures. In our study the highest high peak exposures (83 mg/m^3) were measured during the blasting of hard metal pieces in the PVD-furnace department (Figure 10). Other work tasks in which high peaks were recorded included drying during cleaning of tanks and vessels (67 mg/m^3) in the powder production department and emptying a silo (39 mg/m^3) in the pressing department.

Table 2.Personal air concentration exposure to inhalable and total dust and cobalt (mg/m^3).

	N	AM	SD	GM	GSD	Range	OEL
Inhalable dust	72	0.15	0.37	0.37	2.2	<0.023-3.0	10
Inhalable dust-Co	72	0.0031	0.0083	0.0083	5.0	0.000028-0.056	0.02
Total dust	71	0.13	0.24	0.24	0.082	<0.040-1.8	
Total dust-Co	71	0.0018	0.0041	0.0041	0.00052	<0.000011-0.028	

n: number of measurements
AM: arithmetic mean
SD: standard deviation
GM: geometric mean
GSD: geometric standard deviation
Range: min-max

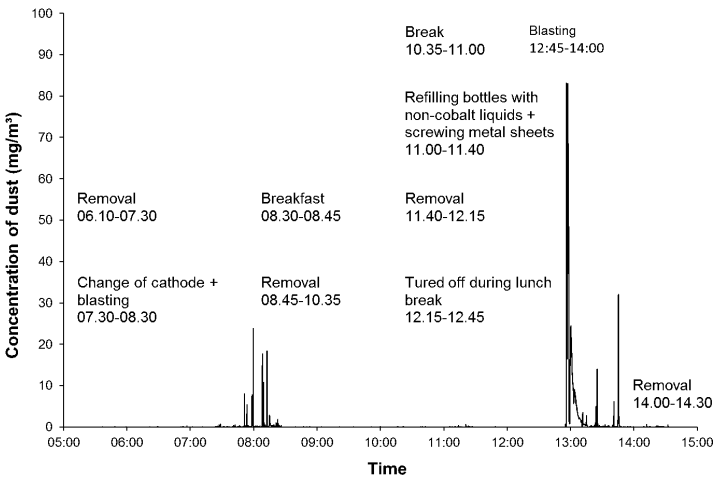

Figure 10. Example of measurements using a DataRAM at the PVD-furnace

Stationary air measurements

The stationary measurements were performed simultaneously as the personal measurements and the sampling equipment was strategically placed at the same departments. The average stationary inhalable dust level was 0.03 mg/m^3 and was lower than the corresponding average for the personal exposure measurements, 0.15 mg/m^3. The corresponding average for inhalable and total cobalt air concentrations was 0.00074 mg/m^3 and 0.00071 mg/m^3, respectively. The lower levels detected in the stationary sampling can be explained by the placement of the sampling equipment. The equipment in our study was located in a far field. When the sampling equipment is in the far field, detection levels can be expected to be lower due to ventilation and air flow. The workers on the other hand is close to the emission source in the near field, which gives considerably higher concentration for the personal exposure measurements.

No data on exposure or stationary sampling of particle number or particle surface area concentrations have been presented for the hard metal industry. However, stationary air measurement data from a survey representing a blend of Swedish industries showed particle number concentration ranging from 12 000 to 22 000 p/cm^3 and particle surface area concentrations ranging from 45 to 3 800 μm^2/cm^3 [212]. Our air concentration levels for both particle number and particle surface are much lower, well reflecting the difference in average total dust concentration, 0.033 mg/m^3 for our hard metal industry compared to 1.1 mg/m^3 of total dust for the Swedish study.

Relationships between different particle fractions

We have also investigated the possibility of using stationary measurements as proxies for personal exposure measurements for different particle air concentration fractions in a hard metal production facility. This is of special interest as there is a potential link between a specific particle fraction and its target area resulting in different respiratory problems, and other negative potential health effects as well as early markers of disease, i.e., inflammatory and coagulatory markers. In parallel to investigating different particle sizes, mass-, area –and particle numbers in our hard metal industry, inflammatory markers were measured and correlated to all our exposure measures presented in a report by Westberg et al. [213]. Furthermore, a correlation between inflammatory markers and exposure has been shown, associated with increases in mortality and hospital admissions due to cardiovascular diseases. The underlying mechanisms for systematic inflammatory response known to occur in cardiovascular diseases studied in human and animal

experiments, suggest a relationship between exposure and inflammatory response [214]. Recent findings by Hedbrandt et al. from measurements conducted on quartz exposed workers in an iron foundry showed that IL-18 and IL-RA (interleukin1 receptor antagonist, encoded by the *IL1RN* gene) gave good correlation with specific particle dust fractions [215]. This further indicate the importance of studying the relationships between particle fractions and the possibility of using them as proxies for future risk assessment studies.

