Dermal absorption of chemicals and toxicity - the role of filaggrin genetics

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EMELIE RIETZ LILJEDAHL
DEPARTMENT OF LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY

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During a day, most people come into contact with many different chemicals via their skin, at work or through consumer products. In this thesis we investigated if genetic variation in the gene encoding the skin barrier protein filaggrin affects how much chemicals we take up through the skin, and if filaggrin genetics is associated to some systemic effects in the body related to chemical exposure.

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Emelie Rietz Liljedahl

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Dermal exposure to chemicals is an increasing problem, and daily we are exposed to chemicals via the skin both in our occupations and from consumer products. The skin barrier is an important protection to chemical exposure, but appr. 10% of the Scandinavian population carry loss-of-function mutations (so called null mutations) in an important skin barrier protein, filaggrin (gene name *FLG*), which may result in an impaired skin barrier. The copy number variation (CNV 10, 11 or 12 copies) of the repeated sequences in exon 3 of the *FLG* gene may also impact skin barrier function. The aim of this thesis was to investigate if genetic variation of *FLG* lead to an increased uptake of chemicals through the skin, and in turn, increased systemic effects, such as genotoxicity and inflammation. We used two occupational cohorts with high dermal exposure via work, consisting of hairdressers and chimney sweeps respectively, to cross-sectionally investigate associations between *FLG* variation and the degree of exposure and genotoxicity. Further, we performed a human exposure experiment to investigate if *FLG* null carriers take up more chemicals through the skin, using a PAH (pyrene), a pesticide (pyrimethanil), a UV-filter (oxybenzone), and a metal (nickel). We also investigated if the exposure during the experiment led to changes in inflammation-related proteins in blood. Among hairdressers, we found *FLG* null and CNV 10 to be associated with shorter telomeres than wildtype (wt) and non CNV 10 carriers. Among chimney sweeps, *FLG* null was also associated with shorter telomeres and more DNA methylation of CpG-site 03636183 of the lung cancer-related gene *F2RL3* than among *FLG* wt, opposite to our hypothesis. In the human exposure experiment, we found *FLG* null carriers to take up the three organic chemicals through the skin with up to 80% shorter lag times than *FLG* wt carriers with high CNV. *FLG* null carriers showed two times the internal dose of pyrimethanil than wt carriers with high CNV. We found a decrease in internal dose with increasing CNV for all three organic compounds included in the study. We found an increase in inflammation-related protein expression among *FLG* null carriers after exposure, and a decrease among *FLG* wt carriers. To conclude, we have clarified that *FLG* null and low CNV lead to an increased uptake of three organic chemicals through the skin and that the exposure may lead to differences in systemic effects measured as expression of inflammation-related proteins in blood between *FLG* null and wt genotypes. Further we have found associations between *FLG* null and increased genotoxicity and epigenetic changes in two occupational groups with high dermal exposure. We identified low CNV as a potential susceptibility factor for increased dermal absorption. Further studies of relevant genetic susceptibility of the skin in association with dermal exposure to more diverse chemical groups is needed. Our research can be used by policy makers in the setting of limit values for existing and new chemicals in consumer products and in the working environment.

Key words: Skin barrier function, dermal exposure to chemicals, genetic susceptibility, systemic effects

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Till mina föräldrar

Table of Contents

Populärvetenskaplig sammanfattning

Vi utsätts dagligen för kemikalieexponering via huden, på jobbet eller från konsumentprodukter, som kosmetik, kläder och möbler. Huden utgör en skyddande barriär mot kemikalier, mikroorganismer och UV-strålning. Huden är också viktig för att producera olika molekyler, t.ex. vitamin D. För att hudbarriären ska fungera, behöver hudcellerna proteinet filaggrin, som bland annat bidrar till cellernas form och att de håller ihop. Nedbrytningsprodukter av filaggrin bibehåller hudens fukt, vilket också är viktigt för hudbarriärens funktion. I Norra Europa är det relativt vanligt (2-11%) förekommande med variationer i DNA-sekvensen, så kallade mutationer, i filaggrin-genen som gör att filaggrinet förlorar sin funktion. Dessutom kan filaggrin-genens längd variera mellan individer och ge olika längd på filaggrinproteinet, vilket också skulle kunna påverka filaggrinets funktion. Det är dock inte klarlagt om mutationer i filaggrin eller variationer i dess längd påverkar hur mycket kemikalier som tas upp genom huden till blodcirkulationen. Det är inte heller känt om mutationsbärare får större skador av kemikalier i kroppen, såsom ökad inflammation, skador på DNA eller förändringar i metylering av DNA, så kallade epigenetiska förändringar. Skador på DNA kan uppstå i form av kortare ändar på kromosomerna, så kallade telomerer. Graden av DNA metylering styr reglering av proteiner. Både förändrade telomerer och DNA-metylering har kopplats till olika sjukdomar, däribland risk för cancer.

I denna avhandling har vi undersökt hypotesen att mutationsbärare eller bärare av korta filaggrin-gener har en försämrad barriärfunktion i huden så att de tar upp mer kemikalier, och att det i sin tur leder till en ökad inflammation i blodet och mer DNA-skada, mätt som telomerlängd och epigenetiska förändringar. Vi har undersökt detta dels i två tvärsnittsstudier av yrkesexponerade grupper, frisörer och sotare, som har hög hudexponering för kemikalier i sina yrken, dels i en experimentell studie av människor rekryterade utifrån om de var mutationsbärare samt kontroller som var icke mutationsbärare.

Våra resultat visar att mutationsbärare eller bärare av en kort filaggrin-gen bland frisörer och sotare hade kortare telomerer, än icke mutationsbärare eller med lång filaggringen. Vi har också visat att det fanns en ökad risk för metylering av tre olika gener som är viktiga för att cellerna inte ska utvecklas till cancerceller, hos mutationsbärare och bärare av korta filaggrin-gener bland frisörer och kontroller. Vi fann också att mutationsbärare bland sotare och kontroller hade mer metylering än

icke mutationsbärare av en gen som vi förväntade oss finna lägre metylering av, eftersom den i lägre metyleringsgrad visats ge ökad risk för lungcancer.

Genom hudexperimentet på frivilliga försökspersoner kunde vi visa att mutationsbärare hade upp till fem gånger snabbare upptag av tre olika kemikalier genom huden, och att deras nivå av en av kemikalierna i blodet var ca två gånger högre än icke mutationsbärare med längre filaggrin-gener.

En fördel som kan komma av att vara mutationsbärare är att huden verkar kunna producera mer vitamin D vid kontakt med solljus. Detta beror på att nedbrytningsprodukterna av filaggrin verkar som kroppseget UV-filter i huden, och en minskning av funktionellt filaggrin i huden på grund av mutationen leder till mindre kroppseget UV-filter. Vi kunde visa att mutationsbärare hade högre nivåer av vitamin D i blodet, både före och efter hudexperimentet med de tre kemikalierna, än icke mutationsbärare.

Slutligen har vi visat att det efter hudexperimentet fanns skillnader i uttrycket av proteiner kopplade till inflammation, mellan mutationsbärare och icke mutationsbärare. Mutationsbärare visade högre relativa nivåer av proteiner kopplade till inflammation i blodet, och icke mutationsbärare visade lägre relativa nivåer av proteiner kopplade till inflammation i blodet, efter hudexperimentet med de tre kemikalierna.

Denna forskning är viktig för att kunna identifiera känsliga grupper i samhället så att de inte ska utsättas för höga nivåer av kemikalier på sina jobb eller genom produkter som de använder på huden. Forskningen i denna avhandling kan bidra till bestämning av lämpliga tillåtna nivåer av olika kemikalier som används av yrkesgrupper eller i konsumentprodukter.

List of papers

I. **Rietz Liljedahl E**, Wahlberg K, Lidén C, Albin M and Broberg K. 2019. Genetic variants of filaggrin are associated with occupational dermal exposure and blood DNA alterations in hairdressers. *Science of the Total Environment* 653:45-54

II. Wahlberg K, **Rietz Liljedahl E**, Alhamdow A, Lindh C, Lidén C, Albin M, Tinnerberg H, Broberg K. 2019. Filaggrin variations are associated with PAH metabolites in urine and DNA alterations in blood. *Environmental Research* 177:108600

III. **Rietz Liljedahl E**, Johanson G, Korres de Paula H, Faniband M, Assarsson E, Littorin M, Engfeldt M, Lidén C, Julander A, Wahlberg K, Lindh C, Broberg K. 2021. Filaggrin polymorphisms and the uptake of chemicals through the skin – a human experimental study. *Environmental Health Perspectives* 129(1):17002

IV. **Rietz Liljedahl E**, Korres de Paula H, Gliga A, Engfeldt M, Julander A, Lidén C, Lindh C, Broberg K. Immune-related protein expression in blood after dermal exposure to some common chemicals depends on the *filaggrin* genotype. Manuscript in preparation.

List of papers not included in the thesis

I. Mörtstedt, H, Ali N, Kåredal M, Jacobsson H, **Rietz E**, Diab K, Nielsen J, Jönsson BA, Lindh C. Targeted proteomic analysis of nasal lavage fluid in persulfate-challenged hairdressers with bleaching powder-associated rhinitis. *Journal of Proteome Research* 14(2):860-873.

II. Corrales Vargas A, Peñaloza Castañeda J, **Rietz Liljedahl E**, Mora AM, Menezes-Filho JA, Smith DR, Mergler D, Reich B, Giffin A, Hoppin JA, Lindh CH, van Wendel de Joode B. Exposure to common-use pesticides, manganese, lead, and thyroid function in pregnant women from the Infant's Environmental Health Study (ISA), Costa Rica. *Science of the Total Environment,* article in press, available online 28 October 2021, 151288.

Abbreviations

AUC – area under the curve

bp – base pair

CNV – copy number variation

DNA – deoxyribonucleic acid

DOEL – dermal occupational exposure limit

ECHA – European Chemicals Agency

FLG – filaggrin

GC-MS/MS – gas chromatography tandem mass spectrometry

IARC – International Agency for Research on Cancer

ICP-MS – inductively coupled plasma mass spectrometry

LC-MS/MS – liquid chromatography tandem mass spectrometry

Log P – octanol/water partition coefficient

Null – loss-of-function

nt – nucleotide

PAH – polycyclic aromatic hydrocarbons

PCR – polymerase chain reaction

RNA – ribonucleic acid

ROS – reactive oxygen species

REACH – Registration, Evaluation, Authorisation and Restriction of Chemicals

SNP – single nucleotide polymorphism

wt – wildtype

Introduction

Exposure to chemicals is inescapable for most people. We eat, inhale, work with and wear them, sometimes intentionally, but mostly unintentionally. Fortunately, we have an inherent protection against chemical exposure, our skin. The skin is the largest organ of the body and it protects against a diverse group of external threats such as cold, heat, microbes and chemicals. Still some chemicals can penetrate the skin, where they may cause skin disease, such as allergic contact dermatitis, irritant contact dermatitis, urticaria or skin cancer. If the chemicals reach the circulation, they may cause systemic effects, by production of reactive oxidative species (ROS), inducing inflammation, binding to DNA, forming DNA adducts and other mechanisms. Like chemicals, people have different properties, a result of our genetic inheritance. Individual differences in genes involved in absorption, metabolism or excretion of chemicals can make us more or less sensitive to chemical exposure. Genetic variations in genes encoding proteins involved in the skin barrier structure and function, such as filaggrin, could have the potential to make carriers more susceptible to chemical exposure, but knowledge in this area is lacking. Identifying sensitive individuals is important for making informed decisions on acceptable chemical exposure limits, and in the end, to protect the most susceptible individuals in a population.

Genetic susceptibility to chemical exposure

We are all different. Our inheritance determines many of our characteristics (also called phenotypes), along with our environment. Sequence variation of the genetic code gives us differences in how we respond to outside stimuli, whether it is chemical (Edenberg 2007), pathological (Freeman and Milner 2020), or even musical in nature (Tan et al. 2014). This thesis focuses on genetic differences in the skin in relation to chemical exposure. Among the skin's many diverse properties are protection against infection, chemicals and trauma, water retention and pre-vitamin D3 production (Carlson 2019). The genetics of proteins expressed in the skin play a part in how well the skin protects against harmful substances, but genetic susceptibility of the skin is not very well described. Genetic susceptibility, or genetic predisposition, is the increased risk of developing a certain disease or adverse effect as a result of a given genetic variant. Examples of genetic variation are single

nucleotide polymorphisms (SNP), insertions and deletions and copy number variations (CNV). These changes in the DNA may give rise to a different variant of a protein if the exchanged base alters the amino acid of that specific genetic code, or lead to a frameshift that entirely changes the amino acid sequence if bases were inserted or deleted, or change the size of a protein if the CNV varies. Protein products from such genetic variations may be functional, but with increased or decreased activity, or they may lose their function (loss-of-function mutation), giving rise to an increased risk of disease. Polymorphisms are defined as DNA variations where the less common variant occur in more than 1% of the population. The more common variant is traditionally called the wildtype (wt). Polymorphisms can increase the risk of disease, often in combination with other factors such as lifestyle and environmental factors. Polymorphisms in genes encoding proteins involved in metabolism and excretion of xenobiotics can modify an individual's response to chemicals. An example is polymorphisms in the family of metabolic enzymes cytochromes P450, which might affect the internal dose of a chemical or drug, and in turn lead to adverse reactions or decreased drug effects, which in some cases are important for caregivers to consider when medicating patients (Meyer and Zanger 1997). This type of genetic susceptibility, where the internal dose of chemical or metabolite is affected by polymorphisms in genes encoding proteins involved in absorption, metabolism or excretion processes, is important to consider for occupational and environmental chemical exposure. Individuals carrying polymorphisms that make them more susceptible to certain types of chemical exposures are at higher risk of developing disease or adverse effects, such as the lung disease chronic beryllium disease from working with beryllium (polymorphisms in Major Histocompatibility Complex, Class II, DP Beta 1 gene, *HLA-DPB1*) (Richeldi et al. 1993) or bladder cancer from working with aryl amines (polymorphism in N-Acetyltransferase 2 gene, *NAT2*) (Christiani et al. 2008).

Genetic susceptibility of the skin barrier

Genetic susceptibility to dermal exposure is not extensively studied. Studies on the subject include polymorphism in tumour necrosis factor α-chain (gene name *TNF*). Polymorphism in *TNF* has been shown to give two different irritation thresholds for dermal exposure to benzalkonium chloride and sodium dodecyl sulphate, possibly making the individuals carrying the low threshold allele more susceptible to irritant contact dermatitis (Allen et al. 2000). Polymorphisms do not only affect the skin's susceptibility to chemicals but can also affect the protection from other exogenous factors such as sunlight. In a Spanish population, polymorphisms in melanocortin 1 receptor (*MC1R*), interferon regulatory factor 4 (*IRF4*), solute carrier family 45 member 2 (*SLC45A2*), HECT and RLD domain containing E3 ubiquitin protein ligase 2 (*HERC2*) (genes related to the development of skin cancer) were associated with increased skin sensitivity to sunlight, as assessed by questionnaires of e.g. skin type and sun exposure habits (Hernando et al. 2018).

It is important to gain knowledge about differences in dermal absorption, to be able to adequately protect the individuals with the highest uptake. New chemicals in consumer products are rapidly increasing in number (Li and Suh 2019), and occupational dermal exposure is an important exposure route, however previously often overshadowed by inhalation exposure (Boeniger 2003).

