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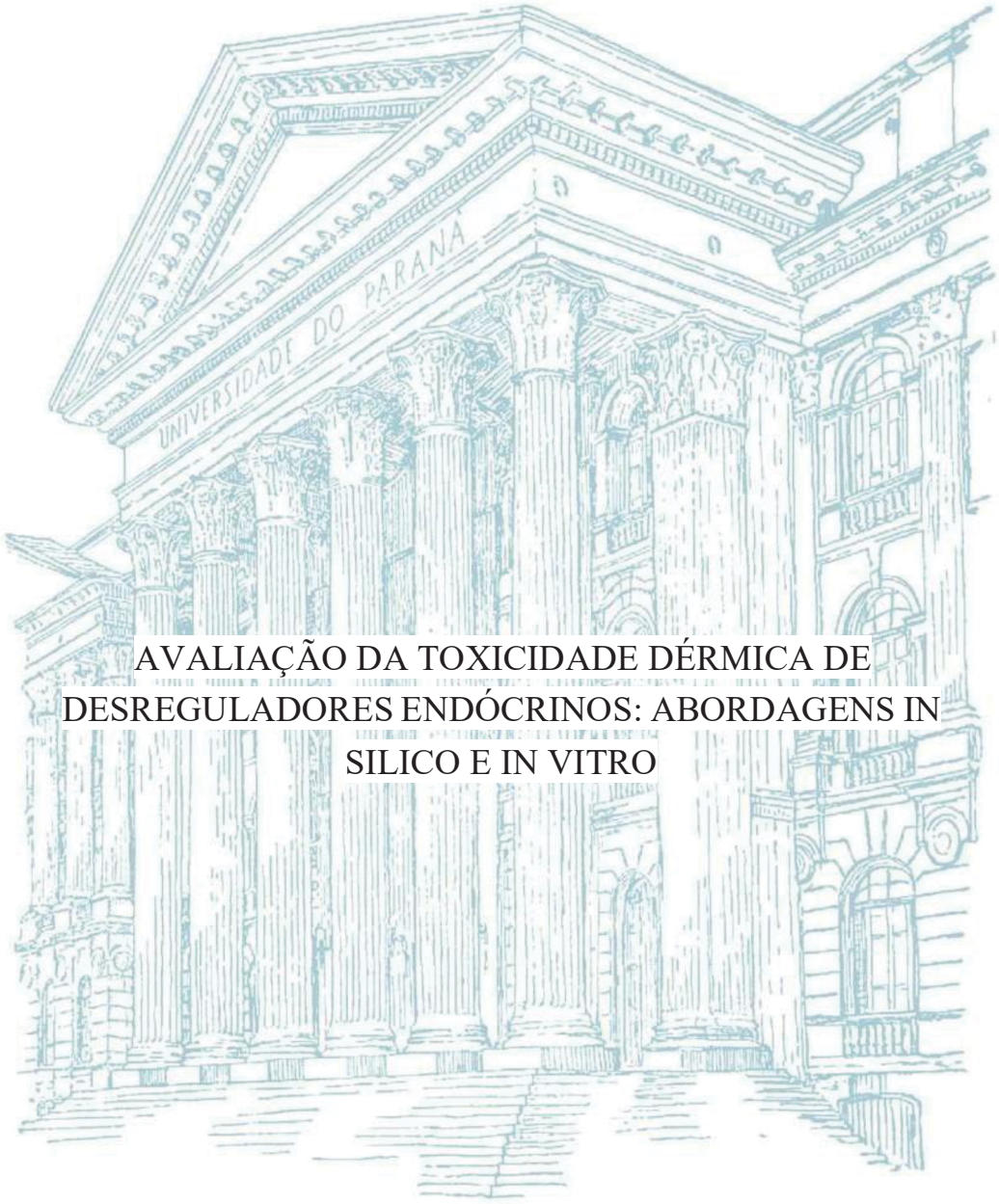
ISISDORIS RODRIGUES DE SOUZA



AVALIAÇÃO DA TOXICIDADE DÉRMICA DE DESREGULADORES ENDÓCRINOS: ABORDAGENS IN SILICO E IN VITRO

CURITIBA

2022

ISISDORIS RODRIGUES DE SOUZA

AVALIAÇÃO DA TOXICIDADE DÉRMICA DE DESREGULADORES ENDÓCRINOS:

ABORDAGENS IN SILICO E IN VITRO

Tese apresentada como requisito parcial à obtenção do grau de Doutora em Genética, no Curso de Pós-Graduação em Genética, Setor de Ciências Biológicas, da Universidade Federal do Paraná.

Orientadora: Profª. Dra. Daniela Morais Leme

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ATA DE SESSÃO PÚBLICA DE DEFESA DE DOUTORADO PARA A OBTENÇÃO DO GRAU DE DOUTORA EM GENÉTICA

No dia vinte e nove de setembro de dois mil e vinte e dois às 14:00 horas, na sala 65, Setor de Ciências Biológicas_Departamento de Genética, foram instaladas as atividades pertinentes ao rito de defesa de tese da doutoranda **ISISDORIS RODRIGUES DE SOUZA**, intitulada**: Avaliação da toxicidade dérmica de desreguladores endócrinos: abordagens *in silico* e *in vitro***, sob orientação da Profa. Dra. DANIELA MORAIS LEME. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação GENÉTICA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: DANIELA MORAIS LEME (UNIVERSIDADE FEDERAL DO PARANÁ), ELISA RAQUEL ANASTÁCIO FERRAZ AVELINO (UNIVERSIDADE FEDERAL FLUMINENSE), SILVIA BERLANGA BARROS (55001108), DANIELA DE ALMEIDA CABRINI (40001016). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de doutora está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, DANIELA MORAIS LEME, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 29 de Setembro de 2022.

Assinatura Eletrônica
07/10/2022 17:08:52.0
DANIELA MORAIS LEME
Presidente da Banca Examinadora

Assinatura Eletrônica
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ELISA RAQUEL ANASTÁCIO FERRAZ AVELINO
Avaliador Externo (UNIVERSIDADE FEDERAL FLUMINENSE)

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MINISTÉRIO DA EDUCAÇÃO
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PROGRAMA DE PÓS-GRADUAÇÃO GENÉTICA - 40001016006P1

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação GENÉTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ISISDORIS RODRIGUES DE SOUZA** intitulada: **Avaliação da toxicidade dérmica de desreguladores endócrinos: abordagens *in silico* e *in vitro***, sob orientação da Profa. Dra. DANIELA MORAIS LEME, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 29 de Setembro de 2022.

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RESUMO

Dificuldades na avaliação toxicológica dos produtos químicos, incluem a variabilidade individual, o lento desenvolvimento das tecnologias de avaliação em comparação com a ampla produção de novos produtos químicos, a dificuldade na tomada de decisões baseadas nos métodos atualmente utilizados e a necessidade de proteger a saúde humana contra efeitos adversos com métodos não animais. Desta forma, o principal objetivo desse trabalho é avaliar uma nova estratégia integrada de teste in silico-in vitro para sensibilização dérmica e genotoxicidade e gerar informações sobre a toxicidade do octilfenol (OP) e do diisopentil ftalato (DiPeP), bem como e promover um levantamento da literatura acerca da influência de variantes genéticas na susceptibilidade à imunotoxicidade dérmica. Assim, o presente trabalho foi dividido em 4 capítulos: (I) Artigo de revisão crítica sobre variantes genética; (II) Artigo original sobre a avaliação da toxicidade dérmica do OP; (III) Artigo original sobre avaliação da toxicidade dermica do DiPeP; (IV) Artigo original sobre danos no DNA e alterações epigenéticas gerados pelo OP. As abordagens *in silico* consideraram o OP como um sensibilizador cutâneo, o que foi confirmado *in vitro*, pelo aumento da expressão de IL-6 (células HaCaT); IL-18 e IL-8 (modelo RHE); e aumento da expressão de CD54 e IL-8 no modelo de ativação de células THP-1. O OP foi previsto como não genotóxico, mutagênico ou carcinogênico pelas ferramentas in silico, porém apresentando um alerta para a ligação ao DNA. *In vitro*, o OP aumentou significativamente a formação de γ-H2AX, 5mC e ROS intracelular em células HaCaT. Indicando potencial de ligação ao DNA e aumento de quebras de dupla fita (DSBs) e metilação do DNA. O DiPeP foi considerado sensibilizador cutâneo apenas *in vitro*, por aumentar a expressão de IL-6, IL-8 e IL-1α e expressão do gene *ILA* em células HaCaT; aumentar a expressão de CD54, IL-8 e TNF-α em células THP-1. Além de agir como um imunomodulador, aumentando a expressão de CD54 induzida por LPS. Desta forma, os resultados atestam o sucesso da utilização das abordagens *in silico-in vitro* através da identificação do potencial de sensibilazão cutânea e genotoxicidade das substâncias testadas.

Palavras-chave: Epiderme Humana Reconstruída (RhE); Sensibilização Cutânea; QSAR; Citocinas Inflamatórias; Micronúcleo; Mutagenicidade; h-CLAT.

ABSTRACT

Difficulties in the toxicological assessment of chemicals include individual variability, the slow development of assessment technologies compared to the widespread production of new chemicals, the difficulty in making decisions based on currently used methods, and the need to protect human health. against adverse effects with non-animal methods. Thus, the main objective of this work is to evaluate a new integrated in silico-in vitro test strategy for dermal sensitization and genotoxicity and generate information on the toxicity of octylphenol (OP) and diisopentyl phthalate (DiPeP), as well as and promote a literature survey on the influence of genetic variants on susceptibility to dermal immunotoxicity. Thus, the present work was divided into 4 chapters: (I) Critical review article on genetic variants; (II) Original article on the assessment of the dermal toxicity of OP; (III) Original article on the assessment of the dermal toxicity of DiPeP; (IV) Original article on DNA damage and epigenetic alterations generated by OP. The in silico approaches considered OP as a skin sensitizer, which was confirmed in vitro by the increased expression of IL-6 (HaCaT cells); IL-18 and IL-8 (RHE model); and increased expression of CD54 and IL-8 in the THP-1 cell activation model. The OP was predicted as non-genotoxic, mutagenic or carcinogenic by the in silico tools, but presenting a warning for DNA binding. In vitro, OP significantly increased the formation of γ-H2AX, 5mC and intracellular ROS in HaCaT cells. Indicating DNA binding potential and increased double-strand breaks (DSBs) and DNA methylation. DiPeP was considered a skin sensitizer only in vitro, as it increases the expression of IL-6, IL-8 and IL-1α and expression of the ILA gene in HaCaT cells; increase the expression of CD54, IL-8 and TNF-α in THP-1 cells. In addition to acting as an immunomodulator, increasing LPS-induced CD54 expression. In this way, the results attest to the successful use of New Methodological Approaches, through the identification of the potential for skin sensitization and genotoxicity of the substances tested.

Keywords: Reconstructed Human Epidermis (RhE); Skin Sensitization; QSAR; Inflammatory Cytokines; Micronucleus; Mutagenicity; h-CLAT

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SUMÁRIO

1. INTRODUÇÃO

Um alto número de produtos químicos estão presentes no dia-a-dia do ser humano e outros organismos vivos (PARISH et al. 2020). Em particular, consumidores e trabalhadores são frequentemente expostos a esses produtos químicos através de produtos cosméticos e domésticos ou em ambientes industriais (BAUCH et al. 2012).

Como a pele serve de interface entre o ambiente e o corpo, esta está continuamente exposta à essas substâncias (CHUONG et al., 2002). A sensibilização dérmica é um dos principais efeitos adversos que podem ocorrer como resultado da exposição da pele, que se manifesta clinicamente como a dermatite alérgica de contato (DAC) (BAUCH et al. 2012). O principal objetivo dos testes toxicológicos é fornecer uma base para a avaliação do perigo e identificação dos riscos à exposição a produtos químicos, garantindo que não ocorram efeitos adversos à saúde humana (BORGERT et al., 2021). A avaliação da capacidade de sensibilização de uma substância tem portanto, sido de grande importância na avaliação de perigos e riscos de substâncias químicas.

Historicamente, os potenciais de sensibilização para as subtâncias geralmente se baseavam nos estudos em animais – como exemplos, existem os testes descritos na OECD 406 (testes em cobaias de acordo com Buehler ou Magnusson & Kligman); OCDE 429 e OCDE 442 (ensaios de linfonodo local murino, LLNA), aceitos pelos órgãos regulatórios (OECD,2022; 2010).

Porém, as questões éticas quanto ao uso de animais levaram a mudanças na legislação sobre o registro, avaliação, autorização e restrição de produtos químicos (REACH, 2006) e em alterações da Diretiva Europeia de Cosméticos (Council Directiva 76/768/CEE, 1976; EU, 2003). A implementação do REACH levantou a discussão do enorme número de testes em animais estimados para avaliação dos vários parâmetros toxicológicos (HARTUNG E ROVIDA, 2009), incluindo a avaliação de risco de sensibilização, um dos parâmetros necessários para registro de produtos químicos no âmbito do REACH. Ao mesmo tempo, o REACH exigiu que todos os esforços fossem feitos para evitar testes em animais e sua utilização apenas como último recurso (European Chemicals Agency, 2011).

Adicionalmente, a 7º Alteração da Diretiva de Cosméticos (76/768/CEE, 1976; EU, 2003) estipulou a eliminação progressiva de testes em animais para fins de avaliação de

segurança de cosméticos e incluiu uma proibição de comercialização concomitante. No contexto regulatório, o uso de animais para a avaliação de segurança de cosméticos para qualquer desfecho toxicológico foi oficialmente banido na União Europeia (EU) a partir de 2013, pela Regulamentação (EC) Nº 1223/2009, independente da disponibilidade de testes alternativos (EU, 2009). Posteriormente, muitos países passaram a adotar legislações semelhantes, como Noruega, Israel, Índia, Nova Zelândia (LAQUIEZE; LORENCINI; GRANJEIRO, 2015).

Ainda assim, todos os anos estima-se que entre 500 a 1.000 novos produtos químicos são lançados e não é realista testar todos eles utilizando-se apenas ensaios *in vitro e in vivo* aceitos e regulamentados (LORENZETTI et al. 2020). Mais de 700 produtos químicos são registrados anualmente para comercialização apenas nos Estados Unidos e aproximadamente 85% não apresentam qualquer dado de saúde ou segurança (KRIMSKY, 2017). A mesma tendência é observada na Europa, onde o REACH não conseguiu gerar o número esperado de novos dados *in vivo* para avaliar o perigo de produtos químicos devido ao alto custo dos testes (KOSTAL et al., 2020; GILBERT, 2011; SOBANSKA et al., 2014). Nesse contexto, a problemática dos testes de toxicidade de produtos químicos gira em torno da necessidade de proteção à saúde humana contra efeitos adversos com métodos alternativos ao uso de animais e o grande número de novos produtos químicos lançados no mercado.

Métodos *in silico* ou computacionais são fundamentais para a avaliação da segurança de produtos químicos sem o uso de animais (WITTWEHR et al. 2020; MADDEN et al. 2020). Atualmente estes métodos são aplicados tanto para fornecer informações sobre exposição quanto para a identificação de perigo, portanto, atuam nos dois pilares da avaliação de risco. Esses métodos utilizam diversas técnicas e abordagem em relação a diferentes desfechos toxicológicos (WITTWEHR et al., 2020; MADDEN et al., 2020; MYATT et al., 2018; JOHNSON et al., 2020). Alternativas utilizando testes *in vitro* e modelos *in silico* são atualmente definidos como Novas Metodologias de Abordagem (New Approach Methodologies - NAMs) (KAVLOCK et al., 2018). No geral, os compostos indentificados como tóxicos em ensaios *in silico* devem ser priorizados para os testes *in vitro* e *in vivo*. Alguns estudos de caso ilustraram essa abordagem, como em bisfenois, resíduos de pesticidas na água, materiais de contato com alimentos, parabenos, aditivos alimentares e medicamentos, nos quais um conjunto de técnicas computacionais foi aplicado (LORENZETTI et al., 2020).

Os métodos *in silico* e *in vitro* são mais rápidos e menos caros, mas apesar disso, ainda há necessidade de maior consenso científico sobre os critérios apropriados e o nível de detalhamento exigido na avaliação e interpretação de NAMs para fins específicos. As principais críticas à utilização dessas abordagens referem-se à interpretação ainda insuficiente a nível molecular de resultados *in vitro* e extrapolações *in vivo* e às incertezas quanto aos modelos *in silico*. Adicionalmente, apesar dos grandes progressos na toxicologia molecular nas últimas duas décadas, ainda há muito desconhecimento em relação às vias moleculares envolvidas nesses processos (CHERKASOV et al., 2014; BENFENATI et al., 2011; KOSTAL et al., 2020).

As atuais lacunas de conhecimento levam à utilização de novas abordagens, como a "Via de Resultados Adversos" ou AOP (Adverse outcome pathways) para determinar a relevância dos resultados (PARISH et al., 2020). A sensibilização dérmica é um dos poucos desfechos toxicológicos para o qual um AOP já foi estabelecido e formalmente descrito . O AOP consiste em quatro eventos chave (KEs), começando com a ligação covalente de sensibilizantes a proteínas dérmicas (KE1 ou haptenação). O segundo evento chave (KE2) é a ativação de queratinócitos epidérmicose inclui respostas inflamatórias, bem como alterações na expressão gênica associadas às vias de sinalização celular específicas, como as vias dependentes do elemento de resposta antioxidante/eletrófilo (ARE). O terceiro evento chave (KE3) é a ativação de células dendríticas (DC), tipicamente avaliada pela expressão de marcadores de superfície celular específicos, transcritos genômicos, quimiocinas e citocinas. O quarto evento chave (KE4), é a ativação e proliferação de células T, que é avaliada indiretamente no ensaio de linfonodo local murino (LLNA) (OECD 442E, 2022).

A aplicação do conceito de AOPs em NAMs, relacionando modelos *in silico* e *in vitro* é uma das necessidades críticas para associar efeitos *in vivo* à eventos bioquímicos responsáveis pela toxicidade, permitindo aplicações independentes desses modelos no futuro (KOSTAL et al., 2020). Seu uso pode aumentar a confiança e reduzir significativamente os atrasos na implementação de novos métodos, possivelmente mais rápidos e eficientes para avaliar o potencial de efeitos adversos.

1.1. OBJETIVOS

1.1.1. Objetivo geral

O presente trabalho teve como objetivo gerar informações sobre a toxicidade dérmica dos desreguladores endócrinos octilfenol (OP) e do diisopentil ftalato (DiPeP), bem como avaliar uma nova estratégia integrada de teste in silico-in vitro para sensibilização dérmica. Além disso, este trabalho teve como objetivo promover um levantamento da literatura acerca da influência de variantes genéticas na susceptibilidade à imunotoxicidade dérmica de forma a contribuir futuramente com a incorporação da variabilidade populacional em NAMs.

1.1.2. Objetivos específicos

(1) Avaliar o potencial de sensibilização dérmica do OP e DiPeP por uma estratégia integrada de teste *in silico-in vitro*;
(2) Avaliar o potencial do OP e DiPeP em modular respostas inflamatórias da pele causada por outros agentes estressores;
(3) Avaliar a performance da estratégia integrada de teste in silico-in vitro para a avaliação do potencial de sensibilização dermica de desreguladores endócrinos;
(4) Avaliar o potencial de causar danos no DNA e alterações epigenéticas do OP por NAMs;
(5) Realizar uma revisão da literatura acerca das variantes genéticas que podem influenciar a susceptibilidade individual a respostas inflamatórias e imunológicas da pele causada por substâncias químicas.