To investigate the relationships between exposures and readout, we used a linear regression analysis. Linear regression analysis is used to determine the extent to which there is a linear relationship between a dependent variable and one or more independent variables. In Table 3, the personal correlations including personal air sampling are shown. The analysis shows that personal and stationary inhalable cobalt correlate, but not inhalable cobalt and particle surface area or particle number. The regression analysis further showed wide confidence intervals for personal and stationary inhalable dust predictions, indicating high level of uncertainty for individual predictions. However, the wide confidence intervals are expected though, since prediction intervals predict individual variables rather than parameters of a distribution. When the stationary sampling was analyzed (data is presented in Paper I), only the mass-based measures were correlated, suggesting caution when mass based exposure measurement data should be used as proxies for particle surface area or particle number air concentrations for further dose-response analyses.

Table 3. Linear regression analysis of different particle measures based on stationary area measurements as dependent and personal inhalable cobalt exposure as independent variables [a,b].

Aerosol fraction (Dependent; Independent)	Regression			Prediction	
	β(p)	95% CI (β)	r^2	Predicted concentration [c]	95% CI (γ)[d]
Inhalable Co (S); Inhalable Co (P)	0.88 (<0.001)	0.626-1.14	0.675	0.00530	0.000611-0.0460
Particle surface area (S); Inhalable cobalt (P)	-0.153 (0.127)	-0.353-0.048	0.118	3.49	1.00-12.3
Particle number (S); Inhalable Co (P)	0.015 (0.799)	-0.104-0.134	0.003	2540	944-6900

S = Stationary measurements
P = Personal exposure measurements
β = Regression coefficient
95% CI (β) = 95% confidence interval for β
r2 = correlation coefficient
a All data ln-transformed, except predicted concentrations presented in original units.
b n = 28, except for particle surface area (n = 22) and particle number (n = 27)
c Predicted concentration = predicted individual concentration for inhalable cobalt 0.02 mg/m3.
d CI (y) = 95% confidence interval for predicted dependent metrics, individual concentrations.

Biological monitoring of cobalt in blood, sampling of cobalt on skin and in air and the contribution of skin exposure for the uptake in blood (Paper II).

The aim of the study was to examine whether cobalt skin and inhalable air exposure contributes to uptake determined as cobalt in blood.

The study group

The workers who participated in this study was evenly distributed in age and 50% of them had been working at the plant for 10 years or more. The group consisted of more men than woman. There had been cases of contact allergy and eczema among the workers in this facility, but they had been reassigned to positions in which they did not handle cobalt. No workers participating in this study reported having eczema and skin allergy.

Measurement of cobalt on skin, in blood and in inhalable air

It is intriguing to investigate the importance of skin uptake since air levels are consistently low and the majority of our measurements are below the Swedish OEL of 0.02 mg/m^3 for cobalt in the inhalable air fraction. Despite the low measured air concentrations, biological monitoring of cobalt in blood showed relatively high concentrations.

Measurement of cobalt on skin varied among the participants and was found in all samples from the hands. Skin samples were collected from 62 of the workers who worked normally for two hours without washing their hands, and when the concentration was converted to an hour, the cobalt skin concentrations ranged between 0.046 and 100 μg/cm^2/h. No occupational exposure limit is set for skin exposure, but our skin exposure levels were in line with other studies in which cobalt is handled in the production industry [7, 39]. The highest concentrations in our study were found in the pressing department (AM: 29 μg/cm^2/h) and the powder department (AM: 3 μg/cm^2/h), see Table 4.

Blood samples were taken before the shift and after the shift and the difference represent the uptake of cobalt in blood that particular workday. The levels of cobalt in blood varied between < 3.2 and 110.3 nM and only 1% of the samples exceeded the Biological Exposure Indices, BEI (17 nM) set by the American Conference of Governmental Industrial Hygienists [97]. The personal air exposure measurements of cobalt were conducted for a full work shift and the concentrations in [7] inhalable air were low and ranged

between 0.000028 and 0.056 mg/m^3. Only 6% of the samples exceeded the Swedish Occupational Exposure Level (OEL) (0.02 mg/m^3) [216].