Filaggrin and the skin barrier

The skin barrier – structure and function

The skin is composed of three main layers, the hypodermis, dermis and epidermis (Figure 1). The hypodermis is a layer of fatty tissue and connective tissue that serves as an energy reservoir. The dermis is a layer of connective tissue supplied with blood vessels, sweat glands and nerve endings. Above the dermis is the epidermis, with Langerhans cells, melanocytes and keratinocytes. Keratinocytes are the predominate cell type in the epidermis. They are produced by stem cells in the deepest layer of the epidermis, the stratum basale. Keratinocytes migrate upwards through the four layers (five in the palm of the hands and soles of the feet, which also includes stratum lucidum beneath stratum corneum, Figure 1) of the epidermis to the skin surface over the course of 8-10 weeks in adults (Carlson 2019). While migrating, they differentiate into corneocytes (dead, flattened keratinocytes) and once at the skin surface, they fall off and are replaced by new corneocytes. Migration is faster in injured skin and in younger individuals and slows down with age (Saladin 2010).

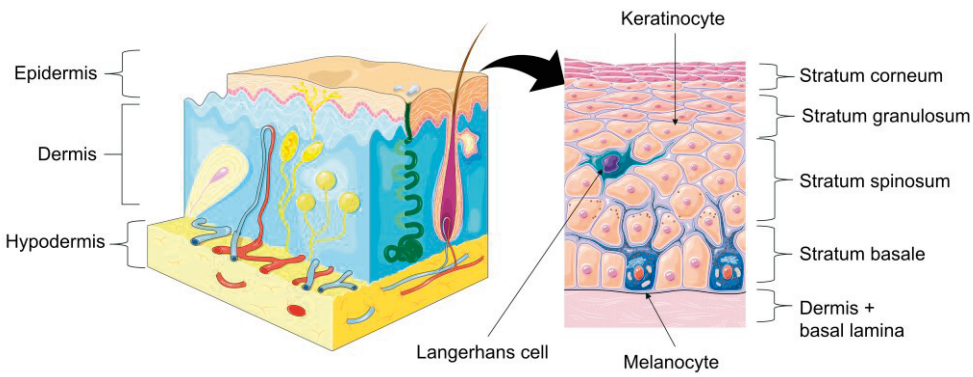


Figure 1. Structure and cell types of the skin. (Adapted from Servier Medical Art, smart.servier.com)

Keratinocytes produce the cytoskeleton protein keratin, thereby their name. While migrating, keratinocytes flatten and produce keratin, membrane-coating granules containing lipids, and cytosolic granules containing a protein called pro-filaggrin.

Filaggrin is an important protein for skin barrier structure and function. When they reach the stratum granulosum the keratinocytes start the process of a specific form of apoptosis called cornification (Candi et al. 2005). The organelles and nucleus disappear and pro-filaggrin is released into the cytosol, which is proteolyzed into filaggrin monomers that bind the keratin filaments 1 and 10 together in strong bundles (Brown and McLean 2012). The membrane-coating vesicles release lipids extracellularly that form a protective barrier along with tight-junctions between the keratinocytes and the intracellular keratin bound together by filaggrin. Filaggrin is also integrated in the cell envelope that replaces the plasma membrane of the cornified keratinocytes, and along with other proteins act as a scaffold for the extracellular lipid layer, keeping it in place (Saladin 2010). The upper layer, the stratum corneum of the epidermis, consists of dense layers of corneocytes, with about 40 % protein content, which is mainly intracellular keratin bound together by filaggrin, 40 % water and 20 % extracellular lipids (Michaels et al. 1975). This barrier retains water in the body and prevents dehydration and absorption of penetrating substances, and it is the first line of defence against microbes and allergens. (Carlson 2019; Saladin 2010). The skin is also an important system for hormone synthesis, such as the conversion of sex steroids (Zouboulis et al. 2007) and production of pre-vitamin D3, which is produced by UVB-light photoisomerization of 7-dehydrocholesterol in the epidermis (Nemanic et al. 1983).

Transport over the skin barrier

Even though the skin barrier protects against water loss, cold, heat, pathogens and chemical exposure, it is not a perfectly tight barrier. Chemicals can move across the stratum corneum, the layer of corneocytes, by passive diffusion, because the cells are dead and thus have no or very limited active transport systems. Diffusion through the stratum corneum is the rate limiting step in skin absorption. The rates of absorption are different depending on the properties of the chemical. Large, highly lipophilic or hydrophilic compounds diffuse very slowly, if at all, through the stratum corneum, while small moderately lipophilic compounds with some hydrophilic regions diffuse faster. Small hydrophilic compounds such as water may diffuse through the cells, whereas most other compounds diffuse intercellularly, through the lipids between the cells (Casarett 2013).

Filaggrin – the gene and the protein

Filaggrin (37 kD, 324 amino acids [aa]) (OMIM 2021) is encoded by the gene *FLG*, which is located on chromosome 1q21.3, spanning about 23000 base pairs (bp) (UCSC 2021). *Filaggrin* is part of the epidermal differentiation complex, a complex of about 50 genes in proximity of each other, that are important for the differentiation and cornification of keratinocytes (Kypriotou et al. 2012; Volz et al. 1993). *Filaggrin* contains three exons (size exon 1: 159 nucleotides [nt], size exon 2 and 3: 12757 -14697 nt) and two introns, of which exon 2 and 3 are protein coding (OMIM 2021). Exon 3 is composed of long repetitive sequences and copy number

variations (CNV; Figure 2) of these repeats result in 10, 11 or 12 copies (referred to as CNV10, CNV11 and CNV12 in this thesis) of filaggrin monomers in the pro-filaggrin protein (400 kDa (Presland et al. 1992).

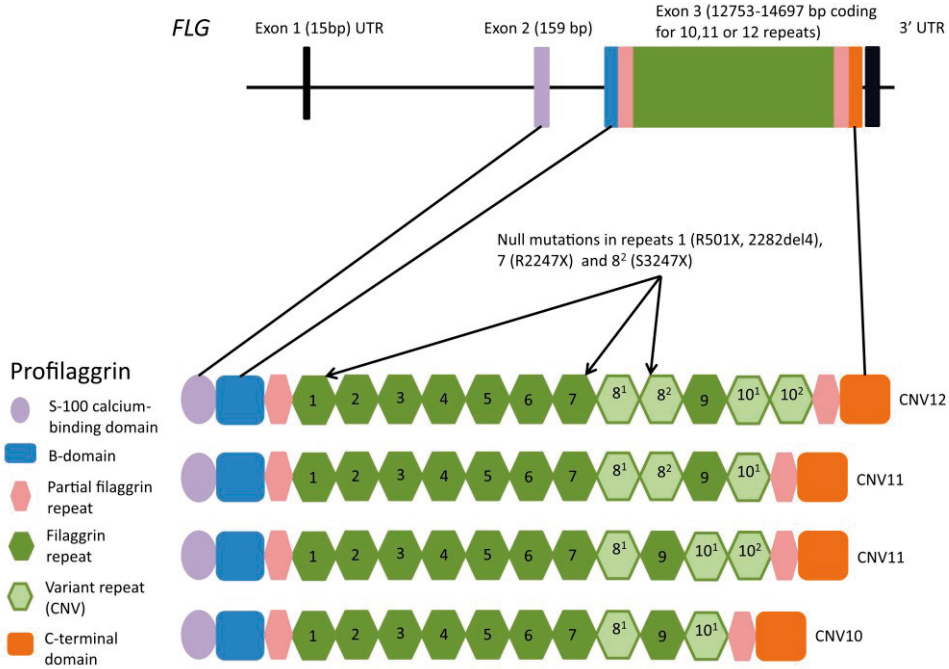


Figure 2. Schematic description of *FLG* and the pro-filaggrin protein, with indications of null mutations and copy number variation (CNV) in the pro-filaggrin protein. Adapted (modified colours and indicated polymorphisms) from Eaaswarkhanth et al. 2016, under license: CC BY-NC 4.0 (Eaaswarkhanth et al. 2016)

Pro-filaggrin consists of an N-terminal containing a calcium-binding domain and a B-domain containing a signal for nucleus localization, encoded by exon 2 of *FLG*. Upon proteolysis of pro-filaggrin into filaggrin monomers, the N-terminal domain relocates into the nucleus. An important step for the cornification of keratinocytes is enucleation (the nucleus disappears). It is hypothesized that the N-terminal domain of pro-filaggrin is important in the process of enucleation (Sandilands et al. 2009). Further, in addition to the filaggrin repeats, exon 3 also encode the C-terminus (Figure 2) which is important for pro-filaggrin proteolysis into filaggrin monomers (Sandilands et al. 2007). Once in the monomeric form, filaggrin aggregates keratin filaments 1 and 10 into tight bundles, which collapses the keratinocytes. Filaggrin is highly expressed in skin, moderately expressed in the oral, oesophageal, vaginal and tonsil mucosa, and expressed at low levels in the cervix and uterine mucosa (TheHumanProteinAtlas 2021). Previous research *in vitro* has indicated that pro-inflammatory cytokines, for example interleukin 17 (IL-17) and interleukin 22 (IL-22), are involved in down-regulating the expression of the *FLG* gene in keratinocytes (Gutowska-Owsiak et al. 2011; Gutowska-Owsiak et

al. 2012) through the MAP17 protein (Noh et al. 2010). Overexpression of IL-17 and IL-22 is seen in the skin disease atopic dermatitis. Interestingly, the aryl hydrocarbon receptor (AHR), a receptor that can bind small molecular weight compounds, have also been suggested to regulate *FLG* transcription (Furue et al. 2015).

Besides functions involved in maintaining the structure of the epidermis, filaggrin also moisturizes the skin by degradation of the filaggrin monomers into free amino acids or natural moisturizing factors (NMF) (Rawlings and Harding 2004). Filaggrin is rich in the amino acid histidine, which is metabolized into trans-urocanic acid. Pyrrolidone-5-carboxylic acid is the other main metabolite of filaggrin. These metabolites are slightly acidic and maintain the acidic pH (below pH 5) of the stratum corneum. The acidic environment has an antimicrobial effect. In addition trans-urocanic acid is a potent UV-filter (Elias and Wakefield 2011).

Filaggrin loss-of-function – consequences for the skin barrier

Population genetics

In Europe 7-10 % of the population carries loss-of-function mutations (also referred to as null mutations) of *FLG*. The most common mutations in Europe are SNP R501X, R2247X, S3247X and deletion 2282del4 present in repeat 1, 7 and 8^2, and 1, respectively (Figure 2). The SNP all result in a premature stop codon whereas the deletion 2282del4, cause a frameshift in the translation of the RNA which also results in a stop codon. The mutations all result in a truncated pro-filaggrin, lacking the c-terminus, without which processing into filaggrin monomers is not possible. The four mutations most common in northern Europe have also been described in North American and Australian populations with European descent, with combined heterozygote frequency from 4-6% (Gao et al. 2009; Tan et al. 2012; Thyssen et al. 2014).

Null mutations in *FLG* is present also in Chinese, Singaporean and Japanese populations, although with other mutations than what are found in Europe (Park et al. 2015; Thyssen et al. 2014). The CNV of *FLG* varies in populations also where *FLG* mutations are not so well described, such as in African American populations (Fulton et al. 2021; Margolis et al. 2020; Quiggle et al. 2015).

Skin barrier dysfunction

The consequence of null mutations in *FLG* is less or no filaggrin in the skin barrier. The skin barrier of *FLG* null subjects carrying two null mutations contains no or very little filaggrin (Brown and McLean 2012). This was shown by studying patients with ichthyosis vulgaris, a disease with a dry and scaly skin phenotype resulting from carrying *FLG* null mutations on both alleles, i.e. being a homozygote for null (Sandilands et al. 2007; Smith et al. 2006). A disorganisation of the keratin filaments

and an abnormal structure of the lipid extracellular matrix was seen in punch biopsies from ichthyosis vulgaris patients, who were homozygote for one of the *FLG* mutations previously mentioned or compound heterozygote for two mutations (Gruber et al. 2011). Carriers of one *FLG* null mutation, i.e. being a heterozygote, have less filaggrin than wild-type carriers (Gruber et al. 2011) and less NMF in the skin (Kezic et al. 2008), and thus a decreased capacity for retaining water in the skin. Trans-epidermal water loss is increased in *FLG* null carriers (Kezic et al. 2008). CNV and the amount of NMF in the skin are positively associated; carriers of higher CNVs have more NMF in the skin (Brown et al. 2012).

FLG null is associated with skin disease, and above all atopic dermatitis. In Sweden, the lifetime prevalence of atopic dermatitis in children is around 10 % (Henriksen et al. 2015), and around 20-50% of atopic dermatitis patients carry a *FLG* null mutation (Brown and Irvine 2008). Carrying null mutations or low CNV of *FLG* has been associated with a higher risk for atopic dermatitis (Brown et al. 2012; Palmer et al. 2006). However, a recent large American study did not find a significant association between low CNV and increased atopic dermatitis risk (Fulton et al. 2021). Null mutations of *FLG* are also associated with irritant contact dermatitis (Visser et al. 2013), allergic contact sensitization (Novak et al. 2008), asthma in association with dermatitis (Chan et al. 2018; Palmer et al. 2006), increased severity of asthma independent of dermatitis status (Palmer et al. 2007), allergic rhinitis (Chan et al. 2018) and food allergy (Marenholz et al. 2017; Venkataraman et al. 2014). The mechanism behind filaggrin polymorphism and allergic disease is hypothesized to result from a defective skin barrier where antigens can penetrate and interact with antigen presenting cells (McAleer and Irvine 2013).

A dysfunctional skin barrier could lead to an increased absorption of chemicals and pathogens. Previous cross-sectional research on Danish men from the general population linked *FLG* null mutations to increased urinary concentrations of phthalates (Joensen et al. 2014), methyl paraben and n-propyl paraben (Joensen et al. 2017).

Despite the negative consequences of a dysfunctional skin barrier, there may be an evolutionary advantage in carrying a *FLG* null allele. Cross-sectional studies have found higher vitamin D levels among *FLG* null carriers (Landeck et al. 2016; Thyssen et al. 2012; Thyssen et al. 2014), hypothetically a consequence of lower levels of the UV-filtering NMF trans-urocanic acid in the skin, permitting a higher production of pre-vitamin D3 in the skin (Thyssen and Elias 2017).

Dermal chemical exposure

Exposure to chemicals in the workplace and through consumer products is an increasing problem. Occupational skin diseases make up 30-40% of all occupational diseases (Alfonso et al. 2017; Julander et al. 2018) and the EU estimates that 90% of all occupational skin diseases are caused by chemicals (EU-OSHA 2013). Contact dermatitis is the most common occupational skin disease in Europe, and is estimated to have an incidence of about 0.5-1.9 cases per 1000 full-time workers per year (Alfonso et al. 2017). Health care workers, hairdressers, and construction workers are among the occupational groups that are affected the most by occupational skin diseases and chemical exposure (Julander et al. 2018), but there is no universal method of determining skin exposure today (WHO 2014).

In the general population, dermal chemical exposure usually is a low dose, chronic exposure. Chemicals present in the general and home environment include preservatives (from personal care products and cleaners), organophosphate flame retardants (from furniture, stuffed toys and textiles), per- or polyfluorinated compounds (from textiles, clothes and shoes), plasticizers (from food containers and toys), pesticides (from food, gardening, pest control or nearby agricultural spraying), polycyclic aromatic hydrocarbons (PAH) (from smoking, wood-burning, candle-burning and grilled food) and UV-filters such as oxybenzone (from plastic and sunscreen) (WHO 2014).