2. REVISÃO BIBLIOGRÁFICA

2.1. CONTAMINAÇÃO POR OCTILFENOL E DIISOPENTIL FTALATO NA POPULAÇÃO

Contaminante emergente é o termo utilizado para definir os compostos provenientes de resíduos industriais, hospitalares, bem como de atividades agrícolas e agropecuárias ou ainda de origem natural no ecossistema (como compostos de plantas e toxinas de algas) recentemente descobertos(ou suspeitos) por apresentarem potencial genoecotóxico ou efeitos adversos aos organismos vivos, mas que ainda não estão incluídos nos programas de monitoramento de rotina (FARRÉ et al., 2008; SAUVÉ E DESROSIERS, 2014). Exemplos de contaminantes emergentes são hormônios sintéticos,anticoncepcionais, nanomateriais, sucralose, herbicidas, metais pesados e até mesmo resíduos de produtos de limpeza e de higiene pessoal, protetores solares, entre outros (FARRÉ et al., 2008; GAFFNEY et al., 2014; RODRIGUEZ et al., 2017).

O perigo iminente dos contaminantes emergentes à saúde humana e ao ambiente tem intensificado os estudos que buscam a compreensão dos efeitos adversos às substâncias (FAWELL et al., 2012; GWENZI et al., 2018).

Em um projeto piloto coordenado pelas Profa. Dra. Marta Margarete Cestari e Profa. Dra. Daniela Morais Leme e realizado pelo Laboratório de Citogenética Animal e Mutagênese Ambiental da UFPR no reservatório dorio Iraí, uma importante fonte de abastecimento urbano de Curitiba (Paraná), detectou-se a presença de contaminantes emergentes em amostra de água. Dentre os contaminantes emergentes detectados podemos citar: ibuprofeno e diclofenaco (fármacos), bisfenol e bisfenol D (ftalatos), octilfenol e nonilfenol (alquilfenóis) e estrona, estradiol e estriol (hormônios estrogênios naturais).

O octilfenol (OP) (C_{14} $H_{22}O$) é um alquilfenol (AP). Os APs são substâncias formadas por um grupamento fenólico ligado a uma cadeia carbônica (Figura 1) e são componentes básicos na produção dos alquilfenóis etoxilatos (APEs) (PRIAC et al., 2017). O OP é utilizado como intermediário na produção de resinas fenólicas para o processamento de borracha, na fabricação de pneus eem tintas (MIYAGAWA et al., 2016). OP é um conhecido desregular endócrino devido à sua atividade estrogênica (WEE E ARIS, 2017) e estudos vêm demonstrando efeitos adversos em espécies de peixes (KUMARAN et al., 2011; GENOVESE et al., 2012; ABD-ELKAREEM et al., 2018).

Em outro estudo piloto de coorte de mulheres gravidas, o “Curitiba Reproductive and Environment Study (CARES)”, realizado em Curitiba para determinar a exposição de mulheres grávidas a ftalatos e outros desreguladores endócrinos foi observada a exposição de gestantes a vários ftalatos e, mas em particular, ao diisopentil ftalato (DiPeP), também conhecido como diisoamil ftalato (BERTONCELLO SOUZA et al., 2018).

O DiPeP é um composto de 5 átomos de carbono na cadeia lateral alquil e é classificado de acordo com o regulamento da União Europeia (CE) Num. 1272/2008 como tóxico para reprodução categoria 1B (toxicidade reprodutiva humana presumida). Embora as fontes de exposição humana ao DiPeP sejam desconhecidas, suas propriedades estruturais e físico-químicas indicam que ele pode ser usado como plastificante e em muitas outras aplicações industriais de nicho (ECHA, 2012). Seu isômero, di-n-pentil ftalato (DnPeP), é conhecido como o ftalato mais potente em relação à inibição da produção de testosterona fetal em ratos, sendo cerca de 3 a 8 vezes mais potente que o DEHP e o di-n-butil ftalato (DnBP) (HANNAS et al., 2011; LIOY et al., 2015). Foi demonstrado que o DiPeP é capaz de reduzir a produção fetal de testosterona em ratos de maneira dose-responsiva e aumentar a distância testículo-bexiga, um marcador relacionado à descida testicular prejudicada (BERTONCELLO SOUZA et al., 2018). Outro estudo recente de biomonitoramento, que recrutou crianças brasileiras de 6 a 14 anos, relatou a presença de monoisopentilftalato, o metabólito monoéster de DiPeP, em amostras de urina de todos os 300 participantes do estudo (ROCHA et al., 2017). Estes estudos indicam a exposição da população ao DiPeP. Até onde sabemos, metabólitos de DiPeP não foram encontrados em estudos de biomonitoramento de outras populações mundiais, sugerindo uma ocorrência única no Brasil, o que resulta em dados limitados sobre possível toxicidade reprodutiva e endócrina de DiPeP (CURI et al., 2019). O DiPeP é o isômero do dipentil ftalato (DnPeP), um ftalato que apresenta maior atividade antiandrogênica em relação a outros ftalatos já estudados (NEUBERT-SILVA et al., 2019). Apesar de vários estudos relacionados ao desenvolvimento e atividade antiandrogênica do DiPeP, não existem estudos sobre o potencial de sensibilização dérmica dessa substância química.

3. DESENVOLVIMENTO

As sessões a seguir foram elaboradas em formato de artigos científicos, como segue:

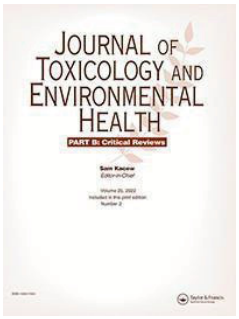
(I) Artigo de revisão crítica, publicado na revista Journal of Toxicology and Environmental Health, Part B (Fator de impacto 8.071 (2021); qualis A1);

(II) Artigo original elaborado a partir dos resultados obtidos da avaliação da toxicidade dérmica do OP;

(III) Artigo original elaborado a partir dos resultados obtidos da avaliação da toxicidade dermica do DiPeP;

(IV) Artigo original elaborado a partir dos resultados da avaliação de danos no DNA e alterações epigenéticas do OP.

3.1 CAPÍTULO I: Genetic variants affecting chemical mediated skin immunotoxicity



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Genetic variants affecting chemical mediated skin immunotoxicity

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ABSTRACT

The skin is an immune-competent organ and this function may be impaired by exposure to chemicals, which may ultimately result in immune-mediated dermal disorders. Interindividual variability to chemical-induced skin immune reactions is associated with intrinsic individual characteristics and their genomes. In the last 30–40 years, several genes influencing susceptibility to skin immune reactions were identified. The aim of this review is to provide information regarding common genetic variations affecting skin immunotoxicity. The polymorphisms selected for this review are related to xenobiotic-metabolizing enzymes (*CYPA1* and *CYPB1* genes), antioxidant defense (*GSTM1, GSTT1*, and *GSTP1* genes), aryl hydrocarbon receptor signaling pathway (*AHR* and *ARNT* genes), skin barrier function transepidermal water loss (*FLG, CASP14*, and *SPINK5* genes), inflammation (*TNF, IL10, IL6, IL18, IL31*, and *TSLP* genes), major histocompatibility complex (MHC) and neuroendocrine system peptides (*CALCA, TRPV1, ACE* genes). These genes present variants associated with skin immune responses and diseases, as well as variants associated with protecting skin immune homeostasis following chemical exposure. The molecular and association studies focusing on these genetic variants may elucidate their functional consequences and contribution in the susceptibility to skin immunotoxicity. Providing information on how genetic variations affect the skin immune system may reduce uncertainties in estimating chemical hazards/risks for human health in the future.

KEYWORDS

Inter-individual variability; polymorphisms; atopic dermatitis susceptibility; skin immune diseases; cytokine genes

Introduction

Immunotoxicity refers to adverse effects on the structure or function of the immune system, or on other systems as a result of immune system dysfunction. Immunotoxicity results from exposure to various chemicals that induce immune suppression or stimulation, leading to chronic inflammation, allergic disease, and autoimmunity (Corsini and Roggen 2017; Lee and Lawrence 2018).

The skin is a complex immune organ acting as a protective barrier against chemicals (Nguyen and Soulika 2019). Although possessing this protective feature, the skin barrier is not always effective and chemicals might penetrate through the skin, making skin prone to suffering from immunotoxicity (Poet and McDougal 2002). Environmental chemicals, cosmetics, dermally-applied drugs, or chemicals from occupational settings, which were initially thought to be safe, mediate skin immunotoxicity (Anderson and Meade 2014; Kim et al. 2021).

Chemicals trigger skin immune response by producing tissue damage and cellular stress, which release molecules such as reactive oxygen species (ROS) and proteins involved in the activation of the immune cells (Zabrodsky 2019). This process may result in activating dendritic cells, T and B lymphocytes, which influence the immune response through release of cytokines and antibody production, respectively. The immune responses triggered by chemicals might indicate an increased incidence of immune-related skin disorders, including inflammation, allergic diseases and autoimmunity. However, the mechanisms underlying various immunopathologies seem limitless, and more studies are warranted to understand the complexities of the biological system and elucidate the influences of genetics and the environment (Lee and Lawrence 2018).

Most information related to chemical-mediated effects on the skin arise from investigations of their potential to induce skin irritation and sensitization

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using *in vivo* Draize rabbit test for dermal irritation and local lymph node *assay* and *in vitro* tests such as *in vitro* skin irritation with Reconstructed Human Epidermis, KeratinoSens assay, h-CLAT assay) (Hardwick et al. 2019). In using this approach for testing chemicals, the influence of the genetic profile on toxicity is not considered. However, the human population is genetically diverse, and skin diseases are affected by individual susceptibility. Genetic variants affecting individual susceptibility to skin diseases were identified, and some of these are known to influence chemical responses in humans. Nevertheless, there is a gap in the comprehension of the influence of genetic variants on skin-mediated immunotoxicity. The aim of this review was to gather information regarding variants for genes that may confer different individual susceptibility to skin immunotoxicity initiated by chemicals. In addition, this gain of knowledge might facilitate a better understanding of the effects of chemicals on human health and providing clues for conducting specific interventions for individuals with differing susceptibilities. This review article also intended to demonstrate evidence that genetic variants influence interindividual susceptibility to chemical-induced skin effects, highlighting the impact of genetic differences on the risk of chemical exposure and the need for predictive genetic testing of individuals at risk.

Methods

The PubMed and Google scholar databases were searched for studies published between 1900 and 2020 that examined genes and their genetic variants related to both skin immunotoxicity and immunological skin disease that may have an association with chemical exposure. For that, several terms were used, isolated or combined, during the search: immunotoxicity; skin immunotoxicity; genes nomenclature; variant numbers; skin diseases associated with chemical exposure, for example, atopic dermatitis; susceptibility; and genetic variants. Only peer-reviewed articles in English were considered for this review.

Particularly to selecting the genes described in this review, gene candidates were first selected based on *in vitro* studies of skin sensitization (KeratinoSens and LuSens assays, and SENS-IS assay). In these studies, the genes selected were genes that had their expression significantly changed in the process of skin sensitization. Then, the selection of genes was refined by only choosing articles based on the considerations next described: (1) genes frequently found in human population studies and related to immunological skin diseases associated with chemical exposure such as atopic dermatitis (AD); (2) most cited genes, which were described in a considerable number of association studies; (3) finally common variants in the selected genes in any human population were chosen. Further, the selection of studies was not limited by the impact factor of journals or the study sample size, nor was the selection of population studies limited by age or gender.

Genes involved in skin immune reactions result from systemic mechanisms of action were also not considered; thus, limiting the review to skin reactions following dermally exposure to chemicals. Genes playing a role only in a particular immunological skin disease or that were involved described only in the mechanism of immunotoxicity of a particular chemical were also not included in this review. These exclusion criteria were defined considering the amount of available information that might help us to infer the contribution of genetic variants to susceptibility to skin immunotoxicity to several chemicals, as well as limit the size of the review. However, it does not mean that those genes not included in this review are not relevant genes within the context of skin disease/immunotoxicity.

To better describe the selected genes and their variants, genes were grouped into 7 categories, as follows: (1) xenobiotic metabolism; (2) oxidative stress; (3) aryl hydrocarbon receptor (AhR) pathway; (4) skin barrier function and transepidermal water loss (TEWL); (5) inflammatory processes; (6) major histocompatibility complex (MHC) and (7) neuroendocrine pathways. The role of each category of genes in the susceptibility to immunotoxicity was discussed based upon the functional consequences of the genetic variants, when molecular data were available. Both *in vivo* and *in vitro* studies addressing the molecular consequences of the genetic variants were considered. The potential

contribution of the genetic variants in susceptibility to skin immunotoxicity was demonstrated, and to illustrate this, tables were formulated.

Skin immunity

The skin is a complex dynamic organ composed of three distinct layers: epidermis, dermis, and subcutaneous fat tissue (Nguyen and Soulika 2019). The first two layers, the epidermis and dermis, play a key role in cutaneous immune responses.

The epidermis is mainly composed of keratinocytes (>90%) but also contains melanocytes and Langerhans cells (LC) (Abdo, Sopko, and Milner 2020), which are antigen-presenting cells (APCs) and take part in the skin immune system. Besides the essential role played by keratinocytes in maintaining the mechanical (Agache and Varchon 2017) and barrier functions of the epidermis, these cells also present sensor receptors able to trigger inflammatory responses, such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Baker et al. 2003; Burian and Yazdi 2018). Upon binding to metabolites of stressed cells or exogenous substances (Lai and Gallo 2008; Patel 2018), these receptors engage in signal transduction pathways that activate several proinflammatory genes (Tabas and Glass 2013). Consequently, keratinocytes express proinflammatory cytokines – small hormone-like peptides involved in autocrine, paracrine, and endocrine signaling to act as immunomodulating agents and activate other cells, inducing an immune response (Zhang and An 2007). Proinflammatory cytokines produced by activated keratinocytes, such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1), induce the expression of the intercellular adhesion molecule-1 (ICAM-1), facilitating blood cell endothelial infiltration. The interferon – γ (IFN-γ) released by T cells might mediate the upregulation of MHC class II (MHC-II) on keratinocytes surface and enable them to promote antigen presentation to $CD4^+$ T cells, as professional APCs (Black et al. 2007; Fan et al. 2003; Kim et al. 2009). In the context of tissue inflammation, keratinocytes directly activate autoreactive $CD4^+$ T cells and participate in autoimmune skin diseases, such as psoriasis (Albanesi et al. 2005; Fan et al. 2003). Keratinocytes also present antigens to $CD8^+$ T effector/memory cells (via MHC class I) in an antigen-specific manner (Black et al. 2007; Kim et al. 2009). Increased ICAM-1 and MHC-II expression on keratinocytes are also associated with many cutaneous diseases including psoriasis, AD, and delayed hypersensitivity reactions (Albanesi et al. 2005; Fan et al. 2003). In addition, keratinocytes might also express CD80 costimulatory molecule when exposed to specific allergens such as nickel chloride, oxazolone and Balsam of Peru and irritants such as treatment with IFN-γ plus 12-O-tetradecanoyl phorbol 13-acetate ester, sodium lauryl sulfate, dimethyl sulfoxide and phenol) (Wakem et al. 2000). CD80 can participate in the breaking of immunologic tolerance of the skin in AD and irritant contact dermatitis (Wakem et al. 2000). Similarly, in addition to playing a role in melanin production, melanocytes also express TLRs, MHC-II and immunoregulatory cytokines (Hong et al. 2015), indicating their function in immune system as nonprofessional APCs.

Langerhans cells (LC) are professional APCs of the epidermis that migrate to lymph nodes and stimulate T lymphocyte responses (Clayton et al. 2017; Quaresma 2019). As a dendritic cell (DC), LC might capture, process, and present antigens to naïve T cells (Klechevsky 2015). During skin sensitization, LC mobilization towards the regional lymph nodes and concomitant maturation (to DCs) are induced and regulated by cutaneous cytokines. Thus, LC process and present the captured antigen on their surface associated with MHC molecules. This complex may be subsequently recognized by naïve T cells, thus instigating clonal expansion of antigen-specific T lymphocytes and enabling development of cellular immunological memory (Divkovic et al. 2005).

Other immune cells, such as $CD4^+$ T cells and γδ T lymphocytes, are also present in the epidermis (Nguyen and Soulika 2019). $CD4^+$ T cells move to the skin from the periphery via cutaneous lymphocyte antigen (CLA) interactions with E-selectin expressed on endothelial cells, under inflammatory conditions (Kantele et al. 1999). These cells play a critical role in mediating immune responses due to the secretion of specific cytokines (Luckheeram et al. 2012; Nguyen and Soulika 2019). After interaction with antigen-MHC complex, naive $CD4^+$T cells differentiate into specific subtypes

depending mainly upon the cytokine milieu of the microenvironment – for example, T helper 1 (Th1) and T helper 2 (Th2), T helper 17 (Th17), regulatory T cell (Treg), follicular helper T cell (TFH), and T helper 9 (Th9), each with a characteristic cytokine profile. Thus, $CD4^+$ T cells carry out multiple functions, ranging from activation of the cells of the innate immune system, B cells, cytotoxic T cells, as well as suppression of immune reaction (Luckheeram et al. 2012).

γδ T lymphocytes are lymphoid cells responsible for secreting keratinocyte growth factor (KGF) and insulin-like growth factor-1 (IGF-1) to maintain keratinocyte populations and migrate to draining lymph nodes after sensing stressed keratinocytes (Nguyen and Soulika 2019). γδ T lymphocytes are not MHC-restricted and recognize soluble antigens, derived from damaged or stressed cells, or those complexed with non-classical MHC molecules (Nguyen and Soulika 2019). After skin exposure to contact allergens, γδ T cells produce high amounts of IL-17 and IL-22 – pro – inflammatory cytokines that promote IL-1β secretion by keratinocytes and enhance the inflammatory process. IL-17 producing cells might participate in inflammatory processes of several skin diseases, such as psoriasis, contact hypersensitivity (CHS), atopic contact dermatitis and AD (Lee et al. 2020).

Innate lymphoid cells (ILCs) are immune cells present on the skin surface and derived from a hematopoietic stem cell-derived common lymphoid precursor cell in the bone marrow. Although, alternative sites of development exist, such as secondary lymphoid organs (Kim 2015; Rafei-Shamsabadi et al. 2019). These cells possess multiple subsets – traditionally classified as ILC1, ILC2 or ILC3- that present different functions depending upon their transcription factor expression profile and/or expression of effector cytokines, promoting either tissue homeostasis or detrimental inflammatory processes at epithelial barrier surfaces and skin disease such as AD and psoriasis (Bielecki et al. 2021; Kim 2015; Rafei-Shamsabadi et al. 2019).

The dermis, in turn, contains fibroblasts as the primary cell type. However, its immune system presents a complex organization with a high concentration of immunocytes, such as dermal dendritic cells (dDCs), macrophages, mast cells, eosinophils, $CD4^+$ and $CD8^+$ T cells, γδ T lymphocytes and B cells (Nguyen and Soulika 2019; Tay et al. 2014).

Dermal macrophages generally originate from embryo-derived progenitors that seed the skin prenatally. Further, the second source of macrophages is the circulating monocytes expressing lymphocyte antigen 6 C (Ly6C+), which reach the skin and mature into skin-resident macrophages in a CCR2-dependent manner (Malissen, Tamoutounour, and Henri 2014). Conversely, the function of $Ly6C^+$ monocytes in circulation remains poorly defined. These monocytes might penetrate non-lymphoid organs without obligatory differentiation into macrophages and upregulate MHC class II expression with subsequent recirculation to lymph nodes, where these cells present antigens to T-cells; thus, being not only precursors of macrophages but also effector cells (Italiani and Boraschi 2014). Mature dermal macrophages display a high degree of plasticity, being able to promote or suppress inflammation in order to maintain homeostasis (Yanez et al. 2017). These cells secrete inflammatory cytokines including TNF-α, IL-1β, and IL-6 or adopt an anti-inflammatory and/or pro-repair phenotype, promoting neovascularization and secreting anti-inflammatory cytokines (Nguyen and Soulika 2019). Although the proinflammatory functions of the macrophages facilitate the resolution of inflammation during the early and short inflammatory phase, the persistence of its activity result in development of chronic inflammatory skin diseases AD (Kasraie and Werfel 2013). Mast cells enter the skin from the bone marrow as progenitors, mature locally in response to stem cell factor derived from keratinocytes and are induced to proliferate by cytokines such as IL-3, IL-4, IL-9 and IL-10 (Nguyen and Soulika 2019). These cells mediate pathogen sensing and release of antimicrobial peptides (Tete et al. 2012). In type I hypersensitivity, a reaction that occurs very rapidly (immediate hypersensitivity reaction), mast cells activated by IgE release histamine and other factors, inducing an inflammatory reaction. From this process, a late-phase reaction lasting several days may occur, and it is characterized by infiltration of tissue with eosinophils and Th2 cells. In contrast, type IV hypersensitivity is a delayed reaction mediated by Th1 cells; and in this reaction, sensitized T cells release

cytokines and chemokines, produce tissue damage that may result in illness such as contact dermatitis (Uzzaman and Seong 2012).