Table 4. Concentrations of cobalt on skin (µg/cm^2/h), in blood (nM), 1-4 samples per person, and cobalt in the inhalable air (mg/m^3) from personal measurements.

	n	AM	Median	SD	Range
Skin-Co (µg/cm^2/h)	62	12	2.0	20.0	0.0046-100
Blood-Co (nM)	72	7.95	6.39	9.43	<3.2-110.3
Inhalable air-Co (mg/m^3)	72	0.0030	0.00074	0.0083	0.000028-0.056

n: number of workers
AM: arithmetic mean
SD: standard deviation
Range: min-max

Uptake of cobalt via the skin

The sampling of cobalt on skin, personal sampling of cobalt in inhalable air and biological monitoring of cobalt in blood was conducted on the day/same work shift for the respective participant. The fact that all samples were taken on the same day made it possible for us to investigate whether the skin exposure had an effect on the uptake on that particular day in relation to the established uptake via inhalation. Uptake via inhalation has been confirmed, but the air levels could not be accounted for the total uptake (measured in blood and urine). Thus, it is suggested that skin contributes to the total uptake [7, 62]. The uptake of cobalt via the skin was also shown in an experiment in which volunteers exposed their hands to cobalt [6]. Further, *in vitro* studies have shown that cobalt can indeed penetrate the skin and cobalt has also been detected inside the skin cells [103, 112].

To investigate whether skin exposure affects the uptake of cobalt, the collected sampling data from skin, air and blood were used. The uptake was defined as the uptake in blood. We used both simple linear regression and multiple linear regression analyses to investigate the uptake. When both the skin and personal inhalation concentrations were included in the multiple regression analysis a lower regression coefficient was obtained for cobalt in blood and skin than for cobalt in blood and inhalable dust (B = 0.010 and 49.1). Significant regression coefficients for both cobalt blood and skin ($p < 0.05$) and for cobalt blood and inhalable air ($p < 0.001$) were determined, see Table 5.

Table 5. Multiple linear regression analysis between cobalt skin exposure, cobalt in inhalable air and uptake expressed as cobalt in blood.

Uptake blood	Exposure	df	r^2	B	CI (B)	P-value
Blood-Co	Skin-Co	60	0.317	0.010	0.003-0.016	<0.01
	Inhalable air-Co			49.1	26.8-71.4	<0.0001

df = degrees of freedom
B = regression coefficient
95% CI (B) = 95% confidence interval for B
r^2 = correlation coefficient

Magnitude of uptake via skin

Confirmation of the effect of skin uptake in regard to the total uptake represented as the blood concentration made us curious about how large the effect from the skin uptake actually was. We therefore developed a mathematical model using the regression formula to explore this, as shown in Table 6. We investigated how a decrease in skin and air levels changed the blood levels. We did this by changing one of the skin or air levels at a studies kept the other constant. Consequently, we saw for example that a twofold increase in cobalt skin exposure at a given air concentration caused an 8% increment in cobalt blood concentration. We also saw that a doubling of inhalable air concentration at a given skin concentration caused an increment of 64% cobalt blood concentration. These finding were interesting and suggests that skin exposure to cobalt in the hard metal industry could affect the total uptake in the same order of magnitude as air exposure. This could further strengthen the importance of dermal exposure when considering preventive measures as exposure to cobalt is a known sensitizer and can cause inflammation in the skin. Occupational exposure limits for dermal exposure as well as dermal exposure assessments are needed.

Table 6. A regression-based model showing the influence of changes in cobalt air and skin concentration on cobalt blood levels.

Blood (nM)	Air (mg/m^3)	Skin ($\mu g/m^2/h$)
0.77	0.01	6
0.83	0.01	12
0.95	0.01	24
1.26	0.02	6
1.32	0.02	12
1.44	0.02	24
2.24	0.04	6
2.30	0.04	12
2.42	0.04	24

Triggering of an alarm system in HaCaT cells due to cobalt exposure (Paper III)

The aim of the study was to compare effects of cobalt on the viability of cultured human keratinocytes (HaCaT) cultured at low or high calcium (the latter yielding a more differentiated cell type) and to determine the pro-inflammatory cytokine release.

HaCaT cell viability

The effect of cobalt on the skin cells is of major concern because when the human skin is exposed to cobalt, the metal will ionize, be taken up through the skin [75, 217] and exert damage on the cells [112, 117]. In our previous study (Paper II), the skin route was found to account for a relatively large amount of the total uptake.

In vitro models with the immortalized human keratinocyte cell line HaCaT are often used in the study of irritants on the skin. The fact that $CoCl_2$ has a negative effect on cell viability has been reviewed [103, 119, 121] but, to our knowledge, no one has investigated whether viable cells located further out in the epidermis react differently than cells located further down in the epidermis when exposed to cobalt. In Papers III and IV, the more basally located cells were represented by the cells cultured in low calcium conditions, and the suprabasally located cells were represented by the cells cultured in high calcium medium. We found that the effects on the metabolic activity in HaCaT cells were dose- and time-dependent for both low and high calcium growth conditions (Figure 11). In addition, the keratinocytes cultured in a low calcium medium were more affected by the $CoCl_2$ exposure than the cells in the high calcium medium.