Occupational and environmental chemicals evaluated in this thesis

This thesis includes two occupational groups with dermal exposure at work, namely hairdressers and chimney sweeps, and we have focused on their exposure to aromatic amines and PAH as they are considered to be main drivers of cancer in these occupations. Aromatic amines are a group of compounds used in hair dyes, and this thesis evaluates exposure to ortho-, para- and meta-toluidine. These chemicals have been classified as skin sensitizing, toxic, possibly carcinogenic and carcinogenic for humans (Database search 2021-09-16, www.echa.europa.eu). Exposure assessment to aromatic amines can be made by analysing haemoglobin adducts of aromatic amines in blood through gas chromatography coupled mass spectrometry (GC-MS) (Johansson et al. 2015).

Soot contains PAH, a large group of compounds present naturally in for example crude oil, but also created from incomplete combustion of biofuel and fossil fuel. Tobacco smoke and grilled food is also a source of PAH (Jansson et al. 2012). Several PAH are classified as carcinogenic, such as benzo[a]pyrene (ECHA 2021c) and benzo[a]anthracene (ECHA 2021a). Some PAH, such as benzo[a]pyrene, become carcinogenic after they have been metabolically activated into diol-epoxides or quinones (Moorthy et al. 2015). Metabolites of PAH (Table 1, page 26)

are excreted in urine, and can be measured by liquid-chromatography coupled mass spectrometry (LC-MS/MS). (Alhamdow et al. 2017b). The urinary metabolites are considered reliable biomarkers for estimation of occupational and environmental exposure to PAH, especially urinary 1-hydroxy-pyrene, which correlates well with total PAH exposure (Levin 1995).

Hairdressers

Hairdressers are dermally exposed to chemicals daily. They work with oxidative hair dye, which may contain aromatic amines (Akyüz and Ata 2008) and bleaching products, which contains persulfates (Gargano et al. 2018), but also with surfactants in shampoo, and with water, so called wet work. Wet work increases the risk of irritation and sensitization of the skin (Jung et al. 2014; Nielsen et al. 2007). Daily exposure to water, surfactants and chemicals has a dehydrating effect on the skin, which may lead to a dysfunctional skin barrier.

Hairdressers have a high frequency of occupational irritant and/or allergic contact dermatitis (Lind et al. 2007). They also have an increased risk of contact urticaria (Foss-Skiftesvik et al. 2017). In addition, using gloves while working may give rise to irritant and allergic contact dermatitis, because of skin occlusion or from skin contact with the material of the glove itself (Kersh et al. 2018; Ramsing and Agner 1996). Hair bleaching products contain persulfate salts, which increase the risk of asthma and rhinitis in hairdressers (Albin et al. 2002; Foss-Skiftesvik et al. 2017; Moscato et al. 2005).

Hairdressers have an increased risk of bladder cancer (Hadkhale et al. 2016), especially if they have been working for more than 10 years (Harling et al. 2010). Cancer cases in hairdressers active prior to 1980 are attributed to carcinogenic aromatic amines being used in hair dyes, until they were banned in the late 1970:s. It is unclear whether hair dye products being produced today contain carcinogenic chemicals, but they may contain new chemicals, similar to the earlier carcinogenic aromatic amines. However, they have not yet been identified and tested. Hairdressers may still be exposed to aromatic amines such as ortho-, para- and meta-toluidine, 2-, 3- and 4-ethylaniline, or 2,3- and 3,4-dimethylaniline. In a study of hairdressers' occupational exposure, more than 50% of the subjects had levels higher than the limit of detection of ortho-, para- and meta-toluidine and 2,3-dimethylaniline (Johansson et al. 2015).

In a study of the same hairdressers as in Johansson et al. 2015, genotoxicity markers and epigenetic changes among the hairdressers (n=295) were compared to controls (n=92) and the hairdressers had shorter telomeres (Li et al. 2016), the end of the chromosomes protecting the DNA (described further below). More hair waving treatments was associated with shorter telomeres in young (<32 years) hairdressers and less methylation of cyclin dependent kinase inhibitor 2A (*CDKN2A*, encoding two proteins involved in cell cycle regulation) in the entire group. Oxidative hair

dyeing and bleaching was associated with less methylation of short stature homeobox 2 (*SHOX2*, encoding a protein involved in transcription regulating). Telomere shortening and methylation of the previously mentioned genes could be involved in the development of bladder cancer.

Chimney sweeps

Chimney sweeps are exposed to soot, solvents, metals, combustion gases and dust. Work tasks include removing soot and dust from chimney sweeps and boilers, cleaning and inspecting ventilation systems and inspecting fire safety, among others (Alhamdow 2019). Working as a chimney sweep is associated with a higher risk of cancer of the liver, lung, pleura, bladder and hematopoietic system (Hogstedt et al. 2013), as well as an increased risk of ischemic heart disease (Jansson et al. 2012). A recent study showed that chimney sweeps had less methylation of the genes encoding aryl hydrocarbon receptor repressor (*AHRR*) and coagulation factor II (thrombin) receptor-like 3 (*F2RL3*) compared with controls (Alhamdow et al. 2018). Hypomethylation of these genes have been linked to lung cancer (Fasanelli et al. 2015). Further, concentrations of proteins involved in inflammation, cell movement and migration were altered in chimney sweeps compared with controls (Alhamdow et al. 2017b; Alhamdow 2019; Alhamdow et al. 2019). This may be relevant for development of cancer and cardiovascular disease. Up to 86.7% of the Swedish chimney sweeps included (n=483) in the study by Alhamdow et al. (2017a) said they use gloves when performing soot-sweeping in private homes or industry, but they may still be exposed via skin in the face, neck and arms, as short sleeved clothes are worn in the summertime (Alhamdow et al. 2017a). In addition, the gloves used are working gloves, and not gloves designed to protect the skin from chemical exposure. Health effects such as eye and respiratory symptoms (Alhamdow et al. 2017a; Alhamdow 2019) and asthma (Li et al. 2008) have been reported among chimney sweeps, but skin diseases such as irritant or allergic contact dermatitis have not been described in chimney sweeps, despite their high dermal exposure.

Chemicals evaluated in the dermal human exposure experiment of this thesis

In this thesis we have chosen a pesticide (pyrimethanil), a PAH (pyrene), a UV-filters (oxybenzone) and a metal (nickel) as examples of occupational and consumer's dermal exposure to chemicals to be evaluated in a dermal exposure experiment. We chose the chemicals because they are of relatively low toxicity among their respective chemical group, and because we had the analytical methods for them readily available. Moreover, their respective metabolism, except for oxybenzone, is well known. Previous human exposure experiments show that pyrimethanil and pyrene are absorbed by the skin and into the systemic circulation, metabolised into 1-hydroxy-pyrimethanil (Faniband et al. 2019) and 1-hydroxy-pyrene (Viau et al. 1995), and excreted in urine. Skin absorption of oxybenzone into the systemic circulation and excretion of the parent compound in urine has also been shown in an experimental setup (Morrison et al. 2017), but metabolism is less

known. Exposure assessments can be made by analysis of the metabolites or parent compound in urine with LC-MS/MS. Exposure assessment to nickel can be made through tape stripping of the skin, followed by measurement of the individual tape strips with inductively coupled plasma mass spectrometry (ICP-MS) (Ahlström et al. 2019). However, the analysis of *FLG* and nickel penetration in skin was not a part of this thesis.

Studies of the general populations' exposure to pesticides (Baudry et al. 2019; Kim et al. 2017; Norén et al. 2020; van Wendel de Joode et al. 2014), PAH (Dodd-Butera et al. 2017; Hoseini et al. 2018; Ma et al. 2019; Orisakwe et al. 2019), oxybenzone (Han et al. 2016) and nickel (Ross-Hansen et al. 2011) show that practically all humans are exposed, via several exposure routes. Dermal exposure to these compounds, apart from occupational exposure, include for example touching soil, dust, surfaces or food contaminated with pesticides or PAH or using sunscreen or touching plastic containing oxybenzone (WHO 2014). In an American population, at least 96% of the included subjects were exposed to oxybenzone during the study period of 10 years (Han et al. 2016), which mainly can be attributed to the use of sunscreen on skin. However, dermal exposure to pyrimethanil is considered to be mainly occupational (Kittas et al. 2014).

Properties of chemicals and skin that affects skin absorption

Different chemical properties can affect the uptake through the skin, such as the molecular size and the lipid solubility, measured by the octanol/water partition coefficient (log P) (Elias and Wakefield 2011). Generalised, a low log P gives a high hydrophilicity and low solubility in skin, a higher log P gives a higher lipophilicity and a high solubility in skin, whereas a very high log P gives a very high lipophilicity and a low solubility in skin. However, *in silico* predictions of skin uptake of chemicals with models based on physiochemical properties including log P are often inaccurate in the absence of experimental data (Kneuer et al. 2018). Other factors such as properties of the vehicle (e.g. pH, solvent *vs* water) that the chemical is dissolved in, the concentration and exposure duration also affects the uptake (Johanson and Rauma 2008; Ngo et al. 2010). Molecular size, log P, and information about what metabolites or parent compounds have been evaluated in this thesis are presented in Table 1. For compounds with qualitative skin notations and International Agency for Research on Cancer (IARC)[1] classifications, these are also included. Chemicals without IARC classifications have not been classified regarding carcinogenicity but may still be.

[1] The classifications are defined as follows: Group 1: carcinogenic to humans, Group 2A: probably carcinogenic to humans, Group 2B: possibly carcinogenic to humans, Group 3: not classifiable as to its carcinogenicity to humans.

Properties of the skin can also affect the uptake of chemicals. High pH, very young age and very old age, occlusion of the exposed area, damaged or atopic skin and light pigmented skin are factors generally considered to increase skin uptake (Elias and Wakefield 2011; Ngo et al. 2010).

Table 1. Summary of chemical properties,measured metabolites or parent compounds, skin notations (accompanying occupational exposure limits) and IARC classifications of chemicals included in the studies.

Chemical group	Compound	Molecular weight (g/mol)	Log $P_{o/w}$	Skin notation	Measured metabolite (abbreviation)	IARC classification
Aromatic amine	Meta-toluidine	107.15	1.40[2]	No	Hb[3]-adduct (hb-meta-toluidine)	N.A.
Aromatic amine	Ortho-toluidine	107.15	1.32[2], 1.43[4]	Yes	Hb-adduct (hb-ortho-toluidine)	Group 1
PAH	Pyrene	202.25	4.88[2,3]	No	1-hydroxy-pyrene (1-OH-PYR)	Group 3
PAH	Phenanthrene	178.23	4.46[2]	No	2-hydroxy-phenanthrene (2-OH-PH)	Group 3
PAH	Benzo[a]-pyrene	252.3	6.39[2], 6.04[3]	Yes[5]	3-hydroxy-benzo[a]pyrene (3-OH-BaP)	Group 1
PAH	Benzo[a]-anthracene	228.3	5.61[3]	Yes[4]	3-hydroxy-benzo[a]anthrazene (3-OH-BaA)	Group 2B
Pesticide	Pyrimethanil	199.25	2.84[2]	No	1-hydroxy-pyrimethanil	N.A[6].
UV-filter	Oxybenzone	228.24	3.79[2]	No	Oxybenzone	N.A.

Limit values for dermal exposure

Occupational chemical exposure limits (OEL) are set from combining knowledge about effects in animals at different doses, human health in relation to exposure to the compound, technical feasibility criteria, and economic criteria. Technical solutions and monetary profit is weighed against the risk of health effects in the workers, which sometimes results in keeping traditional technical solutions although they lead to a higher exposure than necessary or accepting higher risks if enough profit is made (EU-OSHA 2021). Risk assessment for dermal exposure is difficult to perform because so many different factors are involved, such as rate of absorption, skin surface area, skin integrity and relevant exposure scenarios. There are in vitro models available for researching skin absorption, where animal or human skin or synthetic material is used. Another way of predicting the absorption

[2] Human metabolome database (HMDB).

[3] Haemoglobin.

[4] ILO International chemical safety cards (ISCS).

[5] Exposure by all routes should be carefully controlled to levels as low as possible.

[6] Not available (insufficient research about compound).

of a chemical is to model it, and there are several tools available today, relevant for occupational exposure (Kneuer et al. 2018). But models generally perform poorly in the absence of experimental data and predictions do not correspond well with measured data (Kneuer et al. 2018).

Dermal OEL (DOEL) have been derived only for some compounds, such as carcinogens cyclophosphamide and 4,4'-methylene dianiline (MDA), as examples of a proposed procedure (Bos et al. 1998). Instead, a qualitative skin notation (in Sweden, the letter "H", for the Swedish word "hud" meaning skin) (Johanson and Rauma 2008) supplements the OEL if the dermal exposure could contribute significantly to the total body burden of the chemical, but there is no quantitative limit for exposure. Registration, evaluation, authorization, and restriction of chemicals (REACH) legislation demands that the use of DOEL should be implemented. Further, exposure limits exist for the general population in the form of acceptable daily intake levels (ADI), but no such limit values exist for dermal exposure. However, REACH also requires implementation of derived no effect levels (DNEL) for dermal exposure to protect consumers. The basis for determining safe exposure limits are changing from animal studies towards epidemiological studies of human health in relation to chemical exposure, as more such studies are available (ECHA 2021b). More research of dermal exposure is needed to contribute to better limit values.

Toxicity of dermal chemical exposure

Skin toxicity

Dermal exposure to chemicals may lead to diseases of the skin. The most common disease is contact dermatitis, which is subdivided into subgroups: irritant contact dermatitis, allergic contact dermatitis, contact urticaria and protein contact dermatitis. The most common occupational skin diseases are irritant contact dermatitis and allergic contact dermatitis of the hands (Lim and Goon 2007; Lyons et al. 2013; Nettis et al. 2002; Warshaw et al. 2013; Vester et al. 2012; WHO 2014). Mechanisms are non-allergic inflammation for irritant contact dermatitis and a type IV hypersensitivity reaction for allergic contact dermatitis. The prevalence of the different types depends on the type of exposure: irritant or allergenic chemicals and wet work, which all can occur in many occupations. Furthermore, a combination of irritant contact dermatitis and allergic contact dermatitis is often seen in some occupational groups, such as hairdressers, exposed to wet work, irritants, and allergens. Distinction between irritant and allergic contact dermatitis is difficult, because of their similar clinical manifestations. Diagnostics of irritant contact dermatitis is based on a patient's history of exposure to skin irritants and patch

testing to rule out allergic contact dermatitis. Both irritant and allergic contact dermatitis contribute to a decreased function of the skin barrier. A higher frequency of irritant contact dermatitis has been found in *FLG* null carriers compared to non-carriers (de Jongh et al. 2008; Visser et al. 2013).

Dermal exposure to chemicals or radiation can give rise to cancer at the exposure site. Exposure to UV-radiation is the main risk factor for developing skin cancers, such as malignant melanoma (Chang et al. 2009), squamous cell carcinoma and basal cell carcinoma (Carøe et al. 2013) in occupational groups working outdoors (Sena et al. 2016). Higher risks for developing squamous and basal cell cancer have also been linked to exposure to non-solar sources such as pesticides, petroleum products, fiberglass dust, and dry-cleaning products (Gallagher et al. 1996), and arsenic (Cheng et al. 2016).