Eosinophils are found in blood, but normally resident in tissues (Kita 2013), such as skin, although their role in skin homeostasis is not clearly understood thus far (Nguyen and Soulika 2019). Eosinophils present granules loaded with toxic proteins such as major basic protein and eosinophil peroxidase and a variety of preformed cytokines and chemokines released in response to appropriate stimuli (Spencer et al. 2009). These cells also produce all types of prostaglandin D2 (PGD2), a lipid-derived inflammatory mediator, crucial for skin eosinophilic infiltration in hypersensitivity reactions such as AD (He et al. 2010).

The majority of mature B cells reside within lymphoid follicles of the spleen and lymph nodes and it is still unclear if B cells are present in the skin as resident cells (LeBien and Tedder 2008; Nguyen and Soulika 2019). As to T lymphocytes, B lymphocytes move to the skin tissue via CLA, and it may also be dependent upon the CCL20-CCR6 axis (Nguyen and Soulika 2019). These cells are frequently associated with delayed-type hypersensitivity reactions and produce allergen-specific IgM antibodies. The formation of these immune complexes might lead to activation of the complement cascade, resulting in T cell recruitment to the affected skin site (Tsuji et al. 2002). In addition, regulatory B cells suppress autoreactive lymphocyte activation through IL-10 production. These cells may play suppressive role in autoimmune skin diseases such as *Pemphigus vulgaris* and imiquimod-induced murine psoriasis model (Tavakolpour 2018; Yanaba et al. 2013).

In addition to the described skin resident immune cells, the skin also presents a high infiltration of non-resident immunological cells – monocytes, T cells, natural killer (NK) cells, NKT cells, and non-resident DCs (Nguyen and Soulika 2019; Tay et al. 2014). These infiltrating cells respond to signaling molecules released by skin resident cells and participate actively in skin immune reactions (Nguyen and Soulika 2019; Tay et al. 2014). The complex environment of immune-competent cells in the skin demonstrates that this organ responds to xenobiotics through innate and adaptive immunity mechanisms and is susceptible to immunotoxic effects.

A diverse range of chemicals, including fragrances, preservatives, rubber chemicals, epoxy resins, acrylates, medications, metals and many others, produce skin immune reactions. Skin irritation and skin sensitization are relatively common consequences after dermal exposure to chemicals.

Skin sensitization is a response of the adaptive immune system, in which there is a delayed T-cell-mediated allergic response to chemically modified skin proteins where haptens bind to proteins and are recognized by the immune system, termed skin sensitizers (Basketter, Darlenski, and Fluhr 2008). The activation of hapten-specific naïve T cells by haptenated LC that migrate to lymph nodes might generate effector and memory T cells (Divkovic et al. 2005). Subsequent skin contact with a sufficient dose of the chemical might elicit a skin reaction termed allergic contact dermatitis (ACD).

Skin irritation is produced by damage to epidermal cells via direct contact with the chemical substance. Acute disruption of the epidermal barrier results in the release of a preformed pool of cytokines including IL-1α, thus leading to an immediate initial irritant reaction and subsequently induction of barrier repair mechanisms (Kim et al. 2019, 2018). For example, in repeated exposure to sodium lauryl sulfate (SLS), an elevated IL-1 receptor antagonist (IL-1 RA)/ IL-1α ratio was observed, primary to arise in IL-1α RA. Fluhr et al. (2008) proposed that IL1α is derived from damaged keratinocytes during the interaction between irritant and epidermal barrier. IL1α serves as a signal to the release of chemokines CCL20 and CXCL8 that attract a variety of cells including T cells, B cells, neutrophils and immature DCs) mediating the immune response in the skin and resulting in a proinflammatory effect (Fluhr et al. 2008). In addition, irritants – such as SLS, toluene, trichloroacetic acid, sodium hypochlorite, salicylic acid, sodium hydroxide, glycolic acid and benzalkonium chloride – induce CCL21 chemokine up-regulation by dermal lymphatic endothelial cells. CCL21 may enhance the steady-state emigration of $CCR7^{+}$ LC (Eberhard et al. 2004). Skin sensitization- and irritation-derived mechanisms are generally not fully understood.

Genetic susceptibility factors potentially relevant for skin immunotoxicity

Genetic polymorphism refers to the stable coexistence of two or more distinct genotypes for a given trait in a population (Wright 2005). These variations include single nucleotide polymorphisms (SNPs) resulted from substitutions, insertions, and deletions on the DNA (Wright 2005). SNPs are found in genes with roles in the skin barrier that confer a protective effect for humans against aggressors of the external environment, such as chemical substances. Further, SNPs were also identified in genes related to skin metabolism and immunity. These genetic variations in the human population may confer differing susceptibility to immunological skin diseases and skin immunotoxicity (Costa et al. 2020, 2019; Teodoro et al. 2019). Genes related to the process of skin immunotoxicity are presented below, and their variants are discussed regarding their contribution to susceptibility to skin immunotoxicity.

Xenobiotic metabolism: phase I reactions

Xenobiotic metabolism creates a series of intermediates that may exert greater reactivity than parent substance, thus exhibiting the potential to alter biological functions and initiate serious adverse reactions (Marchant 2000). For instance, these intermediate species might be recognized as antigens, triggering immune responses in the skin. In addition, during phase I reactions, prohapten chemicals might also be metabolically activated to electrophilic species, being recognized as haptens that directly activate keratinocytes, initiating the cascade signaling for innate and adaptive immune responses (Albanesi et al. 2005; Kaplan, Igyártó, and Gaspari 2012).

Cytochrome P450 genes

The CYP enzyme superfamily is the most important class of xenobiotic-metabolizing enzymes participating in phase I reactions (Ahmad and Mukhtar 2004; Ginsberg et al. 2009a). These enzymes are highly expressed in the liver (Neafsey et al. 2009b, 2009a); however, these enzymes are also present in the skin, although their expression levels are approximately 300-fold lower than in the liver (Madden et al. 2017). In the skin, these enzymes are primarily expressed by keratinocytes, and CYP genes mainly expressed by this cell type are *CYP1A1, CYP1B1, CYP2B6, CYP2E1*, and *CYP3A* (Ahmad and Mukhtar 2004; Baron et al. 2001). CYP enzymes play a role in the metabolism of most drugs, fatty acids, eicosanoids, sterols, steroids, vitamin A, and vitamin D in the skin (Ahmad and Mukhtar 2004; Ginsberg et al. 2009a; Neafsey et al. 2009b, 2009a). Several prohaptens associated with allergic contact dermatitis act through CYP-mediated conversion to more reactive species (Bergström et al. 2007a; Ott et al. 2009).

Most members of the CYP families are polymorphic (Zhou, Liu, and Chowbay 2009), and each human CYP gene contains an average of 14.6 nonsynonymous SNPs (Wang, Li, and Zhou 2009). The allelic variants, in general, result in altered protein expression or activity, and these functional consequences exert significant effects on xenobiotic metabolism (Wang, Li, and Zhou 2009), influencing the formation of toxic intermediates and consequently affecting responses to immunotoxicants.

CYP1A1 and CYP1B1 genes

CYP1A1 and *CYP1B1* CYP genes play a key role in chemical-mediated toxicity in the skin. CYP1A1 is highly conserved among the CYP enzymes. It is known as one of the most important enzymes in bioactivation of chemicals and predominantly metabolizes exogenous substrates (Walsh, Szklarz, and Scott 2013). CYP1B1 is the most abundantly expressed P450 in human skin and presents a marked inter-individual variability in its constitutive expression (Deeni et al. 2013), making it a target for toxicity investigations addressing individual susceptibility. Both CYP1A1 and CYP1B1 enzymes are aryl hydrocarbon hydroxylases (AHH), which catalyze the conversion of xenobiotics to epoxide intermediates that are further converted to more reactive diol-epoxides with the aid of epoxide hydrolase (Shimada and Fujii-Kuriyama 2004). The expression of *CYP1A1* and *CYP1B1* genes is highly inducible by numerous xenobiotics that act as aryl hydrocarbon receptor (AhR) ligands such as methylcholanthrene, dioxins and β-naphthoflavone. *CYP1A1* and *CYP1B1* found their expression increased in response to exposure to liquor *Carbonis detergens* and some skin sensitizers,

Table 1. Variants in genes for the xenobiotic-metabolizing enzymes and their correlation with individual susceptibility to skin immunotoxicity.

Gene	Genetic variant	Aliases	Mutation	Susceptibility allele or genotype	Functional consequences	Susceptibility to skin immunotoxicity	References
CYP1A1	rs1048943	*CYP1A1*2 C*, 2454A > G	c.1384A > G/ p.Ile462Val	G	Increases enzyme activity and, consequently, the formation of reactive metabolites and ROS	↑	Zanger and Schwab 2013; Cosma et al. 1993
CYP1B1	rs10012	*CYP1B1*6*	c.142 C > G p.Arg48Gly	G	All these variants decrease enzyme activity and, consequently, decrease the formation of reactive metabolites and ROS	↓	Zanger and Schwab 2013
	rs1056827	*CYP1B1*6*	c.355 G > T p.Ala119Ser	T		↓	
	rs1056836	*CYP1B1*6*	c.1294 C > G p.Leu432Val	G		↓	

↑- increased susceptibility to skin immunotoxicity.
↓- decreased susceptibility to skin immunotoxicity.
ROS: reactive oxygen species

such as minoxidil, a therapeutic agent used to treat hair loss and carvoxime (Jäckh et al. 2012; Madden et al. 2017; Nebert et al. 2000; Neis et al. 2010).

The *CYP1A1* gene is located on chromosome 15q22-24 (Hildebrand et al. 1985), while *CYP1B1* is situated on chromosome 2p22-21 (Murray et al. 2001). To date, there are 2400 SNPs described for *CYP1A1* and 3405 SNPs for *CYP1B1* in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/, access date: October 18, 2020). Several SNPs in *CYP1A1* and *CYP1B1* were reported to influence enzyme activity. Approximately 10% of European population carry a variation on *CYP1A1* that increases formation of reactive metabolites (Marchant 2000). These reactive metabolites are recognized as antigens and activate the skin immune system (Bergström et al. 2007a). The variant *CYP1A1**2 C (rs1048943) presents a substitution from adenine to guanine at position c.1384A>G (published as 2454A>G) that leads to an amino-acid substitution from isoleucine to valine at codon 462 (Ile462Val). This substitution enhances CYP1A1 activity 2-fold (Cosma et al. 1993) and, consequently, biotransformation rate in phase I reactions (Zanger and Schwab 2013). In contrast, variants *CYP1B1**6, 142 C > G Arg48Gly (rs10012); 355 G > T Ala119Ser (rs1056827) and 1294 C > G Leu432Val (rs1056836) decrease metabolism (Zanger and Schwab 2013), consequently reducing formation of metabolites, and may represent a lower risk of immune activation and skin immunotoxicity (Table 1).

Oxidative stress: genes encoding antioxidant enzymes

At low to moderate levels, ROS act as signaling and regulatory molecules (Fang, Yang, and Wu 2002; Pham-Huy, He, and Pham-Huy 2008). Thus, ROS influence immune responses by acting as mediators (Fang, Yang, and Wu 2002; Nordberg and Arnér 2001), as well as playing a role in defense against infection due to their antimicrobial properties (Nordberg and Arnér 2001). Nevertheless, high levels of ROS might harm cells (Ji and Li 2016), and oxidative stress initiated in the skin by prooxidant chemicals may result in damages to keratinocytes, which leads to an impairment of the *Stratum corneum* (SC) and, consequently, to a defective skin barrier (Ji and Li 2016). The skin barrier integrity is fundamental to prevent the absorption/penetration of allergens, as well as TEWL and dysregulation of the immune system (Lee et al. 2020).

A variety of environmental chemicals including, formaldehyde, benzo[a]pyrene, toluene, heavy metals and textile dyes exhibit the potential to act as prooxidants in the skin, significantly increasing the levels of ROS (Bergström et al. 2007a; Kwon, Kim, and Lee 2020; Leme et al. 2018), which exacerbate dermal immune responses (Abolhasani et al. 2021). Air pollutants, such as volatile organic compounds (VOCs), nitrogen dioxide (NO_2), particulate matter (PM), carbon monoxide (CO), nitric oxides (NO_x), sulfur oxide (SO_2) and ozone (O_3),

are well recognized as skin prooxidants and adversely impact skin-related disorders, especially in the context of large metropolitan areas (Abolhasani et al. 2021). High levels of ROS are also generated by the Fenton reaction when considering exposure to heavy metals through the generation of ferrous iron (Fe^{2+}) (Knight 2000). In addition, chemicals impair enzymes involved in cellular antioxidant defense including superoxide dismutase, catalase, peroxidase, glutathione transferase, glutathione peroxidase thus increasing ROS levels.

The ROS-mediated damages induced in the skin may be sensed by the pattern recognition receptors (TLRs and NLRs) present in keratinocytes (Biswas 2016). These pattern recognition receptors then initiate a signal transduction pathway, activating genes through the action of transcription factors, such as nuclear factor kappa B (NF-κB). NF-κB is intrinsically related to the activation of genes related to production of antioxidant enzymes, as well as genes of inflammatory cytokines and chemokines (Corsini et al. 2013; Tabas and Glass 2013). Chemokines act as chemoattractants to guide the migration of several immune cells mainly leukocytes, but also lymphocytes, DCs and NK cells. Chemokines also promote angiogenesis and are involved in the maturation, differentiation and activation of lymphocytes and DCs, which may facilitate skin sensitization. Excessive chemokine activation and synthesis are hallmarks of psoriasis and AD (Nedoszytko et al. 2014).

Although activation of immune responses after excessive ROS is a protection mechanism against antigens, it may also generate additional ROS and result in apoptosis and collateral tissue damage (Nedoszytko et al. 2014; Tabas and Glass 2013). The balance of ROS levels in a biological system is strictly controlled by the antioxidant defense system, in which several antioxidant enzymes participate (Amir Aslani and Ghobadi 2016). Polymorphisms in the genes of antioxidant enzymes may confer interindividual variability to antioxidant defense against endogenous and exogenous prooxidant agents, and consequently, may confer differing susceptibility to effects attributed to skin toxicants.

Glutathione S-transferase (GST) genes

The contribution of *GST* gene polymorphisms to the susceptibility to chemical toxicity is related to the role of GST enzymes in the antioxidant defense and inflammation signaling pathways regulating intracellular signal transduction events (Cho et al. 2001; Ginsberg et al. 2009b; Soga, Matsuzawa, and Ichijo 2012). Some GST forms act as glutathione peroxidases, while others exhibit non-enzymatic functions regulating many cellular processes that contribute to the intrinsic ability of cells to survive from oxidative stress (Chiarella 2019). These enzymes detoxify a wide range of structurally different substrates produced through oxidative stress such as peroxides produced from lipids and catecholamines (Berhane et al. 1994; Caccuri et al. 2001).

Individuals with a low level of GST activity display a greater risk of developing skin immune diseases related to xenobiotic exposure than those with high level of GST activity (SheeSheehan et al. 2001). Thus, *GST* variants play an important role in inflammatory skin diseases associated with oxidative stress and cell apoptosis attributed to xenobiotic exposure. This role is demonstrated in studies of arsenic (As) poisoning (McCarty et al. 2007), asthma presenting atopy phenotype (Fryer et al. 2000), allergic contact dermatitis (ACD) (Bertino et al. 2020; Chung, Oh, and Shin 2009), lichen planus (LP) (Cilingir et al. 2018), subacute cutaneous lupus erythematosus (SCLE) (Sontheimer 1989) and chemically-induced or contact/occupational vitiligo (Chiarella 2019).

There are 5 subclasses of the GST superfamily (alpha, pi, mu, theta, and zeta) (Board et al. 1997). GSTM (mu), GSTT (theta), and GSTP (pi) are polymorphically distributed in the human population. The *GSTM1* locus is located on chromosome 1p13.16 (Pearson et al. 1993), and is highly polymorphic, usually presenting homozygous deletion. *GSTT1* maps to chromosome 22q11, and is also frequently deleted (Webb et al. 1996), while *GSTP1* is located on chromosome 11q13 (Ballerini et al. 2003). The inherited homozygous deletions of *GSTM1* and *GSTT1* genes are associated with deficiency of the antioxidant system attributed to absence of both functional enzymes. The homozygous deletion of *GSTM1* (rs4025935) is present in 50% of the European population, while the

homozygous deletion of *GSTT1* (rs71748309) occurs in 10–38% of various ancestries (To-Figueras et al. 2000). Both were associated with an increased risk of toxicity in response to xenobiotics (Hayes and Strange 2000; Strange, Jones, and Fryer 2000).

A potential role of null alleles of *GSTM1* and *GSTT1* in autoimmunity was suggested by Ollier et al. (1996) as this enables accumulation of ROS, resulting in cell death by apoptosis. Apoptosis is associated with skin autoimmunity because it allows keratinocytes to express surface blebs rich in autoantigens, such as the Ro antigen, that trigger production of anti-Ro antibody. Anti-Ro antibody is a type of anti-nuclear autoantibody associated with many autoimmune diseases (Millard, Fryer, and McGregor 2008). Apoptosis, under this context, may lead to a breakdown of the immune tolerance to Ro proteins or an exposure of Ro proteins to circulating anti-Ro antibodies results in autoimmunity (Millard, Fryer, and McGregor 2008; Ollier et al. 1996). Subacute cutaneous lupus erythematosus (SCLE) is an autoimmune skin disease associated with this biological process (Casciola-Rosen and Rosen 1997; Sontheimer 1989). SCLE presents outbreaks of erythematous, annular and/or papulosquamous lesions in a characteristic distribution and presenting Ro/SS-A autoantibodies and granular deposition of IgM, IgG and C3 in a band-like array at the dermal-epidermal junction (Lowe et al. 2011). SCLE was already induced by chemicals that induce ROS, including psoralen with UVA and environmental exposure to hydrazine-containing insecticides (Lowe et al. 2011; Shapiro et al. 2004).

GSTM1 and *GSTT1* null genotypes appear to be associated with a higher risk of LP (Cilingir et al. 2018). LP is a T-cell mediated autoimmune disease with typical clinical presentation of pruritic faintly erythematous to violaceous, flat-topped, polygonal papules distributed mainly over the flexural areas of wrists, arms, and legs. The immunopathogenesis of LP is associated with altered CD8 + T cells, which start to recognize keratinocytes, killing these cells. CD4 + T cells and Langerhans CD1a+ cells and keratinocytes are targets for T-cell mediated destruction via Fas-1 [activating ligand for FAS], perforins and granzyme B (Tas and Altinay 2014). The development of LP after chemical exposure is associated with the concept of antigen mimicry – with the activation of T- or B- cellular immunity with respect to certain cutaneous structures which are similar or analogous to exogenous substances or secondarily and endogenously originated structures (Tas and Altinay 2014). LP was previously associated with exposures to toluene, arsenic, mercury, mustard oil containing allyl-thiocyanate (a photosensitizer), amla oil, henna, hair dyes, color film developing agents derived from p-phenylenediamine (PPDA) (*e.g.*, CD-2 and CD-3) and environmental chemicals (Altman, Perry, and Perry 1961; Lidén 1986; Robles-Méndez et al. 2017; Tas and Altinay 2014). Higher NO serum levels and lipid peroxidation in patients with LP compared to healthy individuals were found, indicating dysfunction of the antioxidant defense system (Aly and Shahin 2010; Sezer et al. 2007). However, the underlying mechanisms involved in LD and the precise role of oxidative stress in this pathogenesis are still unclear.