In order to confirm the appropriate incubation times and concentration of the cells for further experiments, the cells were exposed to $CoCl_2$ concentrations of 5 mM, 2.5 mM, 1 mM, 500 μM, 250 μM, 100 μM, 10 μM and the exposure time was set to 3, 6, 12, 24 and 48 hours. Overall, our findings were in line with other studies in which cobalt concentrations were found be toxic to the cells starting at 400 μM [103, 120].

Since time is a strong factor in the sensitization of cobalt, the exposure time was extended in an experiment using a low, subtoxic level of $CoCl_2$ over a period of 14 days. The cell viability remained the same throughout the experiment, showing no effects at this low level of exposure.

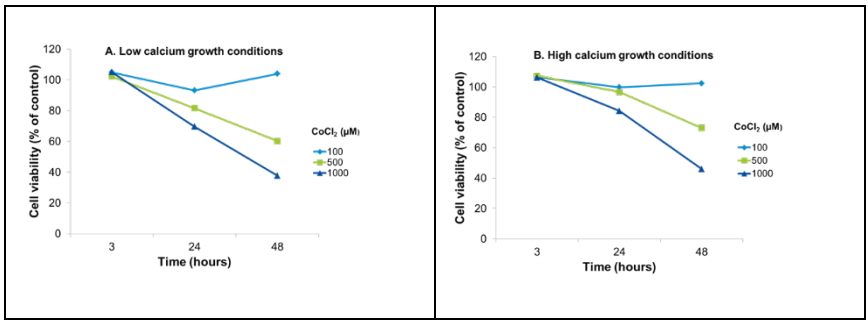

Figure 11. Cell viability for HaCaT cells exposed to $CoCl_2$.

Triggering of the alarm system

When allergen or irritants come in contact with the skin, they will function as a sort of danger signals for the immune system. The immune active cells will respond by producing a variety of inflammatory cytokines and chemokines, triggering an alarm system in the skin. Cobalt is an important skin sensitizer, which is why exposure of an allergen and the production of a "warning signal", i.e. pro-inflammatory cytokines, is needed for sensitization [218-220]. Keratinocytes are the major producers of the “warning signals” in the skin, and they also play an important role in the regulation of inflammation due to their ability to respond to stimuli from the environment.

For the detection of pro-inflammatory cytokines in the *in vitro* exposure experiments on the HaCaT cells in Papers III and IV, cobalt chloride concentrations of 100 µM (determined as a subtoxic level) and 500 and 1000 µM (both determined as toxic to the cells in the viability assay) were used. Since a negative effect on cell viability was already detected at 6 and 24 hours for some concentrations, the two time points of 24 and 48 hours was used. Secretion of IL-6, CXCL8 (IL-8) and CCL2 (MCP-1) from the HaCaT cells in response to the $CoCl_2$ exposure was seen (Paper III). Also, the gene expression for the same cytokines/chemokines was investigated as an early detection of an inflammatory response. Levels of IL-6 mRNA, CXCL8 mRNA and CCL2 mRNA in the HaCaT cells were found after $CoCl_2$ exposure which reflected the cytokine production. Our results were in line with others who also found an increase in the pro-inflammatory cytokines IL-6 and CXCL8 and of IL-6 mRNA at 500 µM $CoCl_2$ [120, 122].

Effects of $CoCl_2$ on the inflammatory response in HaCaT cells

By using the linear regression model (Table 7) and presenting the results as odds ratio for the impact of cobalt exposure time- and concentration, as well as the effects of high- and low calcium growth conditions on the production of IL-6, CXCL8 and CCL2, we could detect the magnitude of the impact, not just whether there was an effect. A time-dependent increase in IL-6 and CXCL8 in response to $CoCl_2$ exposure was detected, but the $CoCl_2$ concentration only affected IL-6 production. The CCL2 levels decreased with increasing $CoCl_2$ concentration, but increased over time.

Comparison of the exposure time of 24 hours with the starting time led to an almost 4-fold increase in IL-6 production, and 48-hour exposure to a 5-fold increment. Increasing the $CoCl_2$ concentration resulted in a 3.5-fold increment for IL-6. For CXCL8 on the other hand, only time affected the production. At 24 hours there was an almost 9-fold increment, and at 48 hours, an 11-fold increment. To our surprise, CCL2 production decreased with increasing $CoCl_2$ concentration, but showed a 6- and 11- fold increment for 24 and 48 hours, respectively. When implementing high and low calcium incubation conditions, representing the more differentiated cells and less differentiated cells, only IL-6 production was affected. The gene expression may reflects an earlier version of the inflammatory response as the pro-inflammatory cytokine production. Both cytokine/chemokine production and mRNA production indicates that keratinocytes are involved in an acute exposure to cobalt.