Systemic toxicity

Dermal exposure can have a role for diseases in other organs than the skin. Recent research in mice has shown that dermal exposure to isocyanates determines the type of asthmatic response. Mice that were dermally sensitized with TDI had mainly a Th2-cell response after airway exposure to TDI, compared to a mixed Th1/Th2-response for mice that were exposed without prior dermal sensitization (Pollaris et al. 2019). Dermal exposure to chemicals can hypothetically also cause cancer in other parts of the body by inducing systemic genotoxicity and inflammation. However, there are often several exposure routes: inhalation, dermal and ingestion exposure, and the contribution of absorption from one route is difficult to separate from the others, making research about only dermal exposure in occupational settings in relation to cancer difficult to perform. Hence, little is known about (non-skin) cancer in relation to dermal exposure.

Further systemic effects include poisonings via skin uptake. Cases where inorganic mercury has been added to skin lightening and acne treatment creams have occurred, however it is not possible to buy such creams through regular commercial channels. The use of such creams has resulted in several cases of mercury poisonings with effects on the central nervous system (Copan et al. 2015)

Early biomarkers of genotoxicity, epigenetic changes and inflammation

Many compounds present in industry and the general environment are known to be carcinogenic. However, cancer takes a long time to develop. Finding markers indicative of carcinogenesis that arise before cancer develops is important, because knowledge about the mechanisms behind cancer may give the chance to intervene, stop a hazardous exposure or adjust limit values. Such a marker is called a biomarker of effect, defined as: an "*indicator signalling an event or condition in a biological*

system or sample and giving a measure of effect", and further: "*a biomarker that, depending on its magnitude, can be recognized as associated with an established or possible health impairment or disease"* (Nordberg et al. 2007). The biomarkers of effect used in this thesis are telomere length (a biomarker of genotoxicity), DNA methylation (a biomarker of epigenetic changes), changes in inflammation-related proteins (biomarkers of inflammation) and mitochondrial DNA copy number (a biomarker of oxidative stress and inflammation).

Telomere length and DNA-methylation

Telomeres are the protective ends of the chromosomes, repetitive sequences of guanine-rich DNA that shorten with age, (Aubert and Lansdorp 2008) in somatic cells which lack expression of the telomere repairing enzyme telomerase (Greider and Blackburn 1985). Telomere shortening is due to the "*end replication problem"*, where DNA polymerase cannot replicate the end of the lagging strand completely, and accordingly the telomeres shorten with each cell division (Muraki et al. 2012). The exposure to exogenous compounds can accelerate the shortening or increase the length of telomeres. Telomere aberrations have been found in buccal cells and leukocytes among patients with different cancer types, such as bladder (Broberg et al. 2005), lung (Doherty et al. 2018) and skin cancer (Srinivas et al. 2019). Shorter telomeres in blood leukocytes have been associated with exposure to copper and perfluoro-octane-sulfonic acid (PFOS) (Vriens et al. 2019), PAH (Pavanello et al. 2010), pesticides (Andreotti et al. 2015) and occupational exposure among hairdressers (Li et al. 2016). Longer telomeres have been associated with exposure to antimony and mercury (Vriens et al. 2019), arsenic (Li et al. 2012) and pesticides (different pesticides show different associations with telomere length) (Andreotti et al. 2015). It is reasonable to presume that the exposures mentioned were a combination of inhalation and dermal exposure routes, however, the authors do not evaluate or discuss this matter. Mechanisms for shortening of telomeric DNA due to exogenous exposure are proposed to be increased oxidative stress, which leads to telomeric DNA damage (Reichert and Stier 2017; von Zglinicki 2002).

Epigenetic changes, such as hypermethylation of tumour suppressor genes is another measure of early cancer-related aberrations of the DNA. Methylation of genes, specifically in the regulatory promoter region, is important for the normal function of transcription. Methyl groups are added to cytosine bases followed by a guanine, without changing the sequence, hence it is an epigenetic phenomenon. Hypermethylation of certain tumour suppressive genes are seen in some cancer types, such as lung and bladder cancer (Abbosh et al. 2008; Kneip et al. 2011), however global genome hypomethylation is generally seen in carcinogenesis (Ehrlich 2009). Changed DNA methylation patterns in leukocytes have previously been associated with exposure to pesticides (van der Plaat et al. 2018), PAH (Alhamdow et al. 2018) and occupational exposure among hairdressers (Li et al. 2016), all of which presumably include both inhalation and dermal exposure routes.

Mitochondrial DNA copy number

Mitochondrial DNA copy number is a proxy for damage to the mitochondrial DNA and is used as a biomarker for oxidative stress. Mitochondria produce ATP and regulate cell metabolism and apoptosis, crucial for the function of all cells in the body. Mitochondrial DNA is circular and only contains genes encoding proteins related for mitochondrial function. Reactive oxygen species (ROS) is produced as a by-product of ATP production (Ott et al. 2007). Mitochondrial DNA (mtDNA) is more sensitive to oxidative stress than chromosomal DNA, since it lacks DNA-repairing enzymes and is not packed on histones as chromosomal DNA, and mtDNA is therefore more exposed (Reuter et al. 2010). Mutations in mtDNA accumulate with age, probably because of the higher transcription rate than nuclear DNA and the proximity to ROS as a by-product of ATP-production (Lawless et al. 2020). The mtDNA lacks introns which means that the accumulated mutations affect the proteins to a higher extent than nuclear DNA. It has been suggested that mtDNA copy number increases as a coping mechanism to damaged DNA and mutations (Filograna et al. 2021), and both increased and decreased mtDNA copy number has been associated with increased risk for different cancer types (Filograna et al. 2021). Changes in mtDNA copy number have been found in several occupationally exposed groups, however with inconsistent results. In asphalt workers and welders (exposed to PAH and particles) mitochondrial DNA copy number was increased (Xu et al. 2017; Xu et al. 2018), but in truck drivers in Beijing (exposed to elemental carbon and particles) mitochondrial DNA copy number was decreased (Hou et al. 2013) compared with controls.

Inflammation

Inflammation is a necessary process for normal healing and recovery of damaged tissue, but if inflammation becomes chronic it can lead to negative health effects. Acute inflammation is the immune system's response to environmental irritants, chemical exposure, pathogens, or trauma. It is characterized by swelling, redness, heat, and pain in tissues. Inflammatory stimuli lead to the activation of different inflammatory signalling pathways (such as NF-κB pathway, MAPK pathway and JAK-STAT pathway) that regulate the transcription of inflammatory cytokines, recruitment of inflammatory cells and release of inflammatory proteins in the blood (Reuter et al. 2010). Exposure to xenobiotics has been identified as an important contributor to the lifelong body burden of low-grade chronic inflammation, termed systemic chronic inflammation (Furman et al. 2019). Some chemicals, including some PAH and pesticides, can induce oxidative stress (Henkler et al. 2012) or interfere with signalling pathways of the immune system (Thompson et al. 2015), and the exposure to such chemicals is, although low grade, chronic in modern society. Systemic chronic inflammation and oxidative stress are mechanisms involved in the development of diseases such as cancer and cardiovascular disease (Reuter et al. 2010; Thompson et al. 2015), especially ischemic heart disease (Ahmed et al. 2019).

Aim

We hypothesized that *FLG* null carriers and low CNV carriers have a higher uptake of chemicals through the skin and show a higher degree of chemical-related inflammation, genotoxicity and epigenetic changes.

The overall aim of this thesis was to clarify if *FLG* genetics affect the dermal uptake and internal dose of chemicals present in the work and general environments, and in turn, the degree of systemic effects of the chemicals. In this thesis, the *FLG* polymorphisms *FLG* null and CNV have been evaluated. The specific aims were to:

- Investigate if *FLG* polymorphism influence the internal dose of aromatic amines and degree of genotoxicity and cancer-related epigenetic changes in hairdressers.
- Investigate if *FLG* polymorphism influence the internal dose of PAH and degree of genotoxicity and cancer-related epigenetic changes in chimney sweeps.
- Clarify if *FLG* polymorphism determine the internal chemical dose, via dermal absorption of a pesticide, a PAH and a UV-filter in a dermal exposure experiment.
- Investigate if the vitamin D levels and systemic inflammatory response differ depending on *FLG* polymorphism in subjects from a dermal exposure experiment.

Materials and methods

Study design

Study I and II

Study I and II were cross-sectional, with exposure assessed and biological samples collected at the same time in two different occupational groups and non-exposed controls.

Study III and IV

We first recruited from the general population according to certain criteria (described under "Study participants"). We then selected subjects for a controlled experiment with an exposure at time point 0, pre-exposure biological samples and several repeated measurements for 48 hours. In study III we used the pre-exposure and repeated urine samples. In study IV we used the pre-exposure and post-exposure serum samples.

Study participants

Study I

The hairdresser study was originally set up to study the hairdressers' exposure to aromatic amines and its relationship to genotoxicity (Johansson et al. 2015). We applied data from the cohort in this thesis to investigate if the genetics of the skin could modify these relationships. Hairdressers (n=292) in Scania and Stockholm, consumers of hair dye (n=32) and controls (n=60) were recruited in 2008-2009. Study participants were included if they were between 18 and 55 years of age, female and non-smoking in the last six months. Hairdressers also had to have worked as a hairdresser for the past six months. The consumers were included if they had dyed their hair every eight weeks. Controls were included if they had not used hair dye during the past year. The consumers and controls were recruited through the Lund University Hospital advertisement boards and intranet, and in Stockholm at university campuses. Participants signed an informed consent form. The study was approved by the regional ethics committee in Lund.

Study II

The chimney sweep cohort was originally set up to study the chimney sweeps' exposure to PAH and the relationship to genotoxicity and other early biomarkers of carcinogenesis as well as cardiovascular disease (Alhamdow et al. 2017b). We applied data from the cohort in this thesis to study the modification of skin genetics on the relationship between PAH and genotoxicity. Chimney sweeps (*n*=151) in southern Sweden were recruited in 2013-2015. The non-smoking control group (*n*=127) was recruited in 2010-2011 among workers at storage warehouses for food stores and in municipalities, without occupational exposure to PAH. More controls from the same companies (*n*=25) were recruited in 2015, and were included if they were smoking, to match the chimney sweeps smoking habits. Participants were male and between 19 and 66 years of age.

Study III

This study was designed to answer some research questions of this thesis. The study was divided in two phases. In phase I we recruited participants (*n*=432) in southern Sweden during spring of 2018, through university campuses in Malmö and Lund, and through a web-based meeting place for researchers and potential participants previously called "Studentkaninen". We included participants if they were non-smoking and over 18 years of age. Participants gave saliva samples from which we extracted DNA and genotyped for *FLG* null. We invited all *FLG* null carriers (*n*=28) to phase II. Four *FLG* null carriers declined participation because of lack of time or no interest to participate, and one did not show up for their appointment on the day of exposure, which resulted in *n*=23 *FLG* null carriers. We then invited sex and age matched controls (*n*=31) to phase II of the study. We included eight extra controls because of the risk of participants quitting the study during phase II.

Study IV

We used samples from the same participants as in study III for analysis of vitamin D levels. We created a subgroup for analysis of inflammatory proteins in serum, consisting of *FLG* null carriers (*n*=22) and *FLG* wt carriers (*n*=22). We excluded the extra controls, as well as one *FLG* null carrier with very few urine samples, and that individual's matched control. The choice of *n*=22 was due to limited funding.

FLG genotyping

Study I, II

DNA was extracted from whole blood in study I, and from whole blood or saliva in study II. DNA quantity and purity were analyzed by NanoDrop ND-1000 spectrophotometer. We used the extracted DNA to identify *FLG* SNP R501X,

R2447X, and S3247X and the deletion 2282del4 using a real time polymerase chain reaction (PCR). We evaluated the PCR results by sequence detection system software. We determined CNV by long range PCR. We visually evaluated results from long range PCR after gel-electrophoresis of PCR products. Six different combinations exist: 10/10, 10/11, 10/12, 11/11, 11/12 or 12/12. In the chimney sweep study, results from the long range PCR were of too low quality to visually determine genotype. Instead, we performed a Taqman-based genotyping on a quantitative PCR (qPCR) system to confirm the presence or absence of CNV12 (the method used is not able to discriminate between CNV10 and CNV11). We confirmed the presence of CNV12 using the same method also in study I.

Study III and IV

We performed the same procedure as described above to identify *FLG* null mutations and determine CNV. For study III we combined the *FLG* genotype with CNV to create a genetic score. The total CNV was calculated for each participant. For example, carriers of CNV 12/12 were assigned a total CNV of 24, while CNV 10/10 carriers were assigned a total CNV of 20. Carriers of *FLG* null were grouped together irrespective of their CNV, while *FLG* wt were divided into two groups, low CNV (CNV total 20-22) and high CNV (CNV total 23-24). We created the score because we hypothesized that the total amount of filaggrin in the skin determines the function of the skin barrier. With the score we could not only compare *FLG* null carriers to wt carriers, but also compare different CNV groups among wt carriers.

Questionnaires

Study I

Questions addressed only to hairdressers regarded work tasks, glove use for different work tasks and working years to identify exposure to aromatic amines and persulfate salts. Further, the hairdressers were asked health related questions to identify symptoms, such as rhinitis and eczema, from occupational chemical exposure. Hairdressers, consumers and controls were asked questions about their level of education, residency, exposure to passive smoking, previous work and hobbies, to identify possible exposure relevant to the study.

Study II

Chimney sweeps and controls were asked about their age, education, history of heart disease and cancer, snuff use and smoking habits, exposure to passive smoking, physical activity, residence, employment history and hobbies with potential PAH exposure. Chimney sweeps were asked about their work tasks and glove use performing different work tasks, both historically and during the past 12 months.

Study III and IV

We sent a questionnaire in Swedish to study participants along with a saliva sampling kit during phase I of study III. The questionnaire was designed to identify smokers and nickel allergic participants. If self-diagnosed nickel allergy was reported, we did not expose the study participant to nickel during phase II.

We gave participants a second questionnaire (Table 2) on the day of exposure. Question 1-8 were based on questions and response alternatives from the Nordic Occupational Skin Questionnaire (NOSQ 2002) (Susitaival et al. 2003).

Table 2. Questionnaire (originally in Swedish, translated by the author) regarding symptoms and diseases of atopic nature and exposure to PAH, pyrimethanil, oxybenzone and toluidine.

Questionaire 2	**No**	**Yes**	**Do not know**
1. Have you ever had an itchy rash that has been coming and going for at least 6 months, and at some time has affected skin creases?			
2. Have you ever had "hay fever" or other symptoms of nasal allergy, e.g. from pollens or animals?			
3. Have your eyes ever shown allergic symptoms, e.g. from pollens or animals?			
	No	**Yes**	
4. Have you ever had asthma			
4.1 If yes, did a physician diagnose it?			
	Free text answer		
4.1.2 If yes, what year?			
	No	**Yes**	
5. Have you ever had hand eczema?			
5.1 If yes, did a physician test you?			
6. Have you ever had eczema on your wristsforearms (excluding fronts of elbows)?			
6.1 If yes, did a physician test you?			
7. Do you have dry skin?			
8. Have you had childhood eczema?			
9. Are you allergic to nickel?			
10. Have you used sunscreen the last days?			
	Free text answer		
10.1 If yes, when?			
	No	**Yes**	
11. Have you ever had skin reactions to sunscreen?			
12. Have you ever had a temporary tattoo?			