GSTM1 and *GSTT1* null genotypes are also associated with vitiligo (Chiarella 2019). Vitiligo is a depigmenting disorder as a result of melanocyte loss, and its etiology is still unclear, although several etiologic hypotheses have been raised. A single dominant pathway in the disease seems unlikely to account for all cases of melanocyte loss, and various etiological factors together may be responsible for functioning melanocytes disappearing from the epidermis (Mathachan et al. 2021). Melanogenesis, the process of pigment melanin production, involves oxidative reactions with generation of some types of ROS (superoxide anion and hydrogen peroxide (H_2O_2) that may have undesirable consequences to the epidermis if the antioxidant enzymes did not adequately degrade ROS. Incomplete H_2O_2, degradation, may lead to dysfunction of the Nrf2-p62 pathway, inhibiting the antioxidant defense system and increasing susceptibility of melanocytes to cytotoxic effects of H_2O_2. Further, H_2O_2 might disrupt melanin synthesis by modifying the binding site of the cytosolic enzyme dihydro pteridin reductase, which leads to an altered biopterin synthesis and recycling – a cofactor for phenylalanine-4- hydroxylase and tyrosine hydroxylase (Hasse et al. 2004). ROS also disrupt melanin synthesis by inhibiting tyrosinase, the enzyme involved in melanin production

(Hwang and Lee 2007; Mathachan et al. 2021). High levels of H_2O_2 also stimulate the expression of TRPM2 (Transient Receptor Potential Cation Channel Subfamily M Member 2), increasing mitochondrial calcium influx and then facilitating mitochondria-dependent apoptosis of melanocytes (Wang, Li, and Li 2019).

Several chemicals that generate ROS were reported as vitiligo promoters including catechols, quinones and their derivatives, sealants/adhesives (4-tert-amylphenol,4- tert-butylphenol), rhododenol present in skin lightening cosmetics, para- phenylenediamine (PPD) found in hair dyes and imiquimod (Allam and Riad 2013; Harris 2017). However, mechanisms for development of vitiligo by these chemicals remains to be elucidated.

In *GSTP1* gene, a non-synonymous amino acid substitution from isoleucine to valine at codon 105 (Ile105Val), (A > G) at position 313 of exon 5 (rs1695) (Ali-Osman et al. 1997; Moyer et al. 2008), either increases or reduces the catalytic activity of GSTP1 depending upon the type of substrate. Non-synonymous amino acid substitution occurs because the Val105 allele results in a steric restriction of the H-site due to shifts in the side chains of several amino acids and possibly accommodates less bulky substrates than Ile105 alloenzyme. As a result, the Val105 displays substrate specificities differ from Ile105 (Ali-Osman et al. 1997). Thus, in rs1695, it is challenging to determine which one is the risk allele, and Val105 may be either protective or a risk factor to allergy. For instance, homozygosity for *GSTP1* Val105 was less common in individuals positive for skin prick test with 7 common aeroallergens house dust mite, house dust, grass mix, tree pollen, cat fur, dog fur, feathers than in those negative for this test, was associated with high ROS generation of Ile105 and a protective effect of Val105 (Millard, Fryer, and McGregor 2008). However, the low enzyme activity of Val105 may favor accumulation of ROS, which explains why this variant is also associated with higher susceptibility to allergy (airway inflammation in atopic asthmatics and AD) reported in several studies (Chung, Oh, and Shin 2009; Hoskins et al. 2013). However, in Russian children, *GSTP1* heterozygous Ile105/Val105 was associated with a lower risk of AD, while both homozygotes were significantly higher in AD patients than in the controls (Safronova et al. 2003).

Oxidative stress is commonly associated with AD, which is an inflammatory skin disease characterized by chronic or relapsing eczematous lesions and pruritus (Leung and Bieber 2003). The inflammation in AD might result from activation of the NF-κB pathway under the induction of oxidative stress. Previously Nedoszytko et al. (2014) noted that NF-κB mediates the expression of proinflammatory cytokines that enhance dermal inflammatory infiltrate and histamine release in the affected skin. The association of xenobiotics with AD development and aggravation is attributed to their capacity to generate oxidative stress in the SC and induce oxidative protein damage, resulting in skin barrier dysfunction and AD aggravation (Ji and Li 2016). Skin barrier defects also play a role in chronicity of AD inflammation (Bertino et al. 2020). Comparative proteomic profiling demonstrated that proteins from the skin barrier (filaggrin-2, corneodesmosin, desmoglein-1, desmocollin-14, and transglutaminase-3) exhibited low expression in lesions of AD patients (Broccardo et al. 2011). Further, an analysis of skin biopsies from AD patients demonstrated that oxidative damages in proteins were higher in the outermost layer of the epidermis (SC) than in the other epidermal layers, suggesting that environmental oxidant chemicals produce marked damage in SC of AD lesions (Niwa et al. 2003). Several urinary biomarkers of oxidative stress, including 8-hydroxy-2′-deoxyguanosine (8-OHdG), nitrite/nitrate and selenium, were found to be higher in children with AD than in normal children (Bertino et al. 2020; Omata et al. 2001). Urinary excretion of oxidative metabolites glycosylation end-products and bilirubin were also significantly elevated in children with AD (Tsukahara et al. 2003), and the blood level of lipid peroxidation was also significantly higher in both children and adults with AD (Amin et al. 2015; Chung, Oh, and Shin 2009). These findings suggest that AD patients display a lower antioxidant defense activity, and thus genetic variants that confer functional consequences decreasing the activity of antioxidant enzymes may be predisposing factors for AD.

Genetic variants on the *GSTP1* gene were also examined under the context of arsenic (As) exposure. *GSTP1* homozygotes for Val105 (GG genotype) compared to homozygotes for Ile105 (AA genotype) were associated with enhanced risk for As-related skin lesions, which was associated with low enzyme activity of Val105 homozygotes (McCarty et al. 2007). In contrast, the *GSTT1* null genotype was associated with a lower risk of As-induced toxicity than the *GSTT1* genotype with functional alleles, indicating that the role of GSTT1 enzyme activity in As-mediated toxicity requires additional investigations (McCarty et al. 2007) (Table 2).

Aryl hydrocarbon receptor (AhR) signaling pathway

Aryl hydrocarbon receptor (AhR) is a cytosolic ligand-activated transcription factor expressed in some cells of the skin, such as keratinocytes, fibroblasts, melanocytes, LC, as well as lymphocytes (Akintobi, Villano, and White 2007; Jux, Kadow, and Esser 2009; Jux et al. 2011; Tauchi et al. 2005; Zhou 2016). In healthy skin, AhR signaling driven by endogenous ligands tryptophan contributes to keratinocyte differentiation, skin barrier function, melanocyte homeostasis, skin pigmentation, and mediates protection against oxidative stress by participating in activation of antioxidant enzyme genes (Esser et al. 2013; Furue et al. 2015; Haarmann-Stemmann, Esser, and Krutmann 2015; Quintana et al. 2008; Van Den Bogaard et al. 2015).

AhR activation is also associated with inflammatory responses in the skin, regulating immunity by mediating T-cell differentiation such as Treg and Th17 cells and production of several cytokines (Luecke et al. 2010; Prigent et al. 2014; Quintana et al. 2008). AhR activation also represents a critical sensor regulating LC activation and LC-mediated T cell polarization (Hong et al. 2020). Therefore, the aberrant expression of AhR is associated with chronic inflammatory skin diseases, such as AD, psoriasis, vitiligo (Haarmann-Stemmann, Esser, and Krutmann 2015), and AD-like phenotypes with pruritus (Tauchi et al. 2005; Van Den Bogaard et al. 2015).

Exogenous substances bind to AhR and induce immune responses through activation of this receptor (Omiecinski et al. 2011), resulting in ROS production and dysregulation of proteins related to cell

Table 2. Variants in genes for the antioxidant enzymes and their correlation with individual susceptibility to skin immunotoxicity.

Gene	Genetic variants	Aliases	Mutation	Susceptibility allele or genotype	Functional consequences	Susceptibility to skin immunotoxicity	Association with skin condition or disorder	References
GSTM1	rs4025935	*null GSTM1*	g.4024_4025del	delGT	Defectiveness in the antioxidant system	↑	SCLE, lichen planus, vitiligo	Casciola-Rosen and Rosen 1997; Sontheimer 1989;Cilingir et al. 2018; Chiarella 2019.
GSTT1	rs71748309	*null GSTT1*	Large deletion		Defectiveness in the antioxidant system	↑	SCLE,lichen planus, vitiligo	Casciola-Rosen and Rosen 1997; Sontheimer 1989;Cilingir et al. 2018; Chiarella 2019; Ollier et al. 1996.
GSTP1	rs1695	-	c.313A > G p. Ile105Val	-	Can either increase or decrease the catalytic activity of GSTP1, depending on the type of substrate	Highly dependent on the chemical nature	AD, arsenic-related skin lesions	Millard, Fryer, and McGregor 2008; Hoskins et al. 2013; Chung, Oh, and Shin 2009

↑- increased susceptibility to skin immunotoxicity.
↓- decreased susceptibility to skin immunotoxicity.
AD: atopic dermatitis; SCLE: Subacute cutaneous lupus erythematosus.

division, differentiation, migration, and apoptosis (Haarmann-Stemmann, Esser, and Krutmann 2015; Napolitano and Patruno 2018). The activation of AhR-mediated transcription in keratinocytes leads to inflammatory skin lesions in mice, immunological imbalance, and may be associated with AD and CHS to polycyclic aromatic hydrocarbons (PAHs) (Tauchi et al. 2005). AhR activation by air pollutants was also associated with skin inflammation in a mouse model (Hidaka et al. 2017).

On the other hand, the lack of AhR activity might protect keratinocytes upon exposure to xenobiotics since AhR induce the expression of CYP enzymes, and in the case of loss of activity, metabolism of chemicals might not be activated, preventing generation of ROS and reactive metabolites (Omiecinski et al. 2011; Vogel et al. 2020). Nevertheless, after introducing ROS inducers, the interaction of AhR and nuclear factor erythroid 2-related factor 2 (Nfr2) activates several cytoprotective genes, displaying AhR activity which is also important in reducing the chemical-mediated toxicity (Dietrich 2016; Haarmann-Stemmann et al. 2012; Tsuji et al. 2012). In this way, AhR activity alone cannot explain the toxicity attributed to a chemical. The consequences of AhR signaling may also depend upon canonical and non-canonical pathways (Fujii-Kuriyama and Mimura 2005; Omiecinski et al. 2011).

The AhR canonical pathway occurs upon a ligand binding to cytosolic AhR, with subsequent translocation to the nucleus. Then AhR dimerizes with its homolog (AhR nuclear translocator (ARNT), which activates DNA-responsive elements termed xenobiotic-response elements (XRE) and induces or represses expression of a wide range of genes. These genes are involved in cell growth, cell differentiation and phase I and phase II xenobiotic metabolism such as *CYP1A1, CYP1B1* and *UGT1A1* (Fujii-Kuriyama and Mimura 2005; Omiecinski et al. 2011). After xenobiotic ligand binding, AhR canonical signal may become dominant and lead to reactive intermediates, anti-apoptosis signaling, pigmentation, aging, and cancer (Esser et al. 2018; Haarmann-Stemmann, Esser, and Krutmann 2015; Hidaka, Fujimura, and Aiba 2019; Parrado et al. 2019; Schurman et al. 2020).

The non-canonical signaling occurs via other cascades, including proinflammatory and immune-modulatory pathways, such as the epidermal growth factor receptor (EGFR) and downstream mitogen-activated protein kinases, NF-κB, signal transducer and activator of transcription (STATs) (Haarmann-Stemmann, Esser, and Krutmann 2015). In inflamed skin, high levels of AhR non-canonical signaling are responsible for impairing keratinocyte differentiation and skin barrier, stimulating cytokine expression and producing oxidative stress and hypopigmentation (Haarmann-Stemmann, Esser, and Krutmann 2015; Kerkvliet 2009; Parrado et al. 2019; Vogel et al. 2014). In contrast, in healthy skin, canonical and non-canonical signaling are tightly balanced (Haarmann-Stemmann, Esser, and Krutmann 2015). Thus, depending upon canonical and non-canonical AhR signaling conditions, AhR agonists or antagonists may induce or prevent inflammation and diseases (Haarmann-Stemmann, Esser, and Krutmann 2015; Parrado et al. 2019; Vogel et al. 2014).

The toxicity attributed to AhR ligands is also time-dependent. It seems that endogenous ligands for AhR are generally rapid and transient inducers and may not be associated with skin inflammation. As an example, AhR high-affinity ligand 6-formylindolo[3,2-b]carbazole (FICZ) induces transcription of *CYP* genes in keratinocytes, is rapidly metabolized by CYP1A1 or CYP1B1, and not associated with inflammation (Hidaka et al. 2017; Wincent et al. 2009). In contrast, various investigators indicated that xenobiotics, which prolonged activation of AhR due to high levels of AhR agonists led to enhanced inflammatory responses and may be responsible for pathological outcomes (Bock 2019). An example is the non-degradable ligand PAH 7,12-dimethylbenz[a]anthracene, which induce AhR target genes (*e.g., ARNT*), inducing pruritus (Hidaka et al. 2017; Murota et al. 2012). Differences in binding affinities of these xenobiotics may also reflect in severity of toxicity (Sorg 2014). Chemical substances that are partially bound to AhR such as ketoconazole inhibit activation of this receptor, reducing metabolic activation of chemicals and initiating Nrf2 antioxidant defense, which subsequently scavenge reactive metabolites (Haarmann-Stemmann et al. 2012).

AhR ligands include air pollutant chemicals particulate matter (PM), *Licor carbonis* detergents used to treat itching, scaling, and flaking of several skin disorders and persistent organic pollutants of environmental concern that are predominantly minor by-products of burning or various industrial processes (Carpenter 2011; Christos and Ju 2013; Larsson et al. 2018; Van Den Bogaard et al. 2013). Industrial unwanted minor components of mixtures, such as polyaromatic chlorinated hydrocarbons dibenzaparadioxins, dibenzofurans, biphenyls, polybrominated compounds, and organometallic compounds organomercury, organotin, and organolead, are AhR ligands (Christos and Ju 2013; Larsson et al. 2018; Van Den Bogaard et al. 2013).

The relationship between AhR activation and skin sensitization led to evaluation of *AHR* expression in skin sensitization testing strategies such as KeratinoSens and LuSens assays. These are cell-based reporter gene assays that mimic keratinocyte activation and differentiate sensitizers from non-sensitizers according to the activation of the cytoprotective Nrf2 system after chemical exposure (Natsch and Emter 2015).

AHR gene and other associated genes

The human *AHR* gene is located on chromosome 7p21.1 (Le Beau et al. 1994). *AHR* polymorphisms occur predominantly in exon 10, a region that encodes a significant portion of the transactivation domain of the receptor responsible for regulating expression of other genes (Harper et al. 2002). The polymorphism at codon 554 (c.1661 G > A) (Arg554Lys) (rs2066853) is the most widely studied in the *AHR* gene (Ren et al. 2019). The Lys554 allele is associated with lower levels of AhR and *ARNT* mRNA expression (Helmig et al. 2011; Ren et al. 2019). The complex AhR-ARNT is essential for activating several genes in the canonical pathway, which involves activation of genes that participate in detoxification mechanisms or phase I and phase II of the xenobiotic metabolism. In this way, *AHR* polymorphisms also influence expression of AhR target genes, including CYP1A1. Heterozygous Arg554/Lys554 or homozygous Lys554/Lys554 individuals in rs2066853 possess increased CYP1A1 activity in lymphocytes, as compared to homozygous Arg554/Arg554 individuals (Smart and Daly 2000). Significant induction of CYP1A1 activity may elevate formation of secondary metabolites and trigger an immune response.

The rs10249788 c.-742 C > T SNP is a noncoding transcription variant located in the *AHR* gene promoter (Li et al. 2019). The promoter region of *AHR* lacks TATA and CCAAT boxes. However, it possesses several putative SP1 transcription factor (SP1) binding sites within a highly GC-rich region (Eguchi et al. 1994), which regulate transcription of genes involved in cell growth and differentiation, apoptosis, and immune responses (Tan and Khachigian 2009). SP1 dominates the maximal constitutive activity and basal expression of the *AHR* gene via binding to these GC-rich motifs (Racky et al. 2004). Any abnormal interaction between SP1 and *AHR* promoter gene is responsible for changes in *AHR* gene expression (Englert et al. 2012). The rs10249788 is situated proximal to a binding site of SP1 (Wang et al. 2015), and the T allele is described as promoting AhR transcriptional activity through facilitating interaction of the promoter with SP1 and providing higher *AHR* expression, compared with the C allele (Wang et al. 2015). The C allele is considered a risk for vitiligo because low *AHR* expression might influence regulation of melanogenic factors, such as tyrosine, and lead to increased number of toxic metabolites and subsequent melanocyte apoptosis in vitiligo (Jimbow et al. 2001; Luecke et al. 2010; Wang et al. 2015).

The combination of rs10249788 (CT + TT) and rs2066853 (AG + AA) genotypes are associated with severely dry skin in patients with AD (Li et al. 2019). As indicated previously, AhR signaling is also essentially involved in keratinocyte differentiation, epidermal stratification, and skin barrier integrity. In part, it occurs because AhR controls the gene expression of filaggrin (FLG), a structural protein with a critical role in developing and maintaining the skin barrier (Li et al. 2019). Polymorphisms that affect AhR function may result in low FLG expression and impair skin barrier function, consequently elevating TEWL, providing a dry skin phenotype (Li et al. 2019). FLG deficiency is also responsible for higher hapten percutaneous penetration because of its structural function in the skin barrier (Kawasaki et al. 2012; Moniaga et al. 2010). Thus, polymorphisms in *AHR*

Table 3. Genetic variants in the Aryl hydrocarbon receptor (AhR) signaling pathway and their correlation with individual susceptibility to skin immunotoxicity.

Gene	Genetic variants	Aliases	Mutation	Susceptibility allele or genotype	Functional consequences	Susceptibility to skin immunotoxicity	Association with skin condition or disorder
AHR	rs2066853	c.1661 G > A p. Arg554Lys	A	Located at the transactivation domain, it is responsible for lower *AHR* levels and induction of CYP1A1 activity	↑	Severe dry skin in patients with AD (the combined rs10249788 (CT + TT) and rs2066853 (AG + AA) genotypes), skin aging	Helmig et al. 2011; Li et al. 2019; Gao et al. 2016; Schnass et al. 2018.
	rs10249788	g.17298523 C > T (Upstream transcript variant)	T	Located at the promoter region, it promotes *AHR* transcriptional activity	↑	Dry skin (the combined rs10249788 (CT + TT) and rs2066853 (AG + AA) genotypes), AD, vitiligo.	Li et al. 2019; Wang et al. 2012
ARNT	rs11204735	g.150869191 T > C (Intron variant)	C	-	↑	All these variants are associated with psoriasis and Scleroderma	Schurman et al. 2020
	rs1889740	g.150827279 C > T (Intron variant)	T	-	↑		

↑- increased susceptibility to skin immunotoxicity.
↓- decreased susceptibility to skin immunotoxicity.

have been associated with AD, vitiligo, and skin aging (Gao et al. 2016; Li et al. 2019; Schnass et al. 2018; Wang et al. 2012) (Table 3).

ARNT, a gene encoding a protein that forms a complex with ligand-bound AhR, also presents genetic variants that influence immune responses in the skin (Mimura et al. 1999). The intron variants rs11204735 and rs1889740 SNPs in this gene may contribute to development of psoriasis and scleroderma, which are autoimmune disorders of the skin (Schurman et al. 2020) (Table 3). Psoriasis is a chronic inflammatory condition of the skin characterized by extensive (lymph)angiogenesis presenting symptoms of dry red skin lesions covered by silvery scales that may bleed or itch, in the most common forms of psoriasis (Benhadou, Mintoff, and Del Marmol 2019). This skin disorder results from constant stimulation of T cells by antigens presented in the skin (Krueger 2002), and cytokines released by activated T cells seem to be responsible for initiating and maintaining psoriasis, in which keratinocyte proliferation is verified (Valdimarsson et al. 1986).