The role of IL-6, CXCL8 and CCL2 in the skin

IL-6 is one of the cytokines associated with skin inflammation and both allergic and irritant dermatitis. IL-6 is involved in growth and differentiation of epidermal cells and is closely linked to wound healing. It also helps regulating the epidermal barrier by thickening of the stratum corneum [221-225].

Pro-inflammatory chemokines are primarily produced by cells to recruit leukocytes to the site of infection or injury. The main role of CXCL8 in inflammation is in the recruitment of neutrophils, although it is also responsible for chemoattractant migration and the activation of monocytes and lymphocytes to the site of inflammation [226, 227]. CCL2 attracts monocytes, leukocytes and natural killer cells and is also suggested to be involved in Th2 polarization [228-231].

Cytokines/chemokines are key modulators of inflammation. The results in Paper III show that cells respond to the danger signal ($CoCl_2$) by producing pro-inflammatory cytokines shown to be involved in skin inflammation

in a dose- and time-dependent manner. The relatively fast production of these cytokines, shown by both gene expression and cytokine secretion, indicates that keratinocytes play a role in an acute inflammatory response to cobalt.

Table 7. Linear regression mixed model analysis of dose- and time trends for IL-6, CXCL8, CCL_2 and high (1.8mM) and low (0.15mM) calcium incubation of HaCaT cells exposed to $CoCl_2$ [a,b].

Cytokine/ chemokine	Exposure time /$CoCl_2$ concentration/ High vs. low calcium incubation	OR	CI 95%
IL-6	30 minutes	1.00	
	24hours	**3.93**	**2.08 - 7.43**
	48 hours	**5.11**	**2.70 - 9.76**
	0 µM	1.00	
	100 µM	1.20	0.58 - 2.49
	500 µM	1.77	0.85 - 3.68
	1000 µM	**3.50**	**1.67 - 7.32**
	Low calcium	1.00	
	High calcium	**2.00**	**1.19 - 3.37**
CXCL8	30 minutes	1.00	
	24 hours	**8.49**	**4.58 - 15.73**
	48 hours	**10.56**	**5.7 - 19.57**
	0 µM	1.00	
	100 µM	1.27	0.63 - 2.58
	500 µM	1.20	0.59 - 2.44
	1000 µM	1.85	0.91 - 3.77
	Low calcium	1.00	
	High calcium	0.87	0.53 - 1.44
CCL2	30 minutes	1.00	
	24 hours	**5.98**	**2.88 - 12.44**
	48 hours	**10.95**	**5.27 - 22.77**
	0 µM	1.00	
	100 µM	0.80	0.35 - 1.84
	500 µM	**0.33**	**0.14 - 0.76**
	1000 µM	**0.26**	**0.11 - 0.61**
	Low calcium	1.00	
	High calcium	1.00	0.55 - 1.80

OR = Odds ratio
CI 95% = 95% confidence interval
[a] All data ln-transformed
[b] n =120

Effects of cobalt exposure on the NLRP3 inflammasome in keratinocytes (Paper IV)

The aim of Paper IV was to investigate dose- and time-resolved conditions in which $CoCl_2$ affect HaCaT-cells to exhibit changes in pro-inflammatory cytokine release and NLRP3 inflammasome activation as a model of the events in the skin following cobalt exposure.

Activation of the NLRP3 inflammasome in HaCaT cells

In the *in vitro* experiments in which HaCaT cells were exposed to $CoCl_2$, IL-1β as well as IL-18 was secreted from the cells. In these experiments, we tested $CoCl_2$ concentrations of 100, 500 and 1000 μM for 24 and 48 hours. However, it was only the highest concentration tested that resulted in a significant secretion of IL-1β and IL-18. The levels of IL-1β were low, but since IL-1β regulates many functions of the immune system, only small amounts seems to be needed for a proper inflammatory response [232, 233]. The secretion of IL-1β and IL-18 is our first indicator that the NLRP3 inflammasome is activated in keratinocytes upon $CoCl_2$ exposure. The inflammasome require the enzyme caspase-1 to cleave pro-IL-1β and pro-IL-18. The proteins are then secreted from the cells [164, 180, 181, 234]. Others have found secretion of IL-1β from HaCaT cells upon $CoCl_2$ exposure, although at a lower $CoCl_2$ level (500 μM) than we did [120, 122].