12.1 If yes, have you ever had skin reactions to a temporary tattoo?					
	No	**Yes**	**Yes, but not all days (party smoker)**		
13. Do you smoke?					
	No	**Yes**			
14. Have you smoked before?					
!5. Do you use snuff?					
16. Are you exposed to passive smoking?					
17. Have you eaten grilled meat or vegetables the last two days?					
18. Have you eaten apples, strawberries or grapes the last two days?					
19. Do you use a stove or fireplace at home?					
	Free text answer				
19.1 If yes, how often do you use it (number of fires/month)					
	Free text answer				
19.2 When did you last use it?					
	No	**Yes**			
20. Have you inhaled smoke from an open fire during the last 24 hours?					
21. Are you exposed to vehicle exhaust in your spare time?					
	Free text answer				
21.1 If yes, describe your exposure					
	0-1 vehicle/min	**2-5 vehicle/min**	**6-10 vehicle/min**	**More than 10 vehicle/min**	**I can't see a street**
22. What is the intensity of traffic in the largest street you can see from a window in your house?					
	0-30 min	**30-60 min**	**1-2 hours**	**More than 2 hours**	
23. What amount of time do you spend outdoors in traffic each day?					
	Free text answer				
24. What is your occupation?					
	No	**Yes**			
25. Do you have an airway infection or other disease?					
	Free text answer				
25.1 If yes, what disease?					
	No	**Yes**			
26. Have you taiken any medication the last 24 hours?					
	Free text answer				
26.1 If yes, what medication?					
	Free text answer				
27. What did you have for breakfast today?					

One part of the questionnaire was designed to explore the participants' medical history of atopic nature, regarding allergies, asthma, and dermatological conditions and symptoms, to be able to associate these symptoms and diseases to the *FLG* genotype. The other part was designed to identify exposure to potential sources of PAH, pyrimethanil, oxybenzone and toluene-2,5-diamine in their daily routines and during the days prior to the experiment. We originally planned that toluene-2,5-diamine be part of the exposure, but it was exchanged to oxybenzone due to issues with solubility in the pilot study.

Experimental exposure situation

Study III and IV

We wanted to avoid background exposure to the pyrimethanil, PAH and oxybenzone. We instructed participants not to include any apples, grapes or strawberries in their diet, not to eat grilled food or smoke cigarettes, and to avoid skin moisturizers and sunscreen for two days prior and two days after the exposure experiment. We exposed participants to the same dose (Table 3, solutions, concentrations, and area), spread out over the areas pictured in Figure 3.

Table 3. List of chemcials used in the exposure experiment and their concentrations and solutions.

Chemical	Solution	Concentration (mg/mL)	Area on skin (cm)
Pyrimethanil	Ethanol	23.5	5x5
Oxybenzone	1:1 Ethanol:acetone	5	5x5
Pyrene	Ethanol	23.5	5x10
Nickel	1:1 1% HNO_3:ethanol	4	2x2

We covered the arms with aluminium foil and mefix tape, or a weighing boat for nickel exposure. We stopped the exposure for nickel during one week because of skin reactions from four participants. We decided to continue with an open nickel exposure instead of an occluded (Figure 4), which resulted in different exposure conditions for nickel exposed participants, which are discussed later.

The exposure lasted for 4 hours. Meanwhile, the participants answered the questionnaire, rested, and were allowed to drink water, coffee, tea and hot chocolate. We stopped the exposure after 4 hours by removing the aluminium foil and washing the exposed area three times with ethanol, and took photographs of the arms, whereafter the participants themselves washed with soap and water. The nickel exposed area was tape stripped after exposure (results are not within the scope of this thesis) and then washed with soap and water.

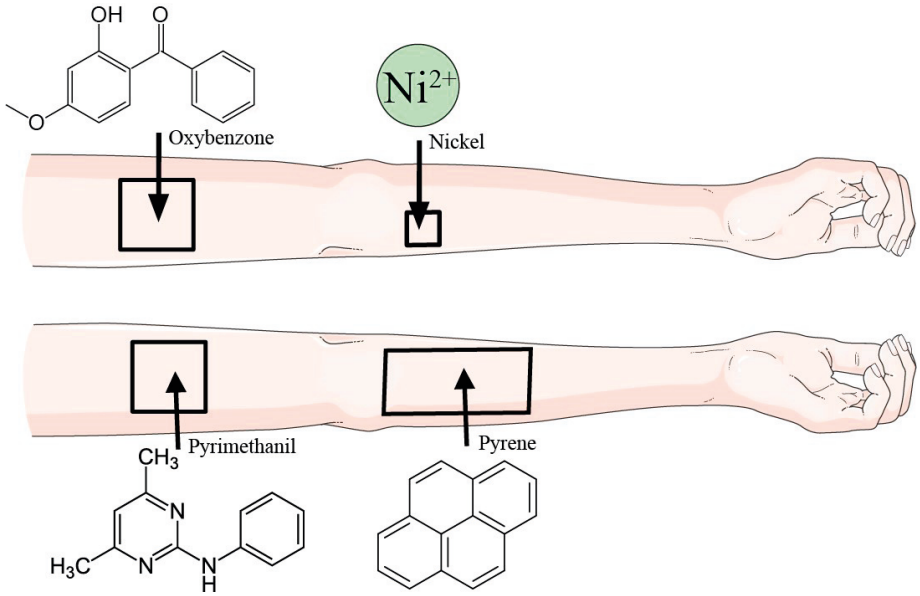
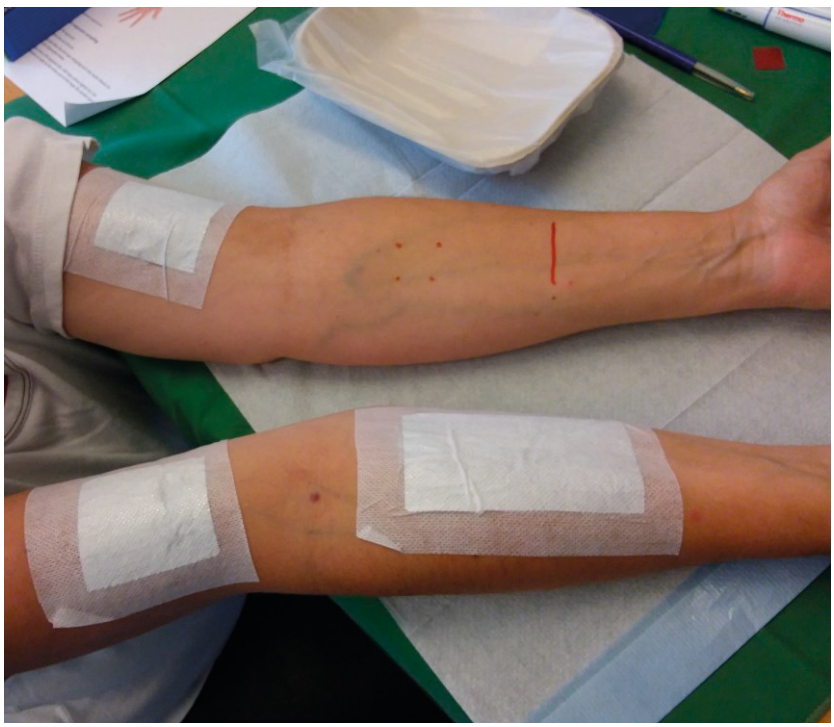


Figure 3. Arms with markings of the exposed areas, including chemicals used and their molecular structure.



Figur 4. Exposed areas of oxybenzone (occluded exposure, upper left arm), nickel (open exposure, lower left arm), pyrimethanil (occluded exposure, upper right arm), and pyrene (occluded exposure, lower right arm).

Biological samples

Study I

Venous non-fasting blood samples were collected from all hairdressers, consumers, and controls for analysis of haemoglobin (hb)-aromatic amine adducts and DNA extraction. Haemoglobin-aromatic amine adducts reflect the exposure the past three to four months, the duration of a red blood cells life cycle. Samples were stored at -20 °C until analysis.

Study II

Post shift venous non-fasting blood and spot urine samples were collected from chimney sweeps and controls, mid-shift in the middle of the week for DNA extraction and PAH analysis. For those not wanting to give a blood sample, a saliva sample was taken. Samples were stored at -20 °C until analysis.

Study III and IV

Before the exposure started the participants collected a full void urine sample and gave venous non-fasting blood samples for chemical metabolite analysis, DNA extraction and measurement of inflammatory proteins and vitamin D. We took blood samples again immediately after the participants had washed their arms after the exposure. We tape stripped the nickel exposed area using 10 individual tapes (results are not included in the thesis). The participants collected full urine voids for 48 hours after the exposure. We centrifuged blood samples and separated them for serum 20-30 minutes after collection. Urine samples were kept in a cooler bag at the participants' homes during the 48-hour sampling period, and then immediately dropped off at the lab. We measured urine samples for volume and density. We took an aliquot (10 mL) for metabolite or parent compound analysis and the remainder was discarded. Samples were stored at -20 °C until analysis.

Exposure assessment

Study I

Hb-adducts were analysed for eight selected aromatic amines previously, in blood samples from hairdressers, consumers and controls using gas-chromatography tandem mass spectrometry (GC-MS/MS) (Johansson et al. 2015). We only included meta- and ortho-toluidine adducts in this thesis, as they were the only ones showing associations with the hairdressers' work tasks (an increase in concentration with weekly number of hair waving and permanent hair dye treatment) in the previous study (Johansson et al. 2015).

Study II

Metabolites of PAH pyrene (Table 1), phenanthrene, benzo[a]pyrene, and benzo[a]anthracene were analysed previously (Alhamdow et al. 2018) in urine from chimney sweeps and controls using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Study III and IV

We analysed metabolites of pyrimethanil, pyrene and oxybenzone (Table 1) in urine samples using LC-MS/MS. We used a QTRAP 5500 in positive ionization mode to analyse 1-hydroxy-pyrimethanil and oxybenzone and a QTRAP 6500+ in negative ionization mode to analyse 1-hydroxy-pyrene. We analysed samples in duplicates in 20 batches. We included quality controls, chemical blanks and calibration standards in each batch. We calculated between-batch precision by comparing duplicate analyses, and the coefficients of variation for the methods were between 4% and 13%. The excretion rate (nmol/h) was calculated and used in toxicokinetic modelling. Nickel concentrations in tapes 1-10 were measured by inductively coupled plasma (ICP)-MS. However, results from the nickel analysis are outside the scope of this thesis, and is presented in Julander et al. 2021 (submitted manuscript)

Toxicokinetic analysis

Study III

We calculated the area under the excretion rate curve (AUC) for 0-40 hours, using the trapezoid rule (Gabrielsson and Weiner 2012), and used as a proxy for internal dose. We used the software monolix (Lixoft, France) for population toxicokinetic analysis. We used a structural model representing three compartments – skin, and central and peripheral compartments. We included the genetics of *FLG* (*FLG* null/*FLG* wt and *FLG* null/*FLG* wt CNV20-22/*FLG* wt CNV23-24) as categorical covariates in the software's integrated statistical model that was used to estimate the lag time (the time it takes for a chemical to penetrate the skin) and rate constant for skin absorption based on the structural model. Input data was urine excretion rate (nmol/h), mid-time point of urine samples (h) and the exposure dose of pyrimethanil, pyrene or oxybenzone, and *FLG* genetics.

Toxicity biomarkers

Telomere length, DNA-methylation and mitochondrial DNA copy number

Study I

Relative telomere length in whole blood was determined previously (Li et al. 2016). Haemoglobin beta chain (*HBB*) was used as a reference gene. Two separate runs, with samples in triplicates, were performed on a qPCR, one targeting telomere repeats and one targeting *HBB*. Copy number of telomere repeats and *HBB* were calculated relative to a standard curve of genomic DNA. Relative telomere length was calculated as telomere product/*HBB* product.

Methylation-sensitive high resolution-melting (MS-HRM) analysis was performed previously (Li et al. 2016) for 11 selected genes found to be hypermethylated in blood leukocytes or tumour tissue of bladder or lung cancer patients. The genes were adenomatous polyposis coli (*APC*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), death associated protein kinase 1 (*DAPK1*), docking protein 1 (*DOK1*), glutathione S transferase pi 1 (*GSTP1*), O-6-methylguanine-DNA methyltransferase (*MGMT*), POU class 4 homeobox 2 (*POU4F2*), Ras association (*RalGDS/AF-6*) domain family member 1A (*RASSF1A*), runt related transcription factor 3 (*RUNX3*), short stature homeobox 2 (*SHOX2*) and twist family basic helix-loop-helix transcription factor 1 (*TWIST1*). Samples were run on a qPCR. Results were divided in methylated (>0 % methylation) or non-methylated because of low DNA methylation levels overall.

Study II

Relative telomere length analysis was performed previously (Alhamdow et al. 2018) on a qPCR system as described for study I. Relative mtDNA copy number was also analysed previously (Alhamdow et al. 2018), similarly to the relative telomere length in two separate runs, one targeting mtDNA and one targeting *HBB* as described in Xu et al. (2017). Standard curves of genomic DNA were used to calculate copy number of mtDNA and *HBB*. Relative mtDNA copy number was calculated as the ratio between mtDNA and *HBB* copy number. DNA methylation analysis of *AHRR* and *F2RL3* was performed previously (Alhamdow et al. 2018). Three CpG sites (referred to as CpG1, 2 and 3) were included for *AHRR* and two CpG-sites (referred to as CpG1 and 2) for *F2RL3*, some of which have previously been associated with smoking and lung cancer (*AHRR* CpG3 and *F2RL3* CpG2) (Baglietto et al. 2017; Fasanelli et al. 2015; Hossain et al. 2015).

Inflammatory proteins

Study IV

We analysed levels of 92 inflammatory proteins in pre-exposure and post-exposure serum from the exposure experiment in study III (*FLG* null *n*=22, *FLG* wt *n*=22). Serum was pipetted into a 96-well plate, sealed, and shipped to OLINK proteomics, Uppsala, Sweden for analysis using proximity extension assay (PEA) technology. PEA utilises antibody pairs labelled with oligonucleotides that hybridise in close proximity of each other and then amplified by DNA polymerase to produce a protein specific DNA-barcode, which is quantified. The results were used to compare the relative levels of inflammatory proteins before and after the dermal exposure between *FLG* null and wt carriers. The results were also used to perform Ingenuity Pathway Analysis (IPA) to predict possible signalling pathways and cellular functions that the proteins may be part of.

Vitamin D analysis

Study IV

We analysed the metabolite of pre-vitamin D3, 25-hydroxy-vitamin-D3 (25(OH)D3 in pre and post exposure serum samples from *FLG* null (*n*=23) carriers and wt (*n*=31) carriers using LC-MS/MS. We used a QTRAP 6500+ in positive ionization mode and analysed quality control samples in all analytical batches. The coefficient of variation for the method was between 7-10%.

Statistical analysis

Study I

We compared *FLG* null heterozygote and CNV10 genotype frequencies between the hairdresser and control groups using the Pearson's Chi-square test. Many individuals had levels below the limit of detection for hb-o-toluidine: *n*(hairdressers)=135, *n*(controls)=45. Hence, we used hb-adducts of o-toluidine (median:0.018 ng/g hb) and m-toluidine (median:0.18 ng/g hb) as dichotomous variables, with the median as cutoff. We used logistic regression to analyse associations between *FLG* null or CNV10 and hb-adducts. We performed unadjusted, age-adjusted and fully adjusted analysis (age, passive smoking, residence and education). Telomere length was used as a continuous variable. We analysed associations between *FLG* null or CNV10 and telomere length using linear regression. We used DNA methylation of individual genes as a dichotomous variable; non-methylated or methylated. Categorizations for DNA methylation

index were: low (≤1 gene methylated), medium (2 genes methylated) and high (≥3 genes methylated). We used logistic and ordinal regression to analyse associations between *FLG* null or CNV10 and DNA methylation of individual genes and DNA methylation index, respectively. We performed unadjusted, age-adjusted and fully adjusted analysis (age, passive smoking, residence and education) for both telomere length and DNA methylation.