Scleroderma is a fibrotic condition characterized by vascular injury and increased accumulation of extracellular matrix proteins in the skin (Yamamoto 2008). Although the underlying mechanisms behind development of scleroderma are yet not completely understood, evidence suggests that overproduction of extracellular matrix by fibroblasts results from complex interactions among endothelial cells, lymphocytes, macrophages and fibroblasts with involvement of several mediators including cytokines, chemokines, and growth factors (Yamamoto 2008). Scleroderma has been associated with human exposure to xenobiotics, such as silica, organic solvents, formaldehyde, epoxy resins and vinyl chloride (Bovenzi et al. 2001).

Thus, the critical role of AhR in mediating xenobiotic toxicity and the association of its activation with altered immunological responses indicate that polymorphisms in *AHR* and related genes, such as *ARNT*, might contribute to development of skin immune responses.

Skin barrier function and TEWL abnormalities

Through a step-wise process of differentiation, keratinocytes form a physical barrier. The structure and function of this barrier are largely dependent on the SC, which is the outermost layer of the epidermis (Fujii 2020). The SC consists of terminally differentiated keratinocytes (called corneocytes) and intercellular lipids, which provide a physical and hydrophobic barrier. Tight junction (TJ) proteins seal adjacent keratinocytes in the *stratum granulosum* (SG), acting as a second barrier beneath the SC (Fujii 2020). The formation of the SC barrier occurs in terms of the following 5 categories: 1) FLG metabolism; 2) cornified envelope; 3) intercellular lipids; 4) corneodesmosome, and 5) corneocyte desquamation (Egawa and Kabashima 2018).

The skin barrier reduces TEWL and protects the skin from exogenous substances. Dysfunctions in the permeability of the skin barrier increase TEWL (Rupec, Boneberger, and Ruzicka 2010) and

enhance dermal absorption of xenobiotics (Egawa and Kabashima 2018). Consequently, chemical substances readily interact with local immune cells, probably triggering immune responses, which may ultimately result in skin inflammation (Egawa and Kabashima 2018). Thus, skin barrier dysfunction is one of the most relevant predisposing factors for developing immune responses in the skin (Lee et al. 2020). In addition, skin barrier dysfunction is associated with an elevated risk of developing several immune diseases, including asthma, allergic rhinitis, and it is a hallmark of AD (Egawa and Kabashima 2018; Rupec, Boneberger, and Ruzicka 2010; Spergel and Paller 2003). Therefore, genes whose products are involved in any of the developing and maintaining processes of the skin barrier may affect the integrity and function of the barrier, resulting in greater susceptibility to chemical toxicity.

Filaggrin (FLG) gene

FLG gene is located on chromosome 1q21.3 that codes for FLG (Osawa, Akiyama, and Shimizu 2011), an abundant protein in the differentiated epidermis. *FLG* gene has more than 40 mutations that result in skin barrier abnormalities (Osawa, Akiyama, and Shimizu 2011). Common loss-of-function mutations are carried by approximately 9% of the European population and are the major predisposing factors for AD (Palmer et al. 2006). The mutations R501X, R2447X (both nonsense mutations), and 2282del4 are frequently associated with dry skin conditions and allergic AD in European children and adolescents (Böhme et al. 2012; González-Tarancón et al. 2020; Lagrelius et al. 2020; Palmer et al. 2006). The SNPs G > A (rs3126085), C > T (rs12144049), T > G (rs471144), T > C (rs4363385) are intron variants in the *FLG* gene, exert transcriptional repression effects (Belyaeva et al. 2020) and may affect FLG metabolism. These SNPs were associated with AD by genome-wide association studies of Asian and European populations (Baurecht et al. 2015; Schaarschmidt et al. 2015; Sun et al. 2011) (Table 4).

The implications of *FLG* mutations in immune-related health outcomes are related to the crucial role of FLG polymer (profilaggrin) in the SG and, at the transition from SG to SC, when it is cleaved by proteases resulting in FLG monomers (Leyvraz et al. 2005; Matsui et al. 2011). In general, these mutations result in abnormally short proFLG molecules, resistant to cleavage, which impair formation of FLG monomers.

The FLG monomers take part in SC formation. In this process, FLG monomers bind to keratin filaments, aggregating them into keratin fibrils organized in parallel bundles to form a matrix that provides rigidity to the overall structure, which are the major constituents of corneocytes (Egawa and Kabashima 2018; Norlén and Al-Amoudi 2004). Corneocytes generate a network within a lipid-rich extracellular matrix and produce compaction of keratinocytes. In the process of compacting keratinocytes, corneocytes are denucleated and flattened, and the intercellular space between them filled with lipids from the lamellar bodies (Egawa and Kabashima 2018). Lamellar bodies are membrane-circumscribed granules produced by keratinocytes from SG and contain lipids, corneodesmosin, and kallikreins (Egawa and Kabashima 2018). These lipids are mainly ceramides, free fatty acids, and cholesterol. The secretion of the content of lamellar bodies into the extracellular space enables the covalent attachment of o-hydroxylated ceramides and fatty acids to cornified envelope proteins, forming a lipid-bound envelope (Hill, Paslin, and Wertz 2006). Then, lysosomal enzymes, which need an acidic pH optimum, degrade the polar lipid precursor to hydrophobic ceramides, generating an intact permeability barrier, and is responsible for the acidic pH of the skin (Doering et al. 1999).

The maintenance of the acidic pH of the skin is possible because FLG is proteolytically cleaved by caspase-14 into amino acids, which are further deaminated into polycarboxylic acids, such as *trans*-urocanic acid (*trans*-UCA) and pyrrolidine carboxylic acid (PCA) (Kezic et al. 2011). PCA is an essential constituent of natural moisturizing factors (NMFs), responsible for retaining water in the SC (Kezic et al. 2011). Both FLG metabolites act as osmolytes, drawing water into corneocytes and accounting for corneocyte hydration (Kezic et al. 2011; Matsui et al. 2011). The hydration, moisturization, and maintenance of acidic pH in the skin are essential for the activity of multiple enzymes that control desquamation, lipid synthesis, and

Table 4. Variants in genes involved in the skin barrier and transepidermal water loss and their correlation with individual susceptibility to skin immunotoxicity.

Gene	Genetic variants	Aliases	Mutation	Susceptibility allele or genotype	Functional consequences	Susceptibility to skin immunotoxicity	Association with skin condition or disorder	References
FLG	rs61816761	R501X	c.1501 C > T p.Arg501Ter	T	All these *FLG* variants represent loss- of-function mutations	↑	All these variants are associated with dry skin, higher transepidermal water loss (TEWL) and AD	Lagrelius et al. 2020; Palmer et al. 2006
	rs558269137	2282del4	c.2282_2285 delCAGT p.Ser761fs	delACTG		↑		
	rs138726443	R2447X	c.7339 C > T p.Arg2447Ter	T		↑		
	rs3126085	-	g.152328341 G > A (Intron variant)	A	All these *FLG* variants repress *FLG* transcription and impaired the skin barrier	↑	All these variants are associated with AD	Belyaeva et al. 2020; Schaarschmidt et al. 2015; Baurecht et al. 2015; Sun et al. 2011
	rs12144049	-	g.152468434 C > T (Intron variant)	T		↑		
	rs471144	-	g.152481779 T > G (Intron variant)	G	All these *FLG* variants repress *FLG* transcription and impaired the skin barrier	↑		Belyaeva et al. 2020; Schaarschmidt et al. 2015; Baurecht et al. 2015; Sun et al. 2011
	rs4363385	-	g.153016845 T > C (Intron variant)	C		↑		
CASP14	rs769277893	-	c.462_463delCA p.Asp154Glufs	delCA	The deletion results in frameshift located at the second amino acid of a small non-catalytic subunit (p11) and a premature stop codon at amino acid position 180, resulting in a truncated protein and impaired the skin barrier	↑	AD, Ichthyosis	Jung et al. 2018; Kirchmeier et al. 2014; Kirchmeier et al. 2017
SPINK5	rs2303067	-	c.1258 G > A p.Lys420Glu	A	All these variants result in a LEKTI protein unable to control serine peptidase activity, which mediates the cleavage of FLG precursor and epidermal desquamation process. It results in an impaired skin barrier, higher TEWL, and decreased inhibition of allergens that are serine proteinases inhibited by LEKTI.	↑	All these variants are associated with AD	Walley et al. 2001; Fortugno et al. 2012; Namkung et al. 2010
	rs2303064	-	c.1156 G > A p.Asp386Asn	A		↑		
	rs2306067	-	g.8683423A>G (Intron variant)	G		↑		
	rs17718511	-	g.148072009A>G (Intron variant)	G		↑		
	rs17860502	-	c.316 G > A p.Asp106Asn	A		↑		
	rs60978485	-	g.148091116A>T (Intron variant)	T		↑		

↑ - increased susceptibility to skin immunotoxicity.
↓ - decreased susceptibility to skin immunotoxicity.
AD: atopic dermatitis

inflammation (McAleer and Irvine 2013). In relation to inflammation, for instance, in skin exposed to UV radiation, *trans*-UCA is naturally converted to *cis*-UCA (Gibbs, Tye, and Norval 2008). *Cis*-UCA reduces the maturation of DCs and increases their capacity to induce regulatory T cells, which is a link between FLG deficiency and immune dysregulation (Gibbs, Tye, and Norval 2008; Leitch et al. 2016).

Loss of FLG function is also related to an enhanced activation of group 2 innate lymphoid cells (ILC2) – cells associated with AD progression. ILC2 express MHC II on their cell surface and secrete IL-5 and IL-13 (effector type 2 cytokines) in response to epithelial cell-derived cytokines IL-25, IL-33 and/or thymic stromal lymphopoietin (TSLP). ILC2 also respond to mast cell mediators, such as prostaglandin D_2 (PGD_2), resulting in ILC2 migration, production of type 2 cytokines and upregulation of IL-33 and IL-25 receptor subunits expression (ST2 and IL-17RA) (Xue et al. 2014). ILC2-derived cytokines, such as IL-13, are necessary to activate and expand DCs, demonstrating the critical role of ILC2 in Th2 response (Rafei-Shamsabadi et al. 2019).

The activation of ILC2 in the skin is also related to low E-cadherin expression. E-cadherin expression is associated with FLG expression on keratinocytes. FLG deficiency decreases the expression of E-cadherin, promoting ILC2 activation (Fallon et al. 2009; Kim 2015; Rafei-Shamsabadi et al. 2019). ILC2 might be involved in proinflammatory responses dependent upon basophil-derived IL-4 (Kim et al. 2014).

Expansion of IL-5-producing ILC2 in skin was demonstrated in FLG-deficient mice, which developed spontaneous AD-like inflammation (Saunders et al. 2016). Saunders et al. (2016) noted an increased number of ILC2 in skin blisters from patients with *FLG* loss-of-function mutation. Bielecki et al. (2021) reported ILC2s shifted into group 3 ILCs (ILC3), inducing skin diseases psoriasis which is related to the ILC3-derived cytokines IL-17 and IL-22.

The importance of FLG in preventing skin immune reactions was confirmed by several other *in vivo* studies. Kawasaki et al. (2012) showed that the skin of FLG-deficient mice exhibited higher antigen penetration, leading to enhanced responses in hapten-induced CHS and higher anti-ovalbumin IgG1 and immunoglobulin E (IgE) serum levels. Moniaga et al. (2010) also reported that FLG-deficient mice developed clinical and histological eczematous skin lesions similar to human AD displaying a defective skin barrier and generating proallergic mice responsive to 1-fluoro-2.4- dinitrobenzene (DNFB) sensitizer and phorbol myristate acetate skin irritant. FLG-deficient mice demonstrate a reduced barrier function with enhanced sensitization to DNFB and skin irritation to croton oil irritant exhibiting increased immune responses (Kawasaki et al. 2012). Dang et al. (2015) using cultured normal human epidermal keratinocytes showed that FLG silencing by short hairpin RNA (shRNA) directly impaired skin barrier function and induced a Th2 immune response, which is recognized as an allergic type of immune response.

In conclusion, *FLG* polymorphisms may be associated with skin immunotoxicity because FLG takes part in SC formation, skin hydration, moisturization, maintenance of the pH optimum (Egawa and Kabashima 2018; Kezic et al. 2011; Matsui et al. 2011) and in the regulation of immune responses (Gibbs, Tye, and Norval 2008; Leitch et al. 2016). *FLG* mutations may influence susceptibility to contact allergy and/or hand eczema when using products that may contain contact allergens, such as moisturizers, topical drugs, and rubber gloves (Lagrelius et al. 2020).

Caspase 14 (CASP14) gene

CASP14 is a protein that, after proteolytic maturation in the SG, degrades FLG monomers to free natural moisturizing factor (NMFs) in the SC (Eckhart and Tschachler 2011). CASP14 is also responsible for activating mesotrypsin, which is necessary for maturation of saposin A – a sphingolipid activator involved in the formation of the skin permeability barrier (Doering et al. 1999; Kirchmeier et al. 2017).

CASP14 is a product of the *CASP14* gene located on chromosome 19p13 (Hoste et al. 2011). This gene is mainly expressed in the suprabasal layers of the epidermis, and its expression increases during the process of keratinocyte differentiation (Rendl et al. 2002). The small deletion c.462_463delCA in *CASP14* leads to a frameshift on the second codon of a small non-catalytic

subunit (p11) and a premature stop codon at amino acid position 180 (p.Asp154Glufs), resulting in a truncated protein, impaired skin barrier, and autosomal recessive inherited ichthyosis (Kirchmeier et al. 2017).

Ichthyoses are a genetically heterogeneous group of skin disorders characterized by systemic inflammatory response and impaired permeability of the skin barrier, which result in abnormal desquamation of the skin (Kirchmeier et al. 2017), with IL-17/IL-22 predominance, similar to the skin compartment (Czarnowicki et al. 2018). Altered function of CASP14 results in inactive mesotrypsin, which may lead to an impaired permeability barrier, one of the main skin conditions of the autosomal recessive ichthyoses. *CASP14* knockout mice present several symptoms of patients with autosomal recessive ichthyoses and AD, such as disturbance of skin permeability barrier, elevated TEWL and hyperlinearity (Kirchmeier et al. 2017). As CASP14 plays important roles in skin barrier integrity, it may predispose it to immune response after chemical exposure.

Serine peptidase inhibitor Kazal-type 5 (SPINK5) gene

SPINK5 is associated with skin barrier and AD phenotype because its protein, termed Lympho-epithelial Kazal-type-related inhibitor (LEKTI), regulates corneodesmosome digestion. The corneodesmosomes are responsible for anucleated corneocytes aggregation, surrounded by a protein envelope filled with water and keratins (Ramesh et al. 2020). Corneodesmosome digestion is an essential event in the epidermal desquamation process. In this process, Kallikrein (KLK)-related peptidases, KLK5 and KLK7, digest corneodesmosomes (Lundwall and Brattsand 2008). Unregulated proteolytic activities of KLKs might result in premature breakdown of the corneodesmosomes and skin barrier dysfunction (Cork et al. 2006). In this way, several protease inhibitors in the skin are essential for regulation of epidermal desquamation processes. LEKTI markedly controls activity of KLK5 and KLK7 (Lundwall and Brattsand 2008), preventing premature breakdown of the corneodesmosomes.

The *SPINK5* gene is located on chromosome 5q32, and mutations in *SPINK5* are involved in developing diseases such as AD and Netherton syndrome, which is a disorder characterized by AD, ichthyosis, and high serum levels of IgE (Fortugno et al. 2012; Lan et al. 2011; Namkung et al. 2010; Walley et al. 2001). The SNP rs2303067 1258 G > A Glu420Lys in *SPINK5* was previously found to be associated with AD (Walley et al. 2001). The two nonsynonymous SNPs rs2303064 G > A Asp386Asn and rs2306067 (A > G) were also associated with AD among Japanese patients (Fortugno et al. 2012). The 4 SNPs rs17718511, rs17860502, rs60978485, rs17718737, and haplotype TAA in the *SPINK5* gene were related to susceptibility to allergic type of AD (Namkung et al. 2010).

The lack of functional LEKTI expression also results in altered proFLG processing (Descargues et al. 2005), which is the major contributor to epidermal barrier function (Fortugno et al. 2012). This occurs because a complex network of serine proteases such as furin, profilaggrin endopeptidase-1 (PEP1), and other proteolytic enzymes and KLKs, act in concert to mediate the multistep cleavage of the FLG precursor (Fortugno et al. 2012), and their activities are influenced by LEKTI functionality. In addition, LEKTI might diminish allergenicity since many allergens are also serine proteinases inhibited by LEKTI proteinase activity (Walley et al. 2001). In this way, mutations in the *SPINK5* gene may result in LEKTI proteins unable to control serine peptidase activity, leading to dysfunctional skin barrier and higher TEWL and inflammatory-type reactions (Ramesh et al. 2020) (Table 4).

Inflammation processes and mediators

Cytokines perform pleiotropic functions to mediate and regulate the immune response and are recognized as biomarkers of immunotoxicity (Elsabahy and Wooley 2013). Cytokines may stimulate immune cells to migrate from blood to the skin, triggering local production of cytokines, enzymes, free radicals, and other molecules, amplifying inflammatory responses (Zhang and An 2007). In addition, cytokines regulate immune cell populations, such as Th1, Th2, Treg and Th17 cells. The balance in these populations is essential in determining the type of immune response following chemical exposure. Many pollutants/toxicants affect Th/Treg populations and functions (Lee and

Lawrence 2018). Individual genetic variation in cytokine genes may influence the cytokine expression pattern in these cells and immune responses following chemical exposure.

Tumor necrosis factor (TNF) gene

TNF-α, the cytokine coded by the *TNF* gene, is proinflammatory and plays a crucial role in skin immune reactions after contact with chemicals (Kimber et al. 2000). One of its best-characterized functions is to stimulate leukocyte recruitment to sites of infection by increasing adhesion molecules in endothelial cells, acting in the disruption of macrovascular and microvascular circulation (Zhang et al. 2009). TNF-α significantly stimulates LC activation, motility and antigen presentation to T cells (Clayton et al. 2017). Cumberbatch and Kimber (1995) in an *in vivo* study demonstrated that anti-TNF-α treatment markedly inhibited the frequency of DC in draining nodes measured 18 hr following exposure to skin allergens (oxazolone and fluorescein isothiocyanate) or non-sensitizing skin irritant SLS. Anti-TNF-α treatment inhibited DC accumulation in lymph nodes and impaired the efficiency of skin sensitization in mice (Cumberbatch and Kimber 1995). Thus, contact sensitization or skin irritation is associated with the TNF-α production by keratinocytes, leading to LC migration in response to skin sensitization or skin irritation. The mobilization depends on TNF-α acting directly on epidermal LC themselves via TNF-R2 receptors and explains the role of TNF- α in immune response to chemicals (Kimber et al. 2000).

TNF-α activates MMP-9 in human skin, promoting matrix remodeling during inflammation (Han et al. 2001; Majtan and Majtan 2011) and increasing leukocyte infiltration to the skin (Zhang et al. 2009) including monocyte/macrophages (Chun et al. 2000). Further, increased TNF-α induces endothelial production of ROS by activation of NADPH oxidase, possibly via the subunits $gp91^{phox}$, NOX-1, $p47^{phox}$ and $p22^{phox}$, resulting in endothelial dysfunction in many pathophysiological conditions (Zhang et al. 2009). This cytokine is produced by dendritic cells, macrophages, Th1 and Th17 cells (Prieto-Pérez et al. 2013), mast cells (Kimber et al. 2000; Lauritano et al. 2020) and keratinocytes (Akdis et al. 2016; Gröne 2002; Li et al. 2007a; Schnuch and Carlsen 2011) and has been associated with AD and psoriasis (Akdis et al. 2016; Li et al. 2007a; Schnuch and Carlsen 2011).