It has been established that the NLRP3 inflammasome is present in HaCaT cells and that the inflammasome is activated in a variety of cells, including HaCaT upon exposure to cobalt [11, 12, 120, 197]. To further support our findings regarding the involvement of the inflammasome, the gene expression of inflammasome components was measured, including the mRNA of *IL1B*, *IL18*, *CASP1*, *NLRP3* and *PYCARD*. The NLRP3 inflammasome is built up of the sensor molecule NLRP3, the adaptor protein ACS and pro-caspase-1. The ASC protein is encoded by the PYCARD gene. We detected gene expression for all the above inflammasome components upon $CoCl_2$ exposure.

Additionally, a significant production of active caspase-1 was detected upon exposure to 1000 μM $CoCl_2$ for 24 hours. This also indicate the activation of the inflammasome upon $CoCl_2$ exposure since it is the active form of caspase-1 which in turn, cleaves pro-IL-1β and pro-IL-18 to their active forms. Our data indicate that cobalt can activate both the first priming signal and the second activating signal of the inflammasome.

Dose- and time-dependent relationships for NLRP3 inflammasome activation

Using a mixed model and choosing to present the results as odds ratio allowed us to determine the magnitude of the effect, not just whether or not there was an affect. Exposure of $CoCl_2$ showed an almost two-fold increase in IL-1β secretion and over six-fold increase in IL-18 secretion from the HaCaT cells (Table 8). This increase was only seen for the highest concentration tested: 1000 μM. Time had no impact on the secretion and neither did high- or low calcium incubation. In concordance, active caspase-1 was significantly increased for the exposed cells and an almost three-fold increase was seen at 1000 μM $CoCl_2$. High- and low calcium incubations did not affect the caspase-1 activity. Moreover, gene expression was also affected by the $CoCl_2$ exposure, and a significant mRNA production of *IL1B* and *IL18* was already seen at 500 μM. Time had an effect on *IL1B* mRNA production, which decreased at 48 hours. mRNA levels of *NLRP3* showed a significant increase at 1000 μM, whereas both *CASP1* and *PYCARD* mRNA levels decreased with time and concentration during CoCl2 exposure. Taken together, these data suggest the involvement of the caspase-1/NLRP3 inflammasome/IL-1β axis in keratinocytes upon exposure to cobalt.

The finding that the mRNA for the inflammasome components caspase-1 (*CASP1* mRNA) and ASC (*PYCARD* mRNA) decreased could in the long term, result in the components being used up as no new components are produced. Such mechanism might exist to control the inflammatory response in the skin. If this is the case, it would indicate a potential "burnout" of the inflammasome and will eventually stop the cleavage of pro-IL-1β and pro-IL-18 to their active forms, leading to no prolonged infiltration of cells to the site of $CoCl_2$ exposure. A similar pattern in mRNA was seen in a study investigating the role of the inflammasome in human immune cells stimulated by the bacterium *Neisseria meningitidis*. Upon stimulation, mRNA levels of *NLRP3* increased, and the mRNA levels of *PYCARD* and *CASP1* were unaffected by the stimulation [235].

Several molecules have been shown to be capable of causing negative regulation of the inflammasome through for example inhibiting the generation of mitochondrial ROS, suppressing or binding to NLRP3 or, through interaction with ASC. Negative regulation of the inflammasome activation is necessary for maintenance of the proper induction of inflammasome function and for preventing a potentially harmful reaction in the host [189]. It has been suggested that autophagy plays a key role in the negative regulation of inflammasome activation through various mechanisms including direct inhibition of the NLRP3 inflammasome, removal of sources of NLRP3

triggers such as mitochondrial DNA, and selective degradation of inflammasome components such as NLRP3 and ASC [161, 236]. Recent findings in a study on foundry workers exposed to quartz showed an increase in IL-18 and IL-1Ra in monocytes in workers' blood when medium and high exposure groups were compared to a low exposure group. Caspase-1 activity, on the other hand decreased for the medium and high exposure groups. The study showed that in the higher exposure groups monocytes showed less responsiveness to the stimulation of inflammasome signal 1 triggers. Hedbrandt et al. suggest that the low responsiveness could indicate that the NLRP3 inflammasome is pre-activated in the higher exposure groups leading to less response in the inflammasome due to additional exposure [215].

To our knowledge, thus far, no one has yet investigated the dose- and time effect on NLRP3 inflammasome activation resulting from $CoCl_2$ exposure in skin cells. We find this self-regulating effect in the keratinocytes very intriguing.

Table 8. Linear regression mixed model analyses for dose- and time trends for IL-1β, IL-18, high (1.8mM) and low (0.15mM) calcium incubation [a,b].