Study II

We compared frequencies of *FLG* null and CNV12 between chimney sweep and control groups using Pearson's chi-square test. We used urinary PAH levels as continuous variables and after natural logarithm (ln) transformation they were approximately normally distributed. We divided chimney sweeps in exposure groups of high (>50 % of the time working with soot sweeping within the last 12 months) and low (<50 % of the time working with soot sweeping within the last 12 months) exposure groups. We chose linear regression to analyse associations between *FLG* null or CNV12 and urinary PAH metabolites. We evaluated unadjusted and adjusted models (age, BMI and smoking). Telomere length and DNA methylation were used as continuous variables. We chose linear regression to analyse associations between *FLG* null or CNV12 and telomere length or DNA methylation. Study group (chimney sweep/control), age and smoking were included as adjustment.

Study III

We divided the wt carriers based on their total CNV, as described under *Toxicokinetic analysis*. We log-transformed the $AUC_{(0\text{-}40h)}$ for pyrimethanil, oxybenzone and pyrene to improve normality and compared differences between *FLG* and CNV genotypes in log $AUC_{(0\text{-}40h)}$ using ANOVA. We transformed the arithmetic mean and confidence intervals of logarithmic $AUC_{(0\text{-}40h)}$ values into linear values to generate the geometric mean and confidence intervals. We tested differences based on *FLG* and CNV genetics for the estimated lag times and absorption rate constants for the three chemicals using the built in statistical model in Monolix, by ANOVA.

Study IV

We excluded 12 of the 92 measured inflammation-related serum proteins due to too low levels (>25% of values below LOD), as recommended by OLINK. All protein concentrations were relative values, Normalized Protein eXpression (NPX), on a $\log^2$ scale, and were transformed to linear NPX ($2^{(NPX)}$) for further calculations. We compared post- *vs* pre-exposure protein concentrations within *FLG* null and wt genotypes using paired samples Wilcoxon signed rank test. We used the Benjamini Hochberg procedure to adjust for multiple testing, at the 0.05 false discovery rate level, and transformed *P*-values into adjusted *P*-values. We compared the median

difference in post- *vs* pre-exposure protein concentrations between *FLG* null and wt genotypes using the Mann-Whitney U-test. We analysed differences in post-*vs* pre-exposure vitamin D concentrations within *FLG* null and wt carriers using paired samples Wilcoxon signed rank test. We compared the pre-exposure and the post-exposure vitamin D levels between *FLG* null and wt carriers using a Mann Whitney U test, and between *FLG* null and low and high CNV using a Kruskal Wallis test. We compared the difference in post- *vs* pre-exposure concentrations between *FLG* null and wt genotypes using the Mann-Whitney U-test.

Ethical considerations

All studies included in this thesis have ethical permission from the Regional Ethics Committee in Lund, Sweden. We gave all participants information about the purpose and process of the studies. We informed them that they had the right to withdraw their participation at any point during the study. We informed participants about what type of samples would be collected and how samples along with personal data would be stored. Genetic information is sensitive data and can seem threatening for participants to share with researchers, so we store our data in a secure system with two-way authentication to which access is only granted for researchers actively working with the project in our lab. We further informed participants about the potential risks of discomfort of sampling. We informed participants that research results would be presented on a group level. Written informed consent was provided from each participant.

After each study results were reported back to the participants. In study I and II participants were given the main results of the studies regarding exposure and risk of cancer and cardiovascular disease (only chimney sweeps). For study III, we sent a summary of the study (a press-release) and a link to the published article.

A physician has been involved in all studies, in case of injury during sampling or if a participant in study I and II showed signs of an occupational disease. In study III a physician was present in case of injury or a reaction to the exposure. If an occupational disease or a reaction to the exposure was discovered, we offered participants a referral to occupational health care or a dermatology clinic.

Main results

Frequency of *FLG* null and CNV

Frequencies of *FLG* null and CNV, and how CNV was reported in the studies differ. A summary of the combined heterozygote *FLG* null frequency for the four mutations included and CNV genotype frequencies is presented in Table 4 for study I-III.

Table 4. Frequencies of *FLG* null and CNV in the studies

Study	Group	*FLG* null (%)	Group	CNV 10/10 (%)	CNV 10/11 (%)	CNV 11/11 (%)	CNV 10/12 (%)	CNV 11/12 (%)	CNV 12/12 (%)
I	Hairdressers (*n*=295)	12 (4.1%)	*FLG* null	2 (16.7)	4 (33.3)	5 (41.7)	1 8.3)	0	0
			FLG wt	15 (5.4)	72 (26)	93 (33.6)	30 (10.8)	49 (17.7)	13 (4.7)
	Controls[7] (*n*=92)	7 (7.6%)	*FLG* null	3 (42.9)	2 (28.6)	1 (14.3)	1 (14.3)	0	0
			FLG wt	10 (11.9)	31 (36.9)	21 (25.0)	5 (6.0)	12 (14.3)	5 (6.0)
II[8]	Chimney Sweeps (*n*=151)	13 (8.6%)	*FLG* null	10 (76.9)			1 (7.7)		
			FLG wt	94 (68.1)			41 (29.7)		
	Controls (*n*=152)	18 (11.8%)	*FLG* null	17 (94.4)			1 (5.6)		
			FLG wt	101(75.4)			29 (21.6)		
III	Phase I participants (*n*=432)	28 (6.5%)	*FLG* null, phase II	4 (17.4)	8 (34.8)	5 (21.7)	1 (4.3)	4 (17.4)	1 (4.3)
			FLG wt, phase II	2 (6.5)	5 (16.1)	11 (35.5)	2 (6.5)	5 (16.1)	3 (9.7)

Study I

We found that hairdressers had a non-significantly lower *FLG* null frequency (4.1%; Table 4) than the control group (7.6 % *P*=0.18). Further, we found that hairdressers had a significantly lower frequency of CNV10 (43.2 %) than controls (56 %, *P*=0.0032).

[7] Includes both consumers and controls

[8] Quality of DNA too low to determine CNV genotype by long range PCR alone. A combination of qPCR and long range PCR was used.

Study II

We found that chimney sweeps had a non-significantly lower *FLG* null frequency (8.6 %; Table 4) than the control group (11.8 %, *P*=0.35). Further we found that chimney sweeps had a non-significantly higher frequency of CNV12 (27.8 %) than the controls (19.7%) (*P*=0.09).

Study III and IV

We found a *FLG* null frequency of 6.5% (Table 4) in phase I, among participants from southern Sweden. We found a frequency of total CNV20-22 (CNV10/10, CNV10/11, CNV11/11, CNV10/12) of 64.6% and CNV23-24 (CNV11/12, CNV12/12) of 25.8% among wt carriers in phase II. We found a frequency of total CNV20-22 of 78.2% and CNV23-24 of 21.7% among *FLG* null carriers in phase II, although we could not determine which allele was expressed.

FLG, dermal absorption and internal dose of chemicals

We found differences in the estimated internal doses of chemicals between *FLG* genotypes including CNV in study I, II and III. The main findings are summarised and presented in Table 5.

Study I

We found that CNV10 carriers in the hairdresser group had a significantly lower risk of elevated hb-o-toluidine adducts in blood than non-carriers (Table 5), but not between *FLG* null and wt and no differences for either genotype for hb-m-toluidine. We found no differences for hb-o-toluidine or hb-m-toluidine between *FLG* genotypes among the controls (not in Table 5; refer to study I)

Study II

We found that CNV12 carriers in the chimney sweep group working mainly with soot sweeping (high PAH exposure) had significantly lower levels of PAH metabolite concentrations compared to non-carriers (Table 5) in linear regression, but not between *FLG* null and wt. We found no differences between *FLG* genotypes among the controls (not in Table 5; refer to study II).

Study III

We found that *FLG* null carriers in phase II excreted 110% higher levels of pyrimethanil metabolite than wt CNV23-24 carriers, and 18% higher levels of pyrimethanil metabolite than wt CNV20-22 carriers, estimated by $AUC_{(0\text{-}40h)}$ (Table 5). For oxybenzone and pyrene, there was also a *FLG* null/CNV dose dependent gradient for $AUC_{(0\text{-}40h}$, but it was not statistically significant.

Table 5. Summary of exposure estimates from study I-III, effect estimates (95% confidence intervals) from logistic and linear regression (log/lin regr) between *FLG* and CNV genotypes in study I-II and comparisons with ANOVA in study III.

Study[9]	Exposure dose	Exposure estimation	Genotype	N above/under median (%)	OR[log regr](95% CI)	*P* [log regr]
I: Hair-dressers *n*=295	Unknown, occupational	Blood hb-o-toluidine	*FLG* null	6/6 (50/50)	0.98 (0.31, 3.11)	0.97
			FLG wt	140/137 (50.5/49.5)	ref	
			CNV10	52/72 (41.9/58.1)	0.53 (0.33, 0.86)	0.010
			No CNV10	93/70 (57.1/42.9)	ref	
		Blood hb-m-toluidine	*FLG* null	7/5 (58.3/41.7)	1.43 (0.44, 4.62)	0.55
			FLG wt	137/140 (49.5/50.5)	ref	
			CNV10	66/58 (53.2/46.8)	1.26 (0.79, 2.01)	0.34
			No CNV10	78/85 (47.9/52.1)	ref	
Study	**Exposure dose**	**Exposure estimation**	**Genotype**	**Concentration (median, µg/g creatinine, IQR)**	**β[lin regr](95% CI)**	***P*** **[lin regr]**
II: Chimney sweeps *n*=151	High occupational soot exposure	Spot urinary 1-hydroxy-pyrene	*FLG* null	0.78 (0.54-4.8)	0.43 (-0.35, 1.2)	0.28
			FLG wt	0.79 (0.37-1.3)	ref	
			CNV12	0.37 (0.29-1.08)	-0.44 (-0.83,-0.053)	0.025
			No CNV12	0.86 (0.52-1.35)	ref	
	Low occupational soot exposure	Spot urinary 1-hydroxy-pyrene	*FLG* null	0.23 (0.10-0.52)	0.28 (-0.42, 0.98)	0.43
			FLG wt	0.16 (0.08-0.36)	ref	
			CNV12	0.14 (0.09-0.39)	0.031 (-0.50, 0.56)	0.91
			No CNV12	0.19 (0.09-0.37)	ref	
Study	**Exposure dose**	**Exposure estimation**	**Genotype**	**$AUC_{(0-40h)}$ (nmol, GM, 95% CI)**		***P*** **[ANOVA]**
III: Phase II participants *n*=54	4.7 mg pyrimethanil	AUC(0-40h) 1-hydroxy-pyrimethanil	*FLG* null	1676, 1253-2243		0.038
			WT CNV 20-22	1421, 1047-1927		
			WT CNV 23-24	799, 494-1294		
	1.1 mg pyrene	AUC(0-40h) 1-hydroxy-pyrene	*FLG* null	28.4, 22.9-35.1		0.195
			WT CNV 20-22	27.2, 21.8-34.0		
			WT CNV 23-24	19.6, 13.8-27.9		
	4.7 mg oxybenzone	AUC(0-40h) Oxybenzone	*FLG* null	1161, 891-1510		0.165
			WT CNV 20-22	1061, 797-1409		
			WT CNV 23-24	711, 459-1104		

Among *FLG* null, we found shorter lag times (Table 6) for skin absorption of pyrimethanil (62% and 79% shorter) and pyrene (14% and 35% shorter), respectively, compared with *FLG* wt CNV20-22 and 23-24. Wildtype carriers with CNV20-22 had 82% shorter lag times for oxybenzone than wt CNV23-24 and *FLG* null had 69% shorter lag times than wt CNV23-24. We found larger absorption rate constants (Table 6) for pyrimethanil (50% larger), pyrene (45% larger), and

[9] For control groups in study I and II, refer to each study

oxybenzone (77% larger) among *FLG* null than *FLG* wt CNV23-24, and similar absorption rate constants to *FLG* null for pyrimethanil, pyrene, and oxybenzone.

Table 6. Comparisons of lag times and rate constants for skin uptake between *FLG* and CNV genotypes in study III.

Study	Exposure dose	Genotype	Lag time (min±SD)	*P*[ANOVA]	Rate constant (h^{-1}±SD)	*P*[ANOVA]
III: Phase II participants *n*=54	4.7 mg pyrimethanil	*FLG* null	8.4±1.2	>0.001	0.19±0.04	0.02
		WT CNV 20-22	22±3.6		0.20±0.05	
		WT CNV 23-24	40±7.2		0.13±0.03	
	1.1 mg pyrene	*FLG* null	54.6±7.2	0.011	0.16±0.05	0.019
		WT CNV 20-22	63.6±9.6		0.14±0.03	
		WT CNV 23-24	84.6±17.4		0.11±0.03	
	4.7 mg oxybenzone	*FLG* null	4.2±0.6	>0.001	0.23±0.07	0.005
		WT CNV 20-22	2.4±0.54		0.22±0.08	
		WT CNV 23-24	13.8±7.8		0.13±0.04	

FLG and toxicity biomarkers

Telomere length, DNA methylation and mitochondrial DNA

Study I

In the hairdresser group, we found that *FLG* null carriers had significantly shorter telomeres (by 23%) than non-carriers in unadjusted linear regression analysis (Table 7), and 15% shorter telomeres than non-carriers (*P*=0.009) in linear regression analysis adjusted for age, passive smoking, residence and education level (not in Table 7, refer to study I). CNV10 carriers had significantly shorter telomeres (by 15%) than non-carriers in unadjusted linear regression analysis (Table 7) and 5% shorter telomeres than non-carriers (*P*=0.048) in linear regression analysis adjusted for age, passive smoking, residence and education level (not in Table 7; refer to study I). We found no differences regarding the DNA methylation index between *FLG* null and wt among hairdressers (Table 7). Among hairdressers, *FLG* null carriers had a higher risk for methylation of *CDKN2A* (OR=6.26, CI=1.13–34.7, *P*=0.036) than non-carriers in linear regression analysis adjusted for age, passive smoking, residence and education level, but not in unadjusted analysis (OR=4.48, CI=0.93–24.5, *P*=0.060). For controls, associations between *FLG* null and telomere length were not observed, but *FLG* null carriers had a higher risk of methylation of *MGMT* (OR=5.30, CI=1.02–27.6, *P*=0.048) than non-carriers in age, passive smoking, residence, and education adjusted linear regression analysis, and in unadjusted analysis (OR=6.48, CI=1.30–32.2, *P*=0.022). CNV10 carriers had a higher risk of methylation of *DAPK1* (OR=2.89, CI=1.08–7.72, *P*=0.034) than non-carriers in age, passive smoking, residence, and education adjusted analysis, but not in unadjusted analysis (OR=2.40, CI=0.98–5.91, *P*=0.057).

Study II

We found that telomere length was significantly shorter in *FLG* null carriers (by 7%) compared with non-carriers (Table 7) in linear regression adjusted for study group (chimney sweep/control) and 8% shorter when adjusted for study group, age and smoking (*P*=0.15; not in Table 7, refer to study II). Methylation of *F2RL3* (CpG2) was 3.4% higher in *FLG* null carriers than non-carriers when adjusting for study group (Table 7) and 2.5% higher after adjusting for study group, age and smoking (*P*=0.019; not in Table 7, refer to study II). We found no associations between *FLG* genotype and *F2RL3* (CpG1), *AHRR* CpG sites or mitochondrial DNA copy number (not included in Table 7).