The *TNF* gene is located on chromosome 6p21.3 within the major histocompatibility complex (MHC) region (Schnuch and Carlsen 2011), which contains several high polymorphic genes (Wastowski et al. 2006). The transition G to A at position −308 (rs1800629) within the promoter region of *TNF* gene is associated with inflammatory conditions (Akdis et al. 2016; Hajeer and Hutchinson 2001). The G allele (*TNF*–308 G) contains an activator protein-2 (AP-2) binding element that suppresses the promoter activity (Kroeger and Abraham 1996). In contrast, the A allele (*TNF*–308A) presents a disrupted AP-2 binding site and provides an increased transcription and elevated secretion of TNF-α (Kroeger and Abraham 1996).

The frequency of AA and GA *TNF*-308 genotypes is significantly higher in individuals sensitized to paraphenylenediamine (PPD) (Blömeke et al. 2009), and *TNF* – 308 GA heterozygous was also associated with skin sensitization to chromate in a cohort study in cement workers (Wang et al. 2007). Allen et al. (2000) observed an elevated frequency of *TNF*–308 A and the *TNF* – 308AA genotype among individuals with a low threshold towards irritants – patch test with aqueous SLS and aqueous benzalkonium chloride (BKC). These studies demonstrated that *TNF*–308A may be a susceptibility factor for contact allergy and irritant contact dermatitis.

Psoriatic patients with *TNF*–308A exhibited *TNF* down-regulation after IL-10 administration, a cytokine with anti-inflammatory property used as a treatment (Reich et al. 2001). Other studies demonstrated higher frequency of *TNF*-308 GG in moderate to severe psoriasis patients or greater frequency of *TNF*-308 G allele in patients with early-onset psoriasis (Karam, Zidan, and Khater 2014; Settin et al. 2009) or found no significant differences among *TNF* – 308 alleles (Craven et al. 2001). These discrepancies may be accounted for differences in populations and linkage disequilibrium (LD) between alleles across the MHC (Wilson et al. 1997).

Other polymorphisms in the promoter regions of the *TNF* gene affecting *TNF* expression were also reported. In the *TNF*-238 (G > A transition at position 238, rs361525), the A allele is associated

with enhanced *TNF* expression and is associated with psoriasis (Li et al. 2007; Prieto-Pérez et al. 2013). The SNP rs1799724 (*TNF*–857 C > T) is also associated with psoriasis in a meta-analysis (Wang and Zhou 2019).

Concerning psoriasis, TNF-α induces the production of proinflammatory cytokines synthesized by activated lymphocytes or keratinocytes, such as IL-1 – a cytokine that stimulates cytokeratin 6 (CK6) expression, a marker of activated and hyperproliferative keratinocytes. TNF-α also stimulates CK6 promoter in kerati- nocytes, and CK6 overexpression influences the formation of the hyperproliferative epithelium found in psoriasis. Although TNF-α might pro- mote apoptosis through binding to the TNF- receptor 1, this cytokine may participate in hyperproliferative psoriatic lesions because this constituent may stimulate activation of NF-κB by increasing degradation of I-κB, an NF-κB inhibitor. NF-κB is a nuclear transcription factor of cytokines including TNF-α, IL-6 and IL-8, and also ICAM1, VCAM-1 and E-selectin. NF- κB not only inhibits apoptosis of keratinocytes but also enhances inflammatory response (Victor and Gottlieb 2002). The effects of TNF-α in leukocytes dermal infiltration, dermal capillary density and abnormal proliferation and differen- tiation of keratinocytes – which is increased is characteristic of psoriasis – might account for why several *TNF* polymorphisms were correlated with this disease.

Interleukin-10 (IL-10) gene

IL-10 is a predominantly anti-inflammatory and immunosuppressive cytokine (Fiorentino et al. 1991; Saxena et al. 2014) that also acts as a proinflammatory cytokine (Sharif et al. 2004). As an anti-inflammatory cytokine, this constituent prevents extensive tissue damage after inflammation (Schnuch and Carlsen 2011) and is critical in preventing exaggerated T-cells response following skin exposure to contact sensitizers (Boyman, Werfel, and Akdis 2012).

The IL-10 proinflammatory roles may appear during an active inflammatory response such as during experimental endotoxemia, after tissue transplantation, chronic obstructive pulmonary disease; silicosis, and in systemic lupus erythematosus (Anlar et al. 2017; Harting et al. 2012; Mühl 2013). The proinflammatory activity is mediated by type I IFNs. IFN-a confers a proinflammatory gain of function on IL-10, leading to IL-10 activation of expression of STAT1-dependent genes, IFN-y-inducible CXCL10, and monokine induced by IFN-y (CXCL9) (Sharif et al. 2004). This cytokine may present immunostimulatory properties on $CD4^+$, $CD8^+$ T cells, macrophages and/or NK cells, resulting in increased IFN-y production (Lauw et al. 2000; Sharif et al. 2004).

The cutaneous macrophages are the primary IL-10 sources and stimulated by various biological molecules such as endotoxin, catecholamines, and TNF-α to produce this cytokine (Isac and Jiquan 2019). IL-10 is also expressed by Th2 cells and Treg cells (Isac and Jiquan 2019) and in minor amounts by B cells, monocytes, NK cells, and DCs (Döcke et al. 2009).

The *IL10* gene is located on 1q31–q32, and its promoter region is highly polymorphic (Eskdale et al. 1997). *IL10* gene polymorphisms were investigated as candidate genetic biomarkers to predict skin immune reactions and diseases (Asadullah, Sterry, and Volk 2003; Settin et al. 2009; Sohn et al. 2007; Zhao, Chen, and Li 2019). In particular, there are three frequently investigated SNPs in the promoter region of *IL10*: – 1082 G/A (rs1800896), – 819 C/T (rs1800871), and – 592 C/A (rs1800872) (Isac and Jiquan 2019).

The *IL10*-1082 G (rs1800896) variant was associated with higher risk of skin inflammation (Karam, Zidan, and Khater 2014; Lee et al. 2012; Settin et al. 2009). Craven et al. (2001) indicated that the heterozygous GA genotype is a risk factor for psoriasis and not the homozygous GG genotype. Investigators found no marked association between the G allele and psoriasis indicating that the SNP effect is population-dependent in East Asian, Egyptian and Indian populations where the allele G was associated with psoriasis, whereas in European and Thai populations, there was no significant correlation (Baran et al. 2008; Isac and Jiquan 2019; Wongpiyabovorn et al. 2008). In addition, a meta-analysis study considered that the *IL10*–1082 GG genotype represents a risk for AD among Asian populations (Zhao, Chen, and Li 2019).

IL10 – 819 C/T (rs1800871) and *IL10*–592 C/A (rs1800872) were also associated with inflammatory skin diseases. A meta-analysis study demonstrated that the *IL10*–819 GG genotype was correlated with AD among European populations (Zhao, Chen, and Li 2019). *IL10*–819 TT is a low IL-10 producing genotype and increases 3.5- fold the hypersensitivity response in *parthenium dermatitis* (Khatri et al. 2011). The C allele produces higher IL-10 levels when in heterozygous genotype (CT) than in homozygous genotype (CC) (Khatri et al. 2011). The *IL10*–592 A allele was related to eosinophil activation, allergic inflammation, lower *IL10* expression, and AD phenotype in children (Sohn et al. 2007).

The – 1082 G/A and – 592 C/A polymorphisms are located within a putative ETS and STAT3 transcription factor binding sites, respectively, and – 819 C/T lies within a putative positive regulatory region (Guzowski et al. 2005; Turner et al. 1997). However, Karam, Zidan, and Khater (2014) noted the involvement of these polymorphisms in regulatory mechanisms of Th1/Th2 balance. *IL10* expression may play a critical role in T cell skin infiltration and response. Roers et al. (2004) showed that mice with deficient IL-10 production by T cells, Treg cells, or LC exert enhanced T-cell recall in response to contact sensitizers and irritants 2,4-dinitrochlorobenzene and croton oil, respectively. IL-10 suppresses T cell responses to skin allergens, mainly through inhibition of APCs' activity and expression of proinflammatory cytokines, such as TNF-α and IL-1β. IL-10 also inhibits cytokines that play a crucial role in T cell differentiation, such as IFN-γ and IL-12 (Asadullah, Sterry, and Volk 2003; Reich et al. 2001). The overexpression of proinflammatory Th1 cytokines was noted in psoriasis. Subcutaneous IL-10 administration (8 μg/kg/day) in psoriatic patients resulted in immunosuppressive effects as evidenced by depressed monocytic HLA-DR expression, TNF-α and IL-12 secretion capacity, IL-12 plasma levels, and responsiveness to recall antigens as well as a shift toward a Th2 cytokine pattern including increasing proportion of IL-4 and IL-5 producing T cells, and selective rise in IgE serum levels (Asadullah et al. 1998). The impact of IL-10 on inhibition of these cytokines may explain why low IL-10 producing genotypes are believed to be of pathophysiological importance.

Interleukin −6 (IL-6) gene

IL-6 can play both a pro- and anti-inflammatory role (Del Giudice and Gangestad 2018). IL-6 anti-inflammatory activities are mediated by classical signaling (activation of cells via the membrane-bound IL-6 R and gp130), whereas proinflammatory responses are mediated by trans-signaling (activation of cells that only express gp130 via the IL-6/sIL-6 R complex) (Scheller et al. 2011). Interestingly, only a few cells express the membrane-bound IL-6 Rα and respond to IL-6 in the classical way, whereas a large variety of cell types express the co-receptor molecule gp130 and are activated through trans-signaling (Klebow et al. 2016; Scheller et al. 2011). In this way, IL-6 predominantly exerts its inflammatory property in cells and activates the immune system by recruiting monocytes to the inflamed area (Kaplanski et al. 2003). IL-6 is produced by many cells including keratinocytes, fibroblasts, macrophages, endothelial cells, and Th17 cells in response to various stimuli (Grossman et al. 1989). IL-6 participates in T cell differentiation, activation, and inhibition of T cell apoptosis (Akira et al. 1990; Curnow et al. 2004; Scheller et al. 2011), playing a crucial role in augmentation T cell response. This cytokine also controls the Treg/Th17 balance by influencing Treg cellular differentiation and maintenance of Th17 cells (Jones et al. 2010; Neurath and Finotto 2011; Scheller et al. 2011). Treg cells are responsible for suppressing or downregulating induction of effector T (Teff) cells (Bettelli et al. 2006).

Concerns regarding the effects of *IL6* genetic variants in the immune response to chemicals have been raised after the association of IL-6 serum levels with AD and psoriasis (Yang et al. 2017). IL-6 is highly expressed by $CD31^+$ endothelial cells and $CD11c^+$ dermal DCs in lesional psoriatic skin. In this disease, T memory/effector cells (Tmem/eff) are chronically activated and poorly suppressed by Treg cells. IL-6 enables Tmem/eff cells to escape from Treg-mediated suppression, acting on T cell subsets that express the IL-6 R. Further, IL-6Rα expression is elevated in psoriatic Teff and Treg cells compared to normal cells, and both cell populations phosphorylate STAT3 in response to IL-6. STAT3 phosphorylation contributes to Th17 differentiation, which is present within lesional tissue and contributes to IL-

6-mediated resistance to Treg suppression. The T lymphocytes trafficking into lesional psoriatic skin encounter high IL-6 levels originating from endothelial cells, DCs, and Th17 cells, enabling cutaneous T cell escape from Treg suppression and Th17 participation in inflammation (Goodman et al. 2009).

The *IL6* gene is located on human chromosome 7p15 (Yasukawa et al. 1987). The most investigated polymorphism in *IL6* is −174 G > C (rs1800795), located in the promoter region. The G allele is associated with a higher *IL6* expression than C allele and more frequently found in AD and psoriatic patients than in healthy individuals. The GG genotype is also associated with enhanced AD susceptibility and psoriasis (Gharagozlou et al. 2013; Kusumawati 2017). Imboden et al. (2006) demonstrated that the GG genotype is correlated with higher IgE production. Other polymorphisms in the *IL6* gene and their combination, the haplotypes (Samuel et al. 2008; Terry, Loukaci, and Green 2000), might also be determined to understand *IL6* expression. Nevertheless, the haplotypes containing the −174 G allele account for only approximately 5% of the general population, and provide the lowest IL-6 expression levels (Terry, Loukaci, and Green 2000). This may account for why only the −174 G > C polymorphism is considered in population-based studies. The G allele shows higher IL-6 expression than C allele, and despite not being attributed only to −174 G, its frequency in haplotypes provides high *IL6* expression.

The *IL6* nt565 A > G is also a commonly investigated polymorphism. The GA genotype was associated with elevated AD risk in a meta-analysis, while there was no marked relationship between *IL6* nt565GG and *IL6* −174GC genotypes in the same study (Yang et al. 2017). With regard to haplotypes, Gharagozlou et al. (2013) found that the GG haplotype (−174, nt565) was associated with AD. However, other investigators reported no significant association between AD and these two genetic variants of *IL6* (−174 C/G and nt565A/G) (Hoffjan et al. 2004; Reich et al. 2003; Stavric et al. 2012; Westphal et al. 2003), indicating that the consequences of *IL6* polymorphisms may also depend upon population.

Interleukin- 18 (IL-18) gene

IL-18 is a pleiotropic cytokine whose functions in the human immune system are heterogeneous and depend upon the nature of the cytokine milieu and genetic background (Nakanishi et al. 2001). Thus, IL-18 presents both inhibiting and promoting immune effects (Novak et al. 2005). Of note, IL-18 plays a crucial role in controlling the balance between Th1 and Th2 immune responses (Granum and Løvik 2002; Nakanishi, Tsutsui, and Yoshimoto 2010; Nakanishi et al. 2001).

IL-18 might be antiallergic because, in synergy with IL-12, it inhibits IgE synthesis by B cells and promotes Th1 immune responses (Yoshimoto et al. 2000, 1999). When released by mast cells, basophils, and eosinophils, major inducers and effectors or allergic inflammation, IL-18 directly stimulates production of Th2 cytokines and stimulates Th2 differentiation and production of IgE (Hoshino, Wiltrout, and Young 1999; Wang et al. 2001; Yoshimoto et al. 2000, 1999). Of note, IL-18 might also take part in skin inflammation and mediate skin changes in an IgE-independent manner (Konishi et al. 2002).

High IL-18 serum levels are associated with psoriasis and AD (Gangemi et al. 2003; Sedimbi, Hägglöf, and Karlsson 2013; Tanaka et al. 2001). Psoriasis is correlated with an overexpression of Th1 cytokines and relative underexpression of Th2 cytokines. *IL18* gene expression may play an important role in this disease by inducing Th1 response because IL-18 is a potent inductor of synthesis of IFN- γ, TNF-α and other mediators (Gangemi et al. 2003). Further, IL-18 may recruit DCs expressing IL-18 R to inflammatory areas under Th1 conditions as in psoriatic lesions (Lee, Cho, and Park 2015). When synergized with IL-23, IL-18 promotes development and maintenance of Th17 cells that were also implicated in developing the disease. Results from previous studies indicated that IL-18 serum or plasma levels correlate well with the Psoriasis Area and Severity Index (PASI) (Pietrzak et al. 2003; Takahashi et al. 2010). The expression of IL-18 was also elevated in initially active and progressive plaque-type psoriatic lesions (Companjen et al. 2004). Further, in cooperation with IL-23, IL-18 induced severe inflammation and enhanced psoriasis-like epidermal hyperplasia

(Shimoura et al. 2017). Niu et al. (2019) using IL-18 knockout mouse model in imiquimod-induced psoriasis found that IL-18 promoted *IL-17* mRNA expression and decreased IL-4 production, which may then prevent Th2 responses.

In allergic contact dermatitis, extracellular DAMPs activate TLR4/2 resulting in the expression of IL-18 and other cytokines including IL-1b, IL-6, IL-12, IL- IL-23 and TNF-α. IL-18 promotes sensitization and facilitates elicitation of contact allergy via multiple mechanisms, including the recruitment of Th1 and Th17 cells. Th1 cells secrete large amounts of DAMPs and stimulate TLR4, activating innate and adaptive immune responses and triggering a positive feedback loop. Th2 cytokines reduced TLR4 function and antagonized this pathway in AD, but repeated exposure to the allergens resulted in a Th2- type response (McFadden et al. 2013).

Sensitizers and irritants, such as trinitrochlorobenzene and SLS, trigger the immune system and lead to IL-18 activation and production (Lee, Cho, and Park 2015). In addition, Galbiati et al. (2014) demonstrated that the contact allergens *p*-phenylenediamine (PPD), 2,4-dinitrochlorobenzene (DNCB), and citral might induce the release of high-mobility group protein B1 (HMGB1), which via TLR4 induced IL-18 neosynthesis in keratinocytes and mediates an immune response. Several inflammatory skin diseases and atopic allergies are correlated with *IL18* SNPs, and it was suggested as a marker of AD phenotype and severity (Kim et al. 2007; Novak et al. 2005; Sanders and Mishra 2016; Yang et al. 2017) (Table 5).

The *IL18* gene is located at chromosome 11q22.2–22.3, and is composed of 6 exons. *IL18* gene expression is regulated by two distinct promoters: promoter 1 is upstream of exon 1 and promoter 2, upstream of exon 2, with the start codon located in exon 2 (Kalina et al. 2000). *IL18* –137 G > C (rs187238) is situated in a GATA3 binding site in promoter 1, which is a transcription factor directly involved in Th2 differentiation (Kruse et al. 2003; Kuo and Leiden 1999). This polymorphism is associated with high IgE levels (Imboden et al. 2006; Kruse et al. 2003; Trzeciak et al. 2010). Arimitsu et al. (2006) showed that monocytes with −137 GG genotype produce higher IL-18 levels than −137 GC monocytes in response to calcium ionophore A23187 plus phorbol myristate acetate (PMA) or lipopolysaccharide (LPS), concluding that it might contribute to allergic sensitization development. The–137 G allele and GG genotype were associated with AD occurrence (Trzeciak et al. 2010). In contrast, Yang et al. (2017) and Imboden et al. (2006) found that *IL18* −137 CC genotype was significantly associated with AD. However, other investigators failed to find any association between this polymorphism and AD in Egyptian and Japanese populations (Ibrahim et al. 2012; Kato et al. 2009). Kato et al. (2009) demonstrated that the −137 G/C *IL18* was also correlated with psoriasis and that the G allele was detected frequently in psoriatic patients.

The most studied *IL18* polymorphism in promoter 2 is located at position −140 C > G (previously −133 C/G), (rs360721), in exon 2 of the *IL18*. The *IL18* – 140 is situated in an NF-1 binding site in the promoter region (Kruse et al. 2003) and its C to G substitution creates a new potential binding site for STAT (Puthothu et al. 2007). STATs activate various genes involved in inflammation and may alter the *IL18* transcription (Puthothu et al. 2007). The −140 G allele is predominantly associated with high IgE levels and atopic phenotypes (Kruse et al. 2003). Regarding the severity of the immunologic reaction, Ibrahim et al. (2012) found that the −140 G allele and GG genotype were associated with severe AD cases compared to mild to moderate conditions. Variants in the *IL18* gene are in significant linkage disequilibrium, reflecting a functional link between a combination of SNPs. For example, the *IL18* −607 C allele increases production of IL-18 only in the presence of the −137 G allele (Pavlovna et al. 2008).

Interleukin- 31 (IL-31) gene

IL-31 is a member of the gp130/IL-6 family of cytokines (Dillon et al. 2004) and a non-histamine pruritogen that is increased in inflammatory and pruritic processes in allergic skin diseases (Cevikbas et al. 2014). IL-31 is mainly involved in Th2-weighted inflammation and skin-homing of CD45RO1 (memory) cutaneous lymphocyte-associated antigen-positive T cells (Bilsborough et al. 2006; Dillon et al. 2004).