Cytokine	Exposure Time /$CoCl_2$ concentration/ High-low calcium incubation	OR	CI 95%
IL-1β	30 minutes	1.00	
	24hours	0.73	0.46 - 1.16
	48 hours	0.88	0.56 - 1.40
	0 µM	1.00	
	100 µM	1.05	0.62 - 1.79
	500 µM	1.11	0.65 - 1.89
	1000 µM	**1.8**	**1.06 - 3.07**
	Low calcium	1.00	
	High calcium	0.75	0.52 - 1.10
IL-18	30 minutes	1.00	
	24 hours	1.12	0.60 - 2.08
	48 hours	1.63	0.87 - 3.04
	0 µM	1.00	
	100 µM	1.29	0.63 - 2.62
	500 µM	1.51	0.73 - 3.09
	1000 µM	**6.23**	**3.04 - 12.77**
	Low calcium	1.00	
	High calcium	1.04	0.63 - 1.73

OR = Odds ratio
CI 95% = 95% confidence interval
[a] All data ln-transformed
[b] n =120

Role of NLRP3 inflammasome activation in skin inflammation

Cytokines are key modulators of inflammation and play a role in acute and chronic inflammation via a complex network of interactions. Keratinocytes are the main producers of these cytokines in the skin. Cobalt act as a danger signal in the skin cells, triggering an alarm system comprising these cytokines.

Earlier findings suggest that keratinocytes play an important role during the innate phase of the immune responses upon exposure to danger signals, substances that are sensed by the inflammasome, and increased levels of IL-1β and IL-18 have been linked to hyper sensitivity [197, 237]. Further, models have shown that contact hypersensitivity is dependent on the NLRP3 inflammasome and the importance of inflammasome components being presents in contact allergy has been studied [197, 238-240]. The NLRP3 inflammasome has been shown to be of importance during the sensitization phase of contact hypersensitivity [239]. Also, during the early phase of the elicitation, which is characterized by increased vessel permeability and local edema, it is suggested that the NLRP3 inflammasome is of importance. This phase peaks around eight hours after antigen exposure and mediated by for example IL-1β [197]. Our findings show an early production of mRNA and secretion of IL-1β and IL-18, indicating the response during acute inflammation in the skin.

The fact that we can see a reduction in inflammasome components, possibly leading to negative regulation of the inflammasome activity, further supports the idea that the NLRP3 inflammasome could be involved in acute inflammation in the skin after $CoCl_2$ exposure.

To our surprise, time did not appear to be a factor in the activation of the inflammasome. It was only the $CoCl_2$ exposure concentration that lead to an activation of the inflammasome, shown as the secretion of IL-1β, IL-18, active caspase -1 and the production of mRNA. These findings can help shed new light on the NLRP3 inflammasome in skin allergy. It shows that the exposure dose rather than the time of exposure affects the sensitization in skin due to cobalt.

CONCLUSIONS

Overall, the results of this thesis show that cobalt skin exposure in the hard metal industry is important as a route of uptake and that skin cells exhibit inflammatory responses that can affect skin sensitization in a dose- and time-dependent manner.

The main findings and conclusions are summarized as follows:

I Air exposure to cobalt was found in all departments and for all work tasks in a Swedish hard metal production facility. Exposure levels were generally below the Swedish OEL for inhalable cobalt and the highest exposure levels were found in the powder production department and for laboratory and furnace work. We found good correlations for the mass-based measures, thereby allowing us to use the findings for future references.

II When personal inhalable air levels of cobalt, cobalt skin levels and biological monitoring of cobalt in blood were analyzed using a mathematical model, skin exposure was determined to be important as a route of uptake. Our findings suggests that skin exposure to cobalt in the hard metal industry could affect the total uptake in the same order of magnitude as air exposure.

III *In vitro* investigations of cobalt using the human keratinocyte cell line HaCaT, showed that $CoCl_2$ triggered an alarm system in cells and the pro-inflammatory cytokines/chemokines IL-6, CXCL8 and CCL2 were secreted in a dose- and time-dependent manner. Our finding also indicate that the effect of cobalt chloride on cell toxicity occurs throughout the living epidermis.

IV Exposure of HaCaT cells to cobalt chloride results in secretion of the pro-inflammatory cytokines IL-1β and IL-18, which indicates activation of the NLRP3 inflammasome in the cells. Cobalt chloride regulates the inflammasome both as a primer and as an activator. Our mRNA data indicate there is a negative feedback mechanism in the inflammasome due to the exposure. The inflammatory response is generally more dose dependent than time dependent. These findings could be important for understanding the mechanisms of allergic sensitization.

Epilogue

Do our exposure levels potentially cause adverse health effects? All our determined cobalt exposure levels are below the Swedish OEL, although they are in the same order of magnitude. Some studies present data on eye irritation at 3 $\mu g/m^3$, even making levels that are well below the present OELs worth studying.