Table 7. Summary of relative telomere lengths and methylation degrees in study I and II, and effect estimates (95% confidence interval) from linear and ordinal regression (lin/ord regr) between *FLG* and CNV genotypes

Study group	**Genotype**	**Relative telomere length (mean)**	**β[lin regr] (95% CI)**	***P*** **[lin regr]**	**Methylation index *n* 1/2/3 (%)**	**OR[ord regr] (95% CI)**	***P*** **[ord regr]**
Í: Hair-dressers	*FLG* null	0.6335	-0.19 (-0.33, -0.055	0.0060	3/4/3 (30/40/30)	1.19 (0.37, 3.81)	0.77
	FLG wt	0.8244	ref		84/100/66 (34/40/26)	ref	
	CNV10	0.7300	-0.083 (-0.14, -0.027)	0.0036	34/47/30 (31/42/27)	1.19 (0.76, 1.88)	0.45
	No CNV10	0.8547	ref		53/59/37 (36/40/25)	ref	
I: Controls	*FLG* null	0.8593	-0.025 (-0.23, 0.18)	0.81	1/2/3 (17/33/50)	2.61 (0.54, 12.7)	0.23
	FLG wt	0.8838	ref		26/29/21 (34/38/28)	ref	
	CNV10	0.8666	-0.035 (-0.14, 0.073)	0.52	14/17/14 (31/38/31)	1.21 (0.54-2.69)	0.64
	No CNV10	0.9015	ref		13/14/10 (35/38/27)	ref	
Study group	**Genotype/ group**	**Relative telomere length (mean)**	**β[lin regr] (95% CI)**	***P*** **[lin regr]**	**Methylation CpG2 *F2LR3* (mean)**	**β[lin regr] (95% CI)**	***P*** **[lin regr]**
II:Chimney sweeps + controls[10]	*FLG* null	0.533	-0.043 (-0.084, -0.003)	0.037	77.34	2.6 (0.080, 5.0)	0.043
	FLG wt	0.576	ref		74.79	ref	
	Chimney sweep	0.549	0.010 (-0.014, 0.034)	0.409	75.96	-0.205 (-1.6, 1.3	0.786
	Control	0.559	ref		76.17	ref	
II:Chimney sweeps + controls[10]	CNV12	0.565	-0.0086 (-0.037, 0.024)	0.552	75.27	0.31 (-1.4, 2.1)	0.642
	No CNV12	0.574	ref		74.96	ref	
	Chimney sweep	0.573	-0.009 (-0.037, 0.020)	0.529	74.95	0.314 (-1.4, 2.1)	0.642
	Control	0.566	ref		75.30	ref	

[10] Exposure group (chimney sweep/control) was included in the linear regression model.

FLG and serum inflammation-related proteins

Study IV

We found up-regulation of inflammation-related proteins among *FLG* null carriers, and down-regulation among *FLG* wt carriers, after simultaneous dermal exposure to pyrimethanil, pyrene, oxybenzone and nickel. After correction for multiple comparison, we found 30 proteins to be differentially expressed post- *vs* pre-exposure. Eleven proteins were differentially expressed for *FLG* null and 22 for *FLG* wt (3 were common). Of these 30 differentially expressed proteins within the genotype groups, 12 proteins (Table 8) were differentially expressed when comparing the median difference between genotypes. Four proteins were up-regulated for *FLG* null and eight were down-regulated for *FLG* wt.

We used ingenuity pathway analysis to predict differences between *FLG* genotypes in diseases and function related to the differentially expressed proteins. We found differences in chemotaxis between *FLG* null and wt carriers, where *FLG* null carriers had a slight activation of processes involving chemotaxis, but *FLG* wt carriers had a clear inhibition (*FLG* null activation z-score=0.21, *P*=1.52E-15, *FLG* wt activation z-score=-3.68, *P*=7.36E-25).

Table 8. Inflammation-related proteins in study IV (n=44), fold changes post-vs pre-exposure, and comparison between genotypes, and vitamin D concentrations in study IV (n=54) pre resp. post exposure, and comparison between genotypes.

Inflammation-related proteins	**Genotype**				
	***FLG* null**		***FLG* wt**		***P*[diff. between genotypes][11]**
	Fold change	**P[post *vs* pre][12]**	**Fold change**	**P[post *vs* pre][8]**	
HGF	1.04	0.040	0.97	>0.99	0.016
IL18R1	1.04	0.040	1.00	0.946	0.003
OSM	1.24	0.040	1.04	>0.99	0.024
TNFSF12	1.04	0.044	1.05	>0.99	0.031
CCL3	0.89	0.089	0.82	0.008	0.039
CCL11	1.07	>0.99	0.92	0.031	0.010
CCL25	0.97	0.383	0.93	<0.001	0.023
CSF1	1.04	>0.99	0.91	0.029	0.019
IL8	0.96	0.860	0.84	0.011	0.010
CCL7	1.10	>0.99	0.91	0.032	0.049
TNFSF10	0.93	0.700	0.93	<0.001	0.019
VEGFA	0.95	>0.99	0.92	0.010	0.008
Vitamin D	***FLG* null median**	***P*[post *vs* pre][12]**	***FLG* wt median**	***P*[post *vs* pre][8]**	***P*[between genotypes][11]**
Vitamin D conc pre exposure (ng/mL)	34.0	0.038	28.5	0.005	0.004
Vitamin D conc post exposure (ng/mL)	37.6		29.8		0.033

[11] Mann Whitney U-test.

[12] Paired samples Wilcoxon test.

Vitamin D

Study IV

FLG null carriers had 19% and 26% higher vitamin D concentrations than wt carriers, before and after the exposure experiment, respectively (Table 8). The vitamin D concentrations increased with exposure by 11% for *FLG* null carriers, and by 5% for *FLG* wt carriers, but the larger increment among *FLG* null was not statistically significant (*P*[Mann-Whitney]=0.113).

Comparisons of vitamin D levels pre- and post exposure between *FLG* null, wt CNV20-22 and wt CNV23-24 is presented in Table 9 and was not presented in the manuscript for study IV. There was a significant difference between post-exposure concentrations depending on *FLG* null and low and high CNV. Pairwise comparison revealed a significant difference between *FLG* null and wt CNV20-22 (*P*=0.011), but not wt CNV23-24 (*P*=0.130), or between CNV20-22 and CNV23-24 (*P*=0.706)

Table 9. Vitamin D concentrations in study IV (n=54) pre resp. post exposure, and comparison between *FLG* null, wt CNV20-22, and 23-24

Genotype	Vitamin D conc. pre exposure (median, IQR, ng/mL)	*P* [Kruskal Wallis]	Vitamin D conc. post exposure (median, IQR, ng/mL)	*P* [Kruskal Wallis]
FLG null	34.0 (28.1-38.4)	0.116	37.6 (31.9-43.3)	0.031
FLG wt CNV20-22	28.5 (24.5-32.0)		29.6 (25.6-34.4)	
FLG wt CNV23-24	28.2 (22.1-39.3)		32.5 (21.9-39.5)	

Discussion

This thesis showed that *FLG* null and low CNV influence the uptake of some common chemicals via the skin. We also showed that *FLG* null and low CNV modify the systemic toxicity after skin exposure. Dermal exposure to chemicals has long been deemed a less significant exposure route than inhalation exposure, and regulative legislation concerning dermal exposure is therefore lagging, partly because assessing dermal occupational or consumer exposure is difficult. An important step in assessing risk in relation to chemical exposure is to identify susceptibility factors for dermal exposure. We have now collected evidence that the variation in dermal absorption of some chemicals can affect the internal dose with as much as two-fold, depending on *FLG* genetics alone. If the associations between *FLG* null and low CNV groups and increased systemic effects have an impact on human health needs further investigation. When setting limit values policymakers should also consider genetic susceptibility studies, to identify the most susceptible individuals in a population and adjust existing limit values to safe limits for everyone.

FLG polymorphism in different study groups

We found fewer carriers of *FLG* null in the occupational groups included in studies I and II than in the control groups, although not significantly. The frequency in the hairdresser group is also lower than available frequencies (7.6–11 %) for general populations in Denmark, Ireland and Germany (Bandier et al. 2013; Brown et al. 2012; Greisenegger et al. 2010; Joensen et al. 2014; Thyssen et al. 2010; Varbo et al. 2017), but higher than in Finland (2%) (Luukkonen et al. 2017). An explanation for the lower *FLG* null frequency among hairdressers could be a healthy worker effect, which also could explain the lower frequency of CNV10 (43%) carriers among hairdressers than in the control group (56%). *FLG* null carriers may develop occupational skin disease early in their carrier and quit their job as a hairdresser, perhaps already during training, which would decrease the frequency of *FLG* null carriers in occupations with high dermal exposure. This hypothesis was investigated by a Danish research team. They found that the combination of carrying *FLG* null and having eczema as a child was associated with avoiding occupational chemical exposure in adulthood (Bandier et al. 2013).

In study III, where participants were recruited regardless of occupation, we found a frequency of 6.5%, closer to the expected in the Northern European population. Nevertheless, we expect that those *FLG* null carriers with substantial problems with eczema or other skin related issues would not sign up for this study as the overall aim was to identify individuals for a subsequent skin exposure study. Hence there is a possibility for slight selection bias towards individuals without skin diseases, and since 20-50% of patients with atopic dermatitis (Brown and Irvine 2008) carry a *FLG* null mutation, thus a bias towards *FLG* wt.

FLG polymorphism and internal dose of chemicals

Occupational groups

In the cross-sectional studies of hairdressers and chimney sweeps, we found some but not consistent associations between *FLG* polymorphism and the internal dose of the chemicals under study. Among hairdressers, the risk of elevated hb-o-toluidine was decreased for CNV10 carriers, opposite to our hypothesis. An explanation could be if CNV10 carriers used gloves more often than non-carriers, because of, we hypothesized, more sensitive skin. This was tested in sensitivity analysis (refer to study I), but no associations were found between glove use and CNV or *FLG* null. Among chimney sweeps, we found lower levels of PAH in highly exposed CNV12 carriers, according to our hypothesis. We found no significant associations between *FLG* null and internal dose of the chemicals. With so few *FLG* null carriers in our occupational study groups, statistical power could be an issue, especially if effect size of being a *FLG* null carrier is smaller than anticipated, because of possible compensating mechanisms.

Morphology differences of the outermost cell layers found in *FLG* null carriers compared with non-carriers could be such a compensating mechanism. Gruber et al. (2011) showed that patients that were compound heterozygote for *FLG* null (two different *FLG* null mutations) and heterozygote *FLG* null carriers (one *FLG* null mutation and one wildtype) had more layers of cells in the stratum corneum than homozygote wild-type carriers. Hypothetically, more cell layers could be protective against chemical exposure, and compensate slightly for an impaired skin barrier. Another such a compensating mechanism could be gene regulation of *FLG* by the AHR. It has been suggested that AHR can up-regulate the expression of filaggrin by binding both oxidative and antioxidant compounds (Furue et al. 2015; Tsuji et al. 2017), and if *FLG* null carriers absorb more of these compounds, *FLG* on the functional allele may be upregulated in response.

Others have found *FLG* polymorphism to be associated with the internal dose of chemicals. Higher urinary concentrations of phthalate metabolites and n-propyl

were found in 65 Danish male *FLG* null carriers compared with non-carriers in a cross-sectional study (Joensen et al. 2014). Further, also in the same 65 Danish men in another cross-sectional study, higher urinary concentrations of metyl parabens and n-propyl paraben (preservatives used in personal-care products and cosmetics) were found in *FLG* null carriers compared with non-carriers (Joensen et al. 2017). The Danish studies are based on study participants recruited from the general population and the frequency of *FLG* null carriers was higher (7.5 %) than in our hairdresser group, possibly resulting in higher power for detecting differences in concentrations of the chemicals depending on *FLG* null.

Exposure experiment

We confirmed our hypothesis, that both *FLG* and low CNV impact the uptake of chemicals through the skin. We saw a 1.5-2-fold higher internal dose (estimated by $AUC_{(0\text{-}40h)}$) among *FLG* null carriers for all three chemicals, and a dose dependent effect by CNV among wt carriers, however only statistically significant for pyrimethanil. We showed that the lag time for absorption of the chemicals included in the study was 1.5-5 times shorter for *FLG* null carriers (or low CNV among wt carriers in one case – oxybenzone), and absorption rate constants were 1.5 times larger (and similar as wt carriers with low CNV), compared with wt carriers with high CNV. The combined results of higher internal doses, shorter lag times and higher rate constants confirm that *FLG* polymorphism had an impact on the uptake of the three chemicals through the skin. We argue that it is possible to generalise the results to an occupational exposure situation or a consumer exposure because we performed a study where the exposure time, dose and area were controlled, and we could follow the urinary excretion over time, which meant we could assess the internal dose. However, we can only generalise to exposures involving the chemicals from this study, if the exposure is of low dose, and the exposure is restricted to the arms and hands. Furthermore, the variation in internal dose between individuals within the genetic groups of study III was large, showing that factors, known and unknown, influence skin absorption, not only *FLG* polymorphism. Age is one such factor that could affect the skin uptake (Ngo et al. 2010), although we had age matched controls in the experimental study. Washing or rubbing of the skin can also affect skin uptake (Law et al. 2020), which is why we had a standardised washing procedure for participants after the exposure experiment. In the exposure experiment we included mainly Northern Europeans and genotyped them for the four most common null mutations in this population. The generalisability to other populations of different ancestry needs further study, both because other *FLG* null mutations are present in other populations, and since differences in skin barrier function between populations depending on pigmentation of the skin have been reported (Elias and Wakefield 2011). However, CNV of *FLG* varies also in other populations, such as Ethiopian (Fernandez et al. 2017) and African-American

populations (Margolis et al. 2020). Similar studies on other populations with different *FLG* mutations and skin types are warranted.

Regardless of *FLG* null, anyone with a compromised skin barrier due to for example atopic dermatitis may absorb more chemicals through both non-lesional and lesional skin (especially relevant for occupations linked to skin disease, such as hairdressers). Previous research in atopic dermatitis patients show that double the absorption is expected in lesional and non-lesional skin (Halling-Overgaard et al. 2017). In this thesis, we have shown that also *FLG* null carriers, regardless of whether they have atopic dermatitis, absorb up to twice as much of the chemicals included in the study, and that low CNV, which is more common than *FLG* null in the population also is a risk factor for higher absorption. A report from the EU for the safe use of pesticides in relation to dermal absorption (EFSA 2017) states that the increased absorption in skin among atopic individuals is covered by the safety factors included when calculating safe exposure levels for operators. However, *FLG* null carriers may absorb twice as much of not only the chemicals they are exposed to at the workplace, but also chemicals from the consumer products they use. It could be possible that twice the total body burden of the mixture exposure leads to negative health effects for *FLG* null carriers compared with *FLG* wt carriers, and this question needs further investigation.

FLG polymorphism and toxicity

Telomere length and DNA-methylation

We found that *FLG* null was associated with more genotoxicity in the form of shorter telomeres among only the hairdressers in study I, and in study II among both chimney sweeps and controls. In study I, also only among hairdressers, CNV10 was associated with shorter telomeres. We cannot conclude that *FLG* null carriers working in occupations with high dermal exposure have more DNA-damage than *FLG* null carriers working in occupations with low dermal exposure. The finding that *FLG* polymorphism is associated with telomere length among hairdressers, but not among the controls may be due to the hairdressers' higher dermal exposure to chemicals. It could also be due to a relatively small control group in the study, and lower power to detect differences.