Th2 cells are the primary IL-31 source in the skin (Saleem et al. 2017). However, this cytokine is also expressed by keratinocytes, fibroblasts, DCs,

Table 5. The contribution of genes participating in inflammation processes in skin immunotoxicity.

Gene	Genetic variants	Aliases	Mutation	Susceptibility allele or genotype	Functional consequences	Susceptibility to skin immunotoxicity	Association with skin condition or disorder	References
TNF	rs1800629	*TNF* −308	g.31575254 G > A (Upstream transcript variant)	A	Located at the promoter region, it results in an altered AP-2 (activator protein-2) binding element, increasing the secretion of TNF-α	↑	Psoriasis, AD Irritant contact dermatitis	Li et al. 2007a; Schnuch and Carlsen 2011; Akdis et al. 2016; Allen et al. 2000
	rs361525	*TNF*-238	g.31575324 G > A (Upstream transcript variant)	A	Located at the promoter region, it increases the expression of *TNF*	↑	Psoriasis	Prieto-Pérez et al. 2013; Li et al. 2007
	rs1799724	*TNF* - 857	g.31574705 C > T (Upstream transcript variant)	T	Located at the promoter region of the *TNF*, it increases the expression of *TNF*	↑	Psoriasis	Wang and Zhou 2019
IL10	rs1800896	*IL10*-1082	g.3943A>G (Upstream transcript variant)	A	Located at the promoter region, specifically within a putative ETS transcription factor binding site, it is related to a lower level of *IL10* expression	↑	Psoriasis	Lee et al. 2012; Karam, Zidan, and Khater 2014; Settin et al. 2009; Craven et al. 2001; Isac and Jiquan 2019; Baran et al. 2008; Wongpiyabovorn et al. 2008
	rs1800871	*IL10* - 819	g.4206 T > C (Upstream transcript variant)	T	Located at the regulatory region, it decreases the expression of *IL10*	↑	AD, psoriasis, parthenium dermatitis	Indhumathi et al. 2017; Akdis et al. 2016; Khatri et al. 2011.
	rs1800872	*IL10* −592	g.4433A>C (Upstream transcript variant)	A	Located within the putative STAT-3 binding site in a negative regulatory region, it is related to low level of *IL10* expression	↑	AD	Indhumathi et al. 2017; Akdis et al. 2016; Sohn et al. 2007
IL6	rs1800795	*IL6* −174 G/C	g.22727026 C > G (Upstream transcript variant)	G	Located at the promoter region, it is related to higher level of *IL6* expression	↑	AD, Psoriasis	Gharagozlou et al. 2013; Kusumawati 2017.
	rs1800797	IL6 nt565	g.22726602A > G (Upstream transcript variant)	GA	Located at the promoter region, it is related to high *IL6* expression	↑	AD	Yang et al. 2017

(*Continued*)

Table 5. (Continued).

Gene	Genetic variants	Aliases	Mutation	Susceptibility allele or genotype	Functional consequences	Susceptibility to skin immunotoxicity	Association with skin condition or disorder	References
IL18	rs187238	*IL18* - 137 G/C	g.112164265 C > G (Upstream transcript variant)	G	Located at the promoter 1, specifically at GATA3 binding site, it is related to high degree of Th2 differentiation and IgE levels	↑	Allergic sensitization, AD, psoriasis	Arimitsu et al. 2006; Yang et al. 2017; Imboden et al. 2006; Kato et al. 2009
	rs360721	Published *IL18* – 140 C/G or *IL18* - 133 C/G	g.112155193 G > C (Upstream transcript variant)	G	Located at the promoter region, specifically at NF-1 binding site; it creates a novel binding site for STAT (signal transducer and activator of transcription) proteins that activate a variety of genes involved in inflammation, it also leads to high transcription of *IL18*.	↑	AD	Ibrahim et al. 2012
IL31	rs10847385	*IL31*-631	g.122174803A>C (Upstream transcript variant)	A	Located at the promoter region, it is related to a higher *IL31* expression		↑ Severity of extrinsic AD	Hong et al. 2012
	rs7974857	*IL31* c.183	g.122172724 C > G (Intron variant)	G	-		↑ AD	Lan et al. 2011
	-	*IL31* −2057	-	GA	-		↑ AD, moderate pruritus	Sokołowska-Wojdyło et al. 2012
	-	*IL31* −1066	-	A/AA	Located within a consensus site for members of the GATA transcription factor family. The A allele can block the binding sites for GATA-2 and GATA-3 and, consequently, it can dysregulated *IL31* expression		↑ AD	Sokołowska-Wojdyło et al. 2012
	rs61955584	*IL31*-IVS2 + 12	c.165 + 12A > G (Intron variant)	A/AA	-		↑ AD	Lange et al. 2017
TSLP	rs2289278	-	g.111073450 C > G (Intron variant)	C	-		↑ AD	Wang et al. 2016
	rs1898671	-	g.111072304 C > T (Intron variant)	C	-		↓ Herpetic eczema, AD	Gao et al. 2010; Margolis et al. 2014
	rs1837253	*TSLP* −5717	g.111066174 T > C (Intron variant)	C	Located at the promoter region, it alters transcription factor binding sites and increases the gene and protein expression of *TSLP*		↑ AD	Miyake et al. 2015

↑ – increased susceptibility to skin immunotoxicity.
↓ – decreased susceptibility to skin immunotoxicity.
AD: atopic dermatitis

monocytes/macrophages, mast cells, cutaneous lymphocyte antigen $(CLA)^+$ $CD45RO^+$ memory T cells (Akdis et al. 2016; Bilsborough et al. 2006), and activated eosinophils (Saleem et al. 2017). IL-31 may be released after skin exposure to pathogens or following physical damage to skin tissues by UVB rays, H_2O_2, or staphylococcus enterotoxin B (Cheung et al. 2010; Cornelissen et al. 2011; Kunsleben et al. 2015; Sonkoly et al. 2006). After skin tissue damage occurs, keratinocytes release IL-33 that, in synergy with IL-4, upregulates IL-31 expression through the NF-κB pathway (Maier et al. 2014). Although, in AD, nonlesional skin also express elevated IL-31 mRNA levels (Sonkoly et al. 2006). IL-31 binds to heterodimeric IL-31 receptor A (IL-31RA) and the oncostatin M receptor (OSMR) – a receptor that enhances IL-31 binding affinity to IL-31RA (Akdis et al. 2016; Hermanns 2015). These receptors are found in T cells, keratinocytes, DCs, eosinophils, basophils, macrophages, activated monocytes, and dorsal root ganglia cells (DRG), sensory neurons responsible for the induction of itching (Akdis et al. 2016; Gibbs, Patsinakidis, and Raap 2019).

Dermatologic diseases displaying enhanced *IL31* expression and related proinflammatory and immunomodulatory functions include AD (Bilsborough et al. 2006; Raap et al. 2012), *prurigo nodularis* (Neis et al. 2006), chronic spontaneous urticaria (CsU) (Raap et al. 2010), as well as in a subset of patients with mastocytosis (Hartmann et al. 2013).

The human *IL31* gene is located on chromosome 12q24.31 (Dillon et al. 2004) and presents polymorphisms with a role in impairing skin immune homeostasis. The SNP *IL31*-631 T > G (rs10847385) was associated with IL-31 blood levels, IgE levels and severity of extrinsic AD (Hong et al. 2012). The −631 C allele contributed to impaired function of the *IL31* promoter and lowered IL-31 serum levels. In addition, −631 C allele downregulates the *IL31* expression only under inflammatory conditions suggesting that *IL31* – 631 acts as a disease modifier by influencing inflammatory conditions rather than as a disease risk factor. Using the bioinformatics tool TFSER, Hong et al. (2012) found two putative binding sites near *IL31* −631, including Cap and Heat shock transcription factor (HSF) that might stimulate promoter activity.

IL31 c.183 C > G (rs7974857) is located in intron 2 and the G allele and was associated with AD in the Taiwanese population (Lan et al. 2011). *IL31* −1066 AA genotype and A allele were associated with AD in the Polish population (Sokołowska-Wojdyło et al. 2012). *IL31*-1066 is located within a consensus site for the GATA transcription factor family (GATA1, GATA2, and GATA3) (Schulz et al. 2007). GATA3 plays an important role in inflammatory processes in AD (Schulz et al. 2007).

IL31-2057 GA and AA genotypes were correlated with AD in the Polish population (Sokołowska-Wojdyło et al. 2012), and also associated with moderate pruritus (Sokołowska-Wojdyło et al. 2012). Lange et al. (2017) reported that the *IL31* IVS2 + 12 (rs61955584) A allele and AA genotype were correlated with pruritus and mastocytosis (Lange et al. 2017).

In AD, IL-31 was found to be correlated to total IgE serum, eosinophil cationic protein, and disease severity. IL-31 is also responsible for inducing long-lasting erythema, which is associated with local skin inflammation (Hawro et al. 2014). The lack of immediate itch induction after administration ofIL-31 in healthy controls and AD patients suggests that this cytokine acts indirectly via keratinocytes rather than on skin neuronal endings in this disease (Olszewska et al. 2020). *IL31* is also responsible for keratinocyte differentiation disruption (Cornelissen et al. 2012) and decreasing FLG expression in human keratinocytes (Singh et al. 2016), which might facilitate immune responses and predisposition to AD.

After binding to keratinocytes, IL-31 also enhances chemokine expression (Cheung et al. 2010), including CCL2 and CCL26, which recruit basophils to affected tissue sites (Gibbs, Patsinakidis, and Raap 2019). Thus, elevated expression of *IL31* might facilitate basophil infiltration into skin lesions of AD and in cases of urticaria, previously reported by Ito et al. (2011). Basophils release IL-4 that supports development of Th2 cells and subsequent IL-31 production (Gibbs, Patsinakidis, and Raap 2019). In a mouse model, IL-31 increased epidermal basal-cell proliferation resulting in thickening of the epidermal skin layer but elevated TEWL (Singh et al. 2016) and elicited pruritus or resulting atopic-like dermatitis (Dillon et al. 2004; Grimstad et al. 2009).

Although IL-31 mechanisms of action still require further investigations, studies indicated that *IL31* polymorphisms might influence IL-31 and IgE levels, a possible marker in response to xenobiotics and development of skin diseases (Table 5).

Thymic stromal lymphopoietin (TSLP) gene

TSLP is a secretory cytokine of thymic stromal cells that uses the combination of Janus kinase 1 (JAK1) and Janus kinase 2 (JAK2) to activate signal transducer and activator of transcription 5 (STAT5) (Rochman et al. 2010). STAT5 induces genes that regulate cell proliferation, differentiation, survival, and inflammation (Ando et al. 2014; Hennighausen and Robinson 2008). TSLP is produced by keratinocytes, fibroblasts, epithelial cells, basophils, and stromal cells (Lee et al. 2021; Rochman et al. 2010) and binds to TSLP receptor (TSLPR) (Quentmeier et al. 2001). TSLP receptor is present on DCs, T and B lymphocytes, NK cells, ILC2, eosinophils, basophils, and monocytes (Halim et al. 2012; Kim et al. 2013; Ziegler and Artis 2010).

The polymorphisms in the *TSLP* gene are associated with AD (Han, Roan, and Ziegler 2017; Miyake et al. 2015) and with susceptibility to skin allergy induced by foods (Noti et al. 2014) (Table 5). *TSLP* is located on chromosome 5q22.1, near the atopic cytokine cluster on 5q31 (Miyake et al. 2015). The *TSLP* T > C (rs1837253) is in the promoter region, 5.7 kb upstream of the *TSLP* transcription start site. The rs1837253 T allele may downregulate expression TSLP by inhibiting transcription factors binding to regulatory elements (Hui et al. 2015). The TC and CC genotypes are associated with AD (Miyake et al. 2015). The CC genotype and C allele of the *TSLP* gene C > G (rs2289278) are significantly associated with AD in children (Wang et al. 2016). In contrast, SNP C > T (rs1898671) was correlated with a reduced risk for herpetic eczema (Gao et al. 2010) and AD (Margolis et al. 2014). Individuals with *FLG* loss-of-function mutations and an rs1898671 variant were nearly 5-fold less likely to have persistent AD than those without this variant (Margolis et al. 2014). The association of AD with *TSLP* polymorphisms is attributed to gene product playing a role in the Th1/Th2 balance.

In the absence of TSLP, IL-12 production by DCs triggers Th1 differentiation and OX40 ligand (OX40L) expression in DCs. OX40L stimulate the Th2 differentiation in the absence of IL-12 (Ito et al. 2005). When present, TSLP may induce OX40L expression and inhibit IL-12 production in human DCs, stimulating Th2 differentiation and inhibiting Th1 differentiation (Ito et al. 2005). The Th2 differentiation by this mechanism produces the classical Th2 inflammatory cytokines (IL-4, −5, and −13) but not IL-10. These Th2 cells rather produce high levels of TNF-α. The inflammatory TNF-α highly positive and IL-10–negative Th2 cells are believed to most likely initiator of allergic inflammation, in contrast to IL-10 producing Th2 cells (Fang and Zhu 2020; Ito et al. 2005). TSLP also leads to Th2 cell recruitment (Liu et al. 2007), and influences the maturation of APCs, eosinophilia, and chemotaxis (Wong et al. 2010).

In contrast to lung and gut, TSLP elicits ILC2 non-mediated IL-33 and IL-25 responses in skin and skin-draining lymph nodes. This promotes skin inflammation and ILC2 expansion in sensitized skin (Kim et al. 2013; Leyva-Castillo et al. 2020), promoting high IL-5 and IL-13 levels observed in individuals with AD. ILC2-derived IL13 promotes keratinocyte proliferation and expression of T-cell attracting chemokines CCL17 and CCL22 (Leyva-Castillo et al. 2020). ILC2s might also express OX40L, which is related to local Th2 cell differentiation, producing epidermal hyperplasia and accumulation of $CD4^+$ T-cell in acute and chronic antigen-driven allergic skin inflammation (Leyva-Castillo et al. 2020; Rafei-Shamsabadi et al. 2019). In this manner, TSLP is a characteristic promoter of atopic inflammation leading to chronic Th2 inflammatory responses (Boguniewicz and Leung 2011; Klonowska et al. 2018). The TSLP produced by keratinocytes plays a crucial role in promoting allergen sensitization in the skin, which ultimately triggers the "atopic march" leading to allergic asthma (Leyva-Castillo et al. 2013).

TSLP expression might also play a role in adaptive immune system and skin barrier function (Choa et al. 2021). TSLP expression has been related to T cell migration to SG in mouse model,

an effect required to enhance sebum secretion and improve organism protection against microorganisms, since sebum contains fatty acids with antimicrobial peptides (AMPs) and bactericidal properties. Thus, although TSLP was correlated with skin diseases and allergies, more functional studies are necessary to better understand the biological functions of this gene, as well as its variants.

Major histocompatibility complex (*MHC) genes*

The MHC molecules are cell surface glycoproteins whose function is to present peptide antigens to T cells. T cell activation may elicit different types of immune reactions, playing an essential role in pathogenesis of infectious and allergic diseases (Orentas et al. 1990; Posadas and Pichler 2007). The human genes encoding MHC molecules such as human leukocyte antigen and HLA are located in a region that spans approximately 3.6 Mb on 6p21.3, and includes not only classical HLA class I (*HLA-A, HLA-B* and *HLA-C*) and II genes (*HLA-DRA1, HLA-DRB1, HLA-DPA1, HLA-DPB1, HLA-DQA1* and *HLA-DQB1)* but also a large number of highly polymorphic genes, encoding proteins with immune-related functions, such as *TNF* (Alfirevic and Pirmohamed 2011).

*HLA-B*13:01* and *HLA-B*44* alleles might play a role in the trichloroethylene (TCE) – hypersensitivity syndrome, which initiates hypersensitivity dermatitis and hepatitis (Li et al. 2007a). TCE is a chlorinated solvent used extensively in industrial operations involving metal cleaning and degreasing and a chemical of concern for occupational exposure. Workers exposed to TCE exhibit a number of dermatological manifestations, such as erythema multiforme, exfoliative dermatitis, Stevens-Johnson syndrome, and toxic epidermal necrolysis (Huang and Li 2006).

The C-terminal peptide side chains, which are lodged in pocket F close to one end of the HLA groove, are important for stability of the HLA/peptide complex (Rammensee, Friede, and Stevanović 1995). *HLA-B*13:01, HLA-B*13:02*, and *HLA-B*44* alleles present three different residues in the pocket F (Ile_{94} $Ile_{95}Arg_{97}$, $Thr_{94}Trp_{95}Thr_{97}$ and $Ile_{94}Ile_{95}Arg_{97}$, respectively). Residue 95, a major component of the pocket F, produces marked alterations in the peptide motifs. Thus, residue Ile_{95} present in *HLA-B*13:01* and *HLA-B*44* alleles might play a role in susceptibility to TCE-induced hypersensitivity dermatitis. *HLA-B*13:01* is an Asian allele absent in European and African descendants (predominantly presenting *HLAB* 13:02* allele), which might account for the outbreaks of TCE-induced hypersensitivity dermatitis in Southern China (Li et al. 2007).

Several relationships between genetic variants and skin irritation responses were identified by Yucesoy et al. (2016). These investigators examined 2131 SNPs from 158 genes in the MHC region, and the associations were predominantly identified with SNPs located in (or near) immune-related genes. The *C2* (rs9332739) was associated with skin irritant reaction to SLS. The *IER3* (rs8512), *TRIM40* (rs1573298), *TRIM10* (rs1557608) and *TRIM31* (rs1264701) SNPs were correlated with response to sodium hydroxide (NaOH) under additive, dominant and recessive genetic models. The *HLA-DPB1* (rs9277554, rs3117228, and rs3130188) and *HLA-DPA1* (rs406477) SNPs were associated with responses to BKC. The rs10046277 in *PSMB9* (proteasome subunit beta type 9) and the rs499384 in *ITPR3* (Inositol 1,4,5-Trisphosphate Receptor Type 3) genes showed a relationship with hand dermatitis in the same study. However, due to linkage disequilibrium between SNPs in this highly polymorphic region, a hypothesis of real associations with other SNPs cannot be excluded.

The possible functional role of those SNPs in skin irritation is still unclear. However, these data suggest that the MHC region may contribute to the genetic basis of skin irritant reactions to chemicals, probably because it harbors multiple genes involved in immune regulation during skin inflammatory reactions (Table 6).

Genes involved in the neuroendocrine system

In the skin, peripheral sensory and autonomic nerves control release of inflammatory mediators from dDCs, mast cells, T cells and keratinocytes, thereby modulating inflammatory responses and, in the case of sensory nerves, pruritus (Blake, Jiang, and Chiu 2019). Thus, understanding the effects of variants in genes whose products contribute to neuroendocrine pathways and skin immune responses is relevant in the context of susceptibility to chemical-mediated skin immunotoxicity.

Table 6. Variants in genes within MHC and their correlation with individual susceptibility to skin immunotoxicity.