How do the concentrations we used in our *in vitro* experiments relate to the amount of cobalt that was actually deposited on the hands of the workers at the Swedish hard metal production facility where we conducted all the sampling? In an experiment using human skin and a Franz diffusion cell [75], cobalt in synthetic sweat at a concentration of 67 mg/L was added to the Franz cell and was absorbed through the skin. Cobalt was present in the treated skin after exposure times 1, 2, 4, 8 and 24 hours at a concentration of 13.2 ± 4.5 $\mu g/cm^2$. These data correspond with our skin exposure data (AM 12 $\mu g/cm^2/h$) assuming a steady state of the exposure in the skin. The $CoCl_2$ concentrations used in our *in vitro* exposure experiments were determined from a series of titrations and the testing of cell viability and the concentrations we used were in accordance with the concentrations used in other studies.

We have also compared our *in vitro* test concentrations with the patch test elicitation threshold dose for cobalt chloride studied by Fisher et al. and it was found to be in the range of 0.0663 to 1.95 μg cobalt/cm^2. This is the dose at which 10% of allergic individuals develop allergic contact dermatitis in the patch test dilution series [241]. The elicitation data in the study by Fisher et al. indicates that the workers in our study were exposed to doses that could cause a reaction in the skin. It is important to remember that in our study we did not investigate hypersensitivity, sensitization or skin allergy. We looked at mechanisms of local inflammation in the skin. HaCaT cells were used as proxies for only one cell type in the skin, and for the onset of allergy in the skin, a collaboration between several different cell types is needed. We have shown that the skin route for cobalt uptake is responsible for a relatively large part of the total uptake and that skin cells respond to exposure to $CoCl_2$ by producing several pro-inflammatory cytokines shown to be involved in inflammatory processes in the skin.

The risk of developing cobalt related health issues upon exposure is a highly relevant area of focus as cobalt is found the cathode in lithium-ion batteries, i.e. rechargeable batteries. Such batteries are used in electrical cars and bicycles, and also in cell phones and other electronics using rechargeable batteries. Continuous research regarding the effects the metal has on

humans are important. Not only to minimize the exposure, hopefully by regulations, but also to continue gaining understanding of the mechanisms causing cobalt related health issues.

FUTURE PERSPECTIVES

The findings in the present studies on cobalt exposure, the importance of uptake of cobalt via the skin, and *in vitro* exposure experiments with $CoCl_2$ on the HaCaT cell line showing the involvement of keratinocytes in acute inflammatory response in the skin has paved the way for further investigations, as detailed below:

It would be of great interest to investigate and further evaluate different exposure measures and general inflammatory markers produced in response to cobalt exposure, in particular using cobalt in blood as a marker of exposure.

A dose- and time-dependent secretion of pro-inflammatory cytokines and chemokines cobalt was seen for IL-6, CXCL8/IL-8, CCL2/MCP-1, IL-18 and IL-1β in keratinocytes exposed to $CoCl_2$. These are all known to be involved in the allergic response in the skin and the response comprise a series of complex interactions between several cell types residing in the skin. It would be of great interest to develop a model in which real or artificial skin was used for $CoCl_2$ exposure and to measure the dose- and time effect release of the same pro-inflammatory cytokines and chemokines that we found while using HaCaT cells. In the experiments in this thesis work, only one cell type is used, which is a limitation since the model cannot take into account the interactions between other types of cells.

By using real or artificial skin, we would also be able to compare the basally located cells with cells in the stratum corneum without having to use calcium as a trigger for differentiation.

The use of $CoCl_2$ in our model is based on the fact that cobalt ionizes when it is deposited on the skin and penetrate the skin as ions. It would be interesting to measure pro-inflammatory cytokines and chemokines in skin biopsies from workers in the hard metal production industry as they are exposed to cobalt-containing dust particles. This information would further help us understand the inflammatory effects of cobalt in the skin and identify a more realistic picture of the risk of the exposure and the link to allergic skin disease from such exposure.

As NLRP3 inflammasome downstream activated cytokines have shown to play important roles in allergic dermatitis, targeting the NLRP3 inflammasome with inhibitors could help in the treatment of allergic dermatitis.

However, to confirm whether NLRP3 is a promising target for the treatment of allergic dermatitis, more studies investigating the effects of specific NLRP3 inhibitors or gene therapy could be carried out in various models.

Many of the studies investigating mechanisms, trying to gain understanding of the underlying cause of skin diseases, are conducted using mouse models. Differences exists between human and mice for example the presence of receptors and the binding to them. Applying results from animal models to humans must be made with caution. Therefore, the development of a reliable *in vitro* model where the use of animals are avoided, would of great interest.

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