Exposure to exogenous compounds have been associated with genotoxicity both in the form of shorter and longer telomeres in leukocytes (Clarity et al. 2020; de Souza et al. 2018; Zhang et al. 2013). A meta-analysis of 21 case-control studies (Ma et al. 2011) published on the subject of telomere length and cancer risk showed that shorter telomeres in peripheral cells may be a marker for increased cancer risk. However, as pointed out by Savage et al 2013 (Savage et al. 2013), these studies did not report the treatment status of patients, as chemotherapy can decrease telomere

length in peripheral cells temporarily (Benitez-Buelga et al. 2015). Several recent large prospective studies have found an association between longer, not shorter, telomeres in peripheral cells and risk for pancreas (Luu et al. 2019a), colorectal (Luu et al. 2019b), breast cancer (Campa et al. 2018; Samavat et al. 2019), lung cancer (Yuan et al. 2018) and no association between short leukocyte telomere length and cancer risk (Weischer et al. 2013). What *FLG* null and low CNV mean for cancer risk warrants further investigation.

We found a six times higher risk for methylation of tumour suppressor gene *CDKN2A* for *FLG* null carriers among hairdressers, which is in line with our hypothesis, although confidence intervals were very wide, probably because of the low number of *FLG* null. Among the controls we found a higher risk for methylation of *MGMT* (five times higher risk) and *DAPK1* (three times higher risk), also with wide confidence intervals. Hypermethylation of *CDKN2A* (Hoque et al. 2006), *MGMT* (Jahed et al. 2016) and *DAPK1* (Abbosh et al. 2008) have previously been linked to bladder cancer. Methylation of these genes should be further investigated between *FLG* null and wt carriers in highly dermally exposed occupational groups and general populations. Opposite to our hypothesis, we found higher methylation of *F2RL3* for *FLG* null carriers among chimney sweeps. Hypomethylation of *F2RL3* has previously been associated with lung cancer (Fasanelli et al. 2015), but we found being a *FLG* null carrier was protective for hypomethylation of this gene. The study by Alhamdow et al. 2018 showed less methylation of *F2RL3* among chimney sweeps exposed to PAH. Our unexpected result – in addition to the lower levels of hb-o-toluidine found in CNV10 carriers in the hairdresser study suggests that there may be compensatory mechanisms for an impaired skin barrier, or it may be a chance finding as a result of multiple comparisons. In study I, we found altered risks for methylation of tumour suppressor genes among controls, and in study II, the associations between *FLG* null and methylation did not change depending on being a chimney sweep or control. This implies that everyday non-occupational exposure also influences DNA methylation depending on *FLG* polymorphism. The relationship between *FLG* polymorphism and genotoxicity and epigenetic changes should be further investigated in other dermally exposed occupational groups and general populations.

Inflammatory proteins

We found that there is a *FLG* polymorphism-dependent difference in inflammation-related proteins in blood in response to dermal exposure to three organic chemicals and nickel. We found mostly up-regulation of proteins among *FLG* null but down-regulation among *FLG* wt. The different expression patterns of proteins seemed to result in, as predicted by the pathway analysis, differences in the recruitment – chemotaxis – of immune cells. These results may simply reflect the increased risk among *FLG* null carriers for inflammatory skin disease (Palmer et al. 2006), or they may also indicate that *FLG* null carriers have an increased risk for systemic chronic

inflammation. Indeed, in a study of *n*=59 atopic dermatitis patients, inflammation-related proteins in blood were elevated compared to controls (Brunner et al. 2017), but *FLG* genetics were not reported. Had we taken blood samples also 48 hours after exposure we could have investigated if the effects were long lasting after exposure. We did not have the capacity for taking more blood samples as the nurse responsible was occupied with the ongoing exposure experiment four days per week.

We hypothesized that *FLG* null carriers would have a pro-inflammatory response, but not that wt carriers would have an anti-inflammatory response, which could have been an effect of the properties of the chemicals included. Some PAH (Allan and Sherr 2010; Davila et al. 1995; Laupeze et al. 2002), although not specifically pyrene, have been associated with immunosuppressive properties *in vitro* and *in vivo*. Nickel, oxybenzone and pyrene have been associated with changes in inflammation-related protein levels and cells; exposure to PAH was associated with increased white blood cell count among the general population (Clark et al. 2012) and increased acute phase protein Serum Amyloid A1 among coke oven workers (Hadrup et al. 2019), which are indicators of increased systemic inflammation, or acute inflammation or infection. *In vitro* studies of human macrophages showed that oxybenzone (Ao et al. 2018) and nickel (Åkerlund et al. 2019) could induce increased cytokine expression. However, we cannot elucidate which chemical is responsible for the differential expression patterns we saw. We did not evaluate differences based on CNV genetics in study IV. We did not expect effect sizes to be large, and the size of the sub-group chosen for serum analysis of inflammation-related proteins was smaller than the total group used in study III. We only had a total of 4 participants representing high CNV (total CNV23-24) in study IV.

Vitamin D

We found that vitamin D levels were higher among *FLG* null carriers than wt carriers in our study, which has been reported previously in a cross-sectional study (Thyssen et al. 2012). It has been suggested that increased vitamin D levels are of evolutionary advantage to *FLG* null carriers (Thyssen et al. 2014), which could be one reason for the relatively high prevalence of *FLG* null in Northern Europe, except Finland. We found an increase in vitamin D levels for both genotypes after the 4 h exposure, which could be due to diurnal variation of vitamin D (Jones et al. 2017). The increment was larger among *FLG* null carriers than wt carriers, however not significantly. Although, we cannot exclude that vitamin D levels was affected by the exposure.

We could not establish a link between increased vitamin D levels and the inflammation-related proteins in study IV other than that we found the VDR receptor to be identified as an upstream regulator for some proteins among both *FLG* null and wt. We hypothesized that vitamin D would be a protective factor against inflammation among *FLG* null carriers, since vitamin D is an important co-factor for the immune system. This needs further investigation.

Strengths and limitations

Study I and II

One strength of study I and II is that these studies include a relatively large number of hairdressers and chimney sweeps: about 1.3% of hairdressers working in Sweden in 2009 (SCB 2021a, b) were included in study I, and about 10 % of chimney sweeps working in Sweden in 2015 (SSR 2021) were included in study II. Previous research about differences in skin absorption has focused on *FLG* null, and most often in atopic dermatitis patients. In study I and II we evaluated both *FLG* null and CNV in occupational groups. We investigated associations between *FLG* null and CNV with systemic effects measured as biomarkers of cancer-related DNA changes in blood, which few others have done before.

Limitations of these studies are that they are cross-sectional, limiting conclusions about casual relationships. Further, the exposure assessments in our occupational groups were not optimal. We did not know the actual level of external exposure, which likely differed between individuals and influenced the internal dose, and we measured exposure biomarkers only once. Also, the urinary and hb-adduct biomarker levels reflect uptake via all exposure routes, not only the dermal exposure route, which hampers investigating associations with *FLG* that is mainly expressed in the skin. Tape stripping could have been performed to assess the dermal exposure. This was not feasible at the time of sampling. The urinary biomarkers reflect very recent exposure to PAH, which made it difficult to assess the exposure for chimney sweeps not working with soot related tasks very often. However, in a previous study if these chimney sweeps, there was a correlation between PAH in urine and how much soot sweeping the chimney sweeps had performed during the last months (Alhamdow 2019).

Another limitation is that we measured telomere length and DNA methylation in peripheral cells from blood in both study I and II, and not from cells in tissues where tumours may develop. The DNA methylation patterns in peripheral blood cells vary depending on the cell type (Reinius et al. 2012) and the telomere length of peripheral blood cells may vary over short periods of time (Theodosiou et al. 2019). However, higher incidences of haematopoietic cancer forms have been found among chimney sweeps (Hogstedt et al. 2013), and genotoxic and epigenetic changes in blood cells may be more relevant for prediction of hematopoietic cancer risk.

The control group for hairdressers was small and included both exposed (consumers of hair dye) and non-exposed subjects, for a hypothesized gradient of aromatic amine exposure between hairdressers, consumers, and controls. However, the exposure overall was low and there was not a gradient in the exposure to aromatic amines. The analysis method for hb-adducts of aromatic amines was labour intensive with many steps, which may have introduced errors. Meanwhile, the hb-adducts reflect a longer period of exposure, up to four months.

We did not include exposure biomarkers in analyses regarding telomere length and DNA methylation patterns, for hairdressers because exposure to the chosen aromatic amines was low; many were below the LOD. For chimney sweeps PAH were excluded because no associations between PAH and telomere length or DNA methylation biomarkers were found in previous work (Alhamdow et al. 2018).

Another limitation is that we did not have complete information about CNV in study II. Because of methodological difficulties the CNV genotypes could not be assessed and only carriers of 12 could be distinguished from carriers of 10 or 11. Tape stripping could also have been used to quantify levels of filaggrin and NMF in the skin.

Study III and IV

Strengths of study III and IV include that they were based on a human experimental setting where the dose, area and time of exposure were controlled. The human experiment included multiple sampling occasions. The chemicals chosen for the dermal exposure experiment, study III, represent common environmental and occupational exposures. However, a limitation is that the three organic chemicals were chemically rather similar: pyrene, pyrimethanil and oxybenzone all contain aromatic rings, and are of similar molecular weight but with varying log P. A limitation regarding generalisability is that we only exposed at two anatomical sites of the arms. However, the difference in absorption through different anatomical sites (Lotte et al. 1987) is hypothetically similar regardless of *FLG* null. We measured the known metabolites of the compounds included, except for oxybenzone where we only measured the parent compound. If the compounds have other metabolites, this could affect the estimation of the internal dose in study III.

Another limitation is that we could not distinguish which strand of DNA among *FLG* null carriers that carried the null mutation, which meant we could not group *FLG* null carriers according to CNV. If *FLG* null carriers with low CNV are an even more susceptible group to dermal absorption remains to be seen.

We had issues with the nickel exposure in study III, even though exposure levels were below that used in patch-testing for nickel allergy. We started using occluded nickel exposure, and then stopped the exposure after some participants had a skin reaction. We then reintroduced nickel exposure, but without occlusion. We had accidentally created three exposure groups – one with no nickel exposure, one with medium nickel exposure (open exposure) and one with high nickel exposure (occluded exposure), which is relevant for the results in study IV. However, *FLG* null and wt carriers were evenly spread out between the groups. Regarding skin reactions, four participants noted reactions due to nickel exposure, and in one case pyrene. The participants were evenly spread out over genotype groups and when excluded in sensitivity analysis in study IV, the results for differentially expressed proteins did not change dramatically.

Further, we cannot determine if or which of the chemicals included in the exposure experiment were responsible for the observed differential expression of inflammation-related proteins in study IV. Our aim was not to examine the effect on inflammatory proteins for each chemical individually. For practical reasons we performed the experiment with co-exposure to all four compounds, and to resemble a mixture exposure that is relevant for consumers and occupational groups. However, we may have overlooked some unintentional exposures, such as the vehicle used to administrate the exposure doses and the tape used for occluding the exposure sites.

Future perspectives

Other skin-related genes of potential interest

In this thesis, we focused on the role of filaggrin polymorphism for dermal absorption of chemicals. We have investigated the significance of the four most common null mutations in Northern Europe, as well as CNV, for absorption of chemicals and systemic effects. For future research, it is important to also consider other genes involved in the maintenance of skin barrier structure and function. Examples are Kallikrein-related peptidase 5 (*KLK5*), that is involved in the processing of pro-filaggrin into filaggrin monomers (Sakabe et al. 2013), and caspase-14 (*CASP14*) that is involved in cleaving of filaggrin monomers into natural moisturizing factors (Hoste et al. 2011). Other skin barrier genes of the epidermal differentiation complex could also be of relevance, such as filaggrin 2 (*FLG2*) and hornerin (*HRNR*), which were found down-regulated in hand eczema patients (Molin et al. 2015). Further important molecules for skin barrier integrity include lipids. A decrease in the lipid content of the stratum corneum in atopic dermatitis patients is common, in addition to *FLG* polymorphism (van Smeden and Bouwstra 2016). The decrease in lipid content is due to decreased expression or decreased activity of several different lipid processing enzymes, which can lead to abnormal stratum corneum lipid organization and impair skin barrier structure and function (van Smeden and Bouwstra 2016).

Health effects of dermal exposure

In this thesis we found associations between *FLG* polymorphism and genotoxicity and inflammation. We need more knowledge about the systemic effects of an increased chemical absorption among *FLG* null and low CNV carriers. For example, we could genotype biobank samples for *FLG* polymorphism from confirmed cancer or cardiovascular disease cases, and compare with healthy controls, to investigate if the frequency of *FLG* null and low CNV is overrepresented in the previously mentioned group. Other systemic effects include endocrine disrupting effects for other hormonal systems than the male reproduction system, with which the

previously mentioned Danish study (Joensen et al. 2014) found associations for *FLG* null, for example female reproduction hormones or thyroid hormones that are important for neurodevelopment.

Predicting skin absorption

Further research about skin absorption of chemicals (with different properties) depending on skin genetics in humans is needed and could be used to predict skin absorption in occupational settings or for consumers. A predictive model for skin absorption, such as the dermal Advanced REACH Tool (McNally et al. 2019), but with built-in genotypes of risk genes for dermal absorption, and combined with studies on human health in relation to skin exposure, could be a way forward towards complying to REACH legislation about implementing DOEL:s. Using toxicokinetic models in combination with experimental data is one way of producing data to base such a predictive model on.

Conclusions

In this thesis we investigated whether *FLG* genetics influence the internal dose of chemicals and systemic effects related to chemical exposure. From the results presented in this thesis we can draw the following conclusions:

I. Carrying a *FLG* null mutation was associated with genotoxicity in the form of shorter telomeres among hairdressers, and among chimney sweeps and controls. Further, carrying a low CNV (10 repeats), independent of *FLG* null, was associated with shorter telomeres than high CNV (11 or 12 repeats) carriers in hairdressers.

II. Carrying a *FLG* null mutation was associated with a higher risk of elevated DNA methylation of tumour suppressor genes *CDKN2A* and *MGMT* among hairdressers and controls, respectively. Carrying a low CNV (10 repeats) was associated with a higher risk of elevated DNA methylation of tumour suppressor gene *DAPK1* among controls. Carrying a *FLG* null was associated with more methylation of *F2RL3* among chimney sweeps and controls, contrary to our hypothesis.

III. Carrying a low CNV (10 repeats) among hairdressers was associated with lower risk of elevated hb-o-toluidine levels, contrary to our hypothesis. Carrying a high CNV (12 repeats) among chimney sweeps with high soot exposure was associated with lower levels of PAH in urine compared with low CNV (10 or 11 repeats).

IV. *FLG* null carriers absorbed about twice as much of the chemicals included in study III, with up to 80% shorter lag times for skin absorption than wt carriers with high CNV (a total CNV of 23-24).

V. There was a dose dependent effect of CNV among wt carriers on the absorption of the chemicals included in study III.

VI. *FLG* null carriers in study III had higher vitamin D levels than wt carriers, both before and after the dermal exposure experiment.

VII. Finally, systemic effects in the form of expression of inflammation-related proteins after exposure to some chemicals differed between *FLG* null and wt carriers, with a more pro-inflammatory profile among *FLG* null carriers.

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