Gene	Genetic variants	Mutation	Susceptibility allele or genotype	Functional consequences	Susceptibility to skin immunotoxicity	Association with skin condition or disorder	References
HLA-B	-	-	*HLA-B*1301*	Pocket F with a residue of isoleucine in position 95, causing strong alteration in the peptide motifs	↑	Trichloroethylene-induced hypersensitivity dermatitis	Li et al. 2007
	-	-	*HLA-B*44*		↑		
C2	rs9332739	c.867 G > C p.Glu318Asp	G/ GG	-	↑	ICD to SLS	Yucesoy et al. 2016
IER3	rs8512	g.30743580 G > A (UTR variant)	G/GG	-	↑	ICD to NaOH	Yucesoy et al. 2016
TRIM40	rs1573298	g.30148383 G > C (UTR variant)	G	-	↑	ICD to NaOH	Yucesoy et al. 2016
TRIM10	rs1557608	g.30150805 T > G	AA	-	↑		
TRIM31	rs1264701	g.30098581 G > T	CC	-	↑		
HLA-DPB1	rs9277554	g.33087761 C > G (UTR variant)	G	-	↑	All these variants are associated with ICD to BKC	Yucesoy et al. 2016
	rs3117228	g.33088658 G > T (UTR variant)	C	-	↑		
	rs3130188	g.33089399 T > C (UTR variant)	C	-	↑		
HLA-DPA1	rs406477	g.33037867 T > C	A	-	↑	ICD to BKC	Yucesoy et al. 2016
PSMB9	rs10046277	g.32887304 C > T	G/GG	-	↑	Hand ICD	Yucesoy et al. 2016
ITPR3	rs499384	g.33614313 G > A	G/GG	-	↑	Hand ICD	Yucesoy et al. 2016

↑- increased susceptibility to skin immunotoxicity.
↓- decreased susceptibility to skin immunotoxicity.
ICD: irritant contact dermatitis; SLS: sodium lauryl sulfate; NaOH: sodium hydroxide; BKC: benzalkonium chloride

Calcitonin Related Polypeptide Alpha (CALCA) gene

Calcitonin gene-related peptide (CGRP) is a neuropeptide involved in the process of inflammation, vesicant-induced skin injury and edema (Achanta et al. 2018). Skin irritants promote release of CGRP by sensory receptors (nociceptors) from nerve terminals, which induce vasodilation, edema, and immune cell recruitment (Blake, Jiang, and Chiu 2019). CGRP might also be produced by activated mast cells that, subsequently, take part in an inflammatory vicious cycle, in which other skin local nerves are activated and lead to immune cell recruitment to inflammation site, ultimately promoting skin neurogenic inflammation (Ayasse et al. 2020). Further, CGRP enhances LCs antigen presentation to Th2 cells while inhibiting antigen presentation to Th1 cells (Kabata and Artis 2019).

The human *CALCA* gene is localized on chromosome 11p15.2-p15.1 and codes for calcitonin and CGRP by alternative RNA splicing (Hoovers et al. 1993). A common variant in this gene is located at codon 692 T > C (g.14972978A>G; rs3781719). The TT genotype, associated with high levels of CGRP, contributes to susceptibility to *psoriasis vulgaris* in alcohol-drinking patients (Guo et al. 2015). Although the role of CGRP in pathogenesis of *psoriasis vulgaris* is thus far unclear, it is known that keratinocyte-neuron interactions result in potent feedback loops, exacerbating psoriasis (Blake, Jiang, and Chiu 2019). CGRP induces keratinocyte proliferation and IL-1α and IL-8 expression, which may mediate inflammation and thickened scaly skin in subjects with psoriasis (Blake, Jiang, and Chiu 2019). Moreover, keratinocytes secrete nerve growth factor (NGF) in response to this neuropeptide, which results in neuronal hyperinnervation and penetration of sensory nerves into the uppermost layers of the skin and impairs the skin barrier (Blake, Jiang, and Chiu 2019; Schwendinger-Schreck, Wilson, and Bautista 2015).

Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1) gene

Environmental agents might activate receptors or ion channels such as transient receptor potential (TRP) channels, that act as multimodal sensors for a wide variety of endogenous and exogenous stimuli. Upon activation, TRP transduce electrical and Ca^{2+} signals, resulting in a variety of body reactions to adapt to the environmental changes (Takahashi, Kozai, and Mori 2012). Some of the TRP channels are expressed by sensory nerves and regulate skin neurogenic inflammation and pruritus (Steinhoff et al. 2006; Trier, Mack, and Kim 2019).

TRPV1 is activated downstream of cytokine signaling and by endogenous reactive species including NO and peroxynitrite, oxidized lipids and pH changes that occur during skin inflammation (Ayasse et al. 2020; Blake, Jiang, and Chiu 2019). *TRPV1* is expressed on keratinocytes and sensory nerve fibers, such as C and Aδ, two nerve fibers that mediate sensory irritation (Sun et al. 2016). When activated, TRPV1 exerts an important function in exocytosis of neuropeptides from nerve endings as well as in expression of proinflammatory genes. In an imiquimod-induced psoriasis mice model, the $TRPV1^+$ sensory neurons released CGRP and initiated IL-23 secretion from $CD301b^+$ DCs, resulting in secretion of γδT cell-derived IL-17A and promoting psoriatic skin inflammation (Riol-Blanco et al. 2014). This demonstrates the close relationship between dDCs and $TRPV1^+$ sensory neurons.

TRPV1 activation also promotes release of substance P (SP). SP is a proinflammatory neuropeptide that increases both vasodilation and vascular permeability, upregulates the ICAM-1 expression in human dermal microvascular endothelial cells, stimulates T cell proliferation and enhances expression of proinflammatory cytokines (Kanda and Watanabe 2002; Quinlan et al. 1998; Scholzen and Luger 2004; Weger et al. 2007). SP mediates the chemotaxis of neutrophils, stimulates keratinocyte proliferation, and provokes histamine release from skin mast cells (Ayasse et al. 2020; Borici-Mazi, Kouridakis, and Kontou-Fili 1999). The activation of T cells, mast cells and macrophages by SP, contributes to the imbalance of the immune responses in the early stages of psoriasis (Ayasse et al. 2020). SP might also enhance expression of corticotropin-releasing hormone receptor 1 (CRHR-1) on human mast cell surfaces. Mast cell activation by CRH leads to IL-8, TNF-α, and VEGF secretion and is related to stress induced skin conditions as noted in psoriasis and AD (Asadi et al. 2012). Thus, TRPV1 is an important promoter of neurogenic inflammation, pain, itch and skin diseases (Roosterman et al. 2006); and the capsaicin test –

a test based upon activation of TRPV1 after capsaicin application on the nasolabial fold, is applied to detect skin neurosensitivity (Sun et al. 2016).

Okamoto et al. (2018) investigated several genetic variants in *TRPV1* and found that the rs8065080 homozygous GG genotype (Val-Val phenotype, also known as c.1191A>G) leads to an amino acid substitution (Ile585Val) with concomitant higher capsaicin sensitivity in the Japanese population. Other *TRPV1* intron variant (43,524 G > T (rs4790523), 19,274 G > A (rs117112057), 30,580 G > A (rs57716901), 30,599 G > T (rs61387317), 33,605 G > A (rs8078936), 37,280 C > T (rs57405156), 39,341(rs3826503)) occurred more frequently in capsaicin responsive than in non-responsive group, suggesting that these SNPs might be related to sensory skin irritation to cosmetics (Table 7) (Okamoto et al. 2018; Sun et al. 2016).

The activation of TRPV1 channels also suppresses skin inflammation. TRPV1 activation is required to generate persistent itch in squaric acid dibutylester (SADBE)-induced ADC mice model; however, TRPV1 channels are protective in blocking SADBE-induced ear edema through modulating the function of dermal macrophages (Feng et al. 2017). This also inhibited inflammation process, noted in mouse model of oxazolone-induced CHS (Laverdet et al. 2015). These data provide evidence that skin inflammation and persistent itch are mediated by distinct molecular mechanisms that rely on the type of xenobiotic.

Angiotensin-converting enzyme (ACE) gene

ACE is a zinc metallopeptidase that converts angiotensin I (Ang I) to angiotensin II (Ang II) (Baudin 2002). Active Ang II increases the synthesis of cytokines such as IL-6 and IL-8 and is a potent activator of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme that

Table 7. Variants in genes that affect the neuroendocrine system and their correlation with individual susceptibility to skin immunotoxicity.

Gene	Genetic variants	Mutation	Susceptibility allele or genotype	Functional consequences	Susceptibility to skin immunotoxicity	Association with skin condition or disorder	References
CALCA	rs3781719	g.14972978A > G (Upstream transcript variant)	TT	Located at a promoter region, it promotes high levels of neuropeptide CGRP	↑	Psoriasis vulgaris	Guo et al. 2015.
TRPV1	rs8065080	c.1191A>G (p.Ile585Val)	G/GG	-	↑	Responsive individuals to Capsaicin test with probability to skin neurosensitivity	Okamoto et al. 2018; Sun et al. 2016
	rs117112057	g.22979 G > A (Intron variant)	GA	These genetic variants are located in introns, specifically in the vicinity of splice sites, which may alter protein expression by regulating RNA splicing.	↑		
	rs57716901	g. 34,285 G > A (Intron variant)	AA		↑		
	rs61387317	g.34304 G > T (Intron variant)	TT		↑		
	rs8078936	g.37310 G > A (Intron variant)	AA		↑		
	rs57405156	g.40985 C > T (Intron variant)	TT		↑		
	rs3826503	g.43046 C > T (Intron variant)	TT		↑		
ACE	rs4646994		D/ DD	Causes deletion at the AluYa5 repetitive sequence of ACE gene, which may affect the expression of this gene.	↑	Psoriasis vulgaris; Vitiligo; Acne vulgaris	Elneam, Al-Dhubaibi, and Alrheam 2018; Song et al. 2015; Huang, Huang, and Peng 2017; Almohideb 2020; Basher et al. 2021; Dutt et al. 2018; Sourour et al. 2019.

↑ – increased susceptibility to skin immunotoxicity.

augments ROS production (Cat et al. 2012; Elneam, Al-Dhubaibi, and Alrheam 2018). On the other hand, ACE inactivate bradykinin, which is a potent vasodilator peptide that promotes IL-6, IL-8 and NO synthesis (Elneam, Al-Dhubaibi, and Alrheam 2018; Kuoppala et al. 2000). Both Ang II and bradykinin increase the expression of endothelin-1 (ET-1) (Murphey et al. 2000). ET-1 is a 21-amino acid peptide synthesized by several cell types, including monocytes, which (1) affects vasoregulation, (2) acts as a mitogen for keratinocytes, (3) mediates proinflammatory pathways by synthesizing cytokines IL-6 and monocyte chemoattractant protein-1 (MCP-1), and (4) activates NF-κB (Scholzen et al. 2003; Weger et al. 2007). ACE also degrades SP that is important for skin inflammation (Scholzen et al. 2003). ACE has been identified in different human skin cells including keratinocytes, fibroblasts and endothelial cells (Sorour et al. 2019); thus, ACE plays a role in skin homeostasis and controlling skin inflammatory responses.

The *ACE* gene is located on chromosome 17q23 and has an insertion/deletion (I/D) variant (rs4646994) displaying three genotypes: deletion/deletion (DD), insertion/deletion (ID) and insertion/insertion (II) (Elneam, Al-Dhubaibi, and Alrheam 2018; Weger et al. 2007). The DD genotype confers elevated ACE activity and is responsible for 2-fold higher ACE serum and tissue levels than the II genotype, while the heterozygote genotype (ID) has an intermediate level of this enzyme (Mlak et al. 2016; Tippisetty et al. 2011). Although the underlying causes behind the higher ACE expression in DD genotype are unclear, the deletion is localized within an *alu* element at 287-base pair repetitive sequence in intron 16 of *ACE* (*AluYa5*, some authors describe this change as 288 bp or 289 bp). The *alu* sequences were related to altering gene expression at the post-transcriptional level (alternative splicing, RNA editing and translation regulation) (Häsler and Strub 2006).

The D allele and DD genotype were associated with increased levels of IL-6 and IL-8 and frequently found in patients with severe *psoriasis vulgaris* than those with intermediate and mild disease (Elneam, Al-Dhubaibi, and Alrheam 2018; Huang, Huang, and Peng 2017; Song et al. 2015). Thus, it is likely that the DD genotype might stimulate conversion of Ang I to Ang II and, consequently, IL-6

and IL-8 secretion, triggering an inflammatory response and developing psoriasis. In contrast, *ACE* II genotype and I allele are significantly more prevalent in psoriatic patients compared to controls (Weger et al. 2007; Yang et al. 2013). Previously II genotype was found to be associated with reduced ability to inactivate bradykinin and SP, contributing to development of psoriatic lesions (Murphey et al. 2000; Weger et al. 2007). Discrepant results might result from different types of psoriasis and/or populations in the studies.

Several investigations on the *ACE* I/D variant also demonstrated that DD genotype and D allele may confer a higher risk for vitiligo and Acne Vulgaris (AV) than II genotype. For vitiligo, Egyptian and Asian populations present a higher risk compared to Europeans (Almohideb 2020; Basher et al. 2021). However, as a pleiotropic gene, it has been difficult to understand whether *ACE* I/D variant takes part in the initiation and development of vitiligo. Interestingly, the DD genotype was associated with enhanced ROS production through Ang II, while the II genotype was related to a protective nature against vitiligo due to lower levels of ROS (Jian et al. 2005; Tippisetty et al. 2011). The DD genotype is associated with oxidative stress and angiogenesis, which may facilitate access of cells from the immune system and auto-antibodies to the melanocyte destruction site (Tippisetty et al. 2011).

AV is chronic inflammatory disease of the pilosebaceous unit that involves the overproduction of sebum, hyperkeratinization of sebaceous follicles, *Propionibacterium acnes* colonization, and inflammation (Keyal, Bhatta, and Wang 2016). Although different factors may contribute to the pathogenesis of acne, oxidative stress is one of the most important factors. The high prevalence of the Ang II activator genotype (DD) may support the phenotype of skin inflammation and oxidative stress present in individuals with AV (Sorour et al. 2019).

Conclusions and perspectives

Human activities impact the environment and produce undesirable effects on human health. Increased incidence of immune-related disorders, including allergies, autoimmunity, and inflammatory diseases, is currently verified (Lee and Lawrence 2018). Skin diseases, in general, have multifactorial etiology, in which complex interactions between genetic and environmental factors play an important role and modify disease presentation and severity in manifesting clinical phenotypes (Sacco and Milner 2019). In this context, a variety of host-related factors including age, sex/gender, nutritional, hormonal and central nervous system status, and pathological conditions and chemical-related factors such as nature of the chemical, concentration, type of exposure, chemical reactivity, biotransformation and toxicokinetics are of importance to understand the complex etiology of skin diseases, reducing the risks of developing the disease or developing effective treatments (Corsini and Kimber 2007). The onset of ACD, which is a multifactorial skin disease, depends upon the nature of the chemical, concentration, type of exposure, age, gender, and genetic susceptibility (Corsini and Kimber 2007). In addition, quality of life and psychological distress and emotional dysregulation may influence the incidence of skin diseases (Quinto et al. 2019; Tordeurs et al. 2001).

Much of the difficulty in reducing the incidence of immune skin disorders mediated by chemicals is attributed to lack of knowledge regarding chemical-mediated effects on the immune system, especially when different genetic backgrounds are taken into consideration. Genes are the fulcrum for development and function of immune cells (Lee and Lawrence 2018). The lack of knowledge on the contribution of genetic factors in inter-individual variability to chemical toxicity is also a limiting factor for estimating the risks of chemicals. Predicting all the immunological reactions that different individuals might exhibit to the plethora of chemicals currently available is not possible. Further, failure to identify alterations in the immune system after chemical exposure does not necessarily mean the absence of toxicological risk. Human genetic variability might lead to uncertainties in predicting the risk of immunotoxic responses to chemicals, especially regarding hypersensitivity and autoimmunity.

This review described several genes that modulate skin immune responses (Figure 1). The genetic variants in these genes provide variability

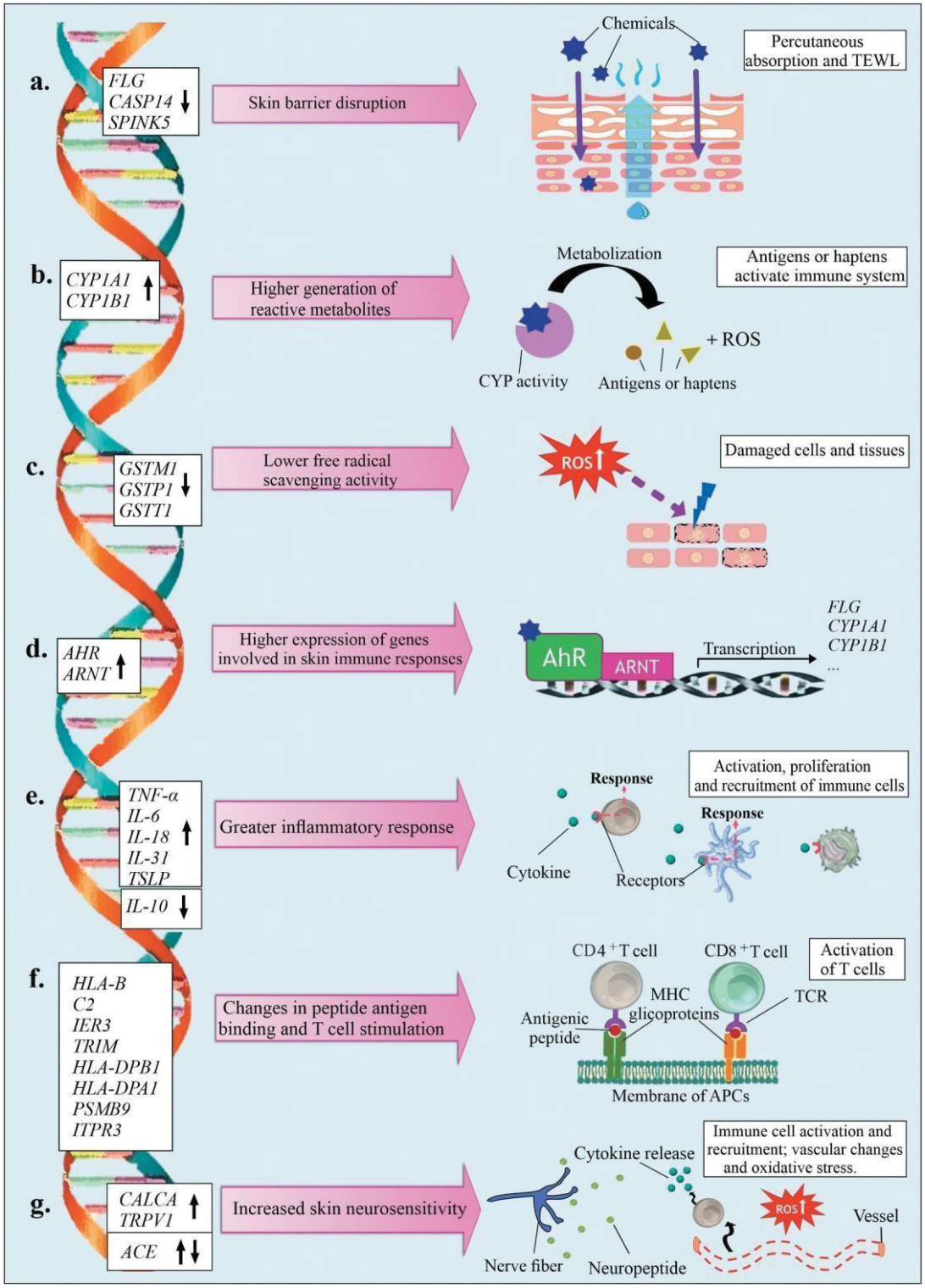

Figure 1. *Cellular and molecular consequences of genetic variants influencing skin immune response to xenobiotics*. Genes in white boxes are associated with key events of toxicity processes. The genetic variants influencing their expression as indicated by the arrow s may affect the skin immune response. a. Higher xenobiotic penetration and TEWL in the skin of low *FLG*, *CASP14*, and *SPINK5* expression genotypes. b. Increased CYP enzyme activity, leading to higher formation of ROS and metabolites that act as antigens or haptens. c. ROS affecting skin cells due to low GSTM1, GSTP1, GSTT1 antioxidant enzyme activity. d. After xenobiotic AhR activation, AhR dimerizes with ARNT and induces the expression of several genes that affect skin immune response. e. Cytokines bind to receptors in immune cells and generate signals that cause inflammatory or anti-inflammatory immune responses depending on the cytokine. f. An example of a variation in MHC: *HLA* genotypes result in differences in peptide antigen binding and T cell stimulation. g. Higher *CALCA*, *TRPV1*, and *ACE* expression affect the release of inflammatory neuropeptides and cytokine production. High *ACE* expression may augment ROS production, and the release of inflammatory cytokines. Low *ACE* expression affects angiogenesis and vasoregulation (facilitating the immune cells access into the skin) and mediates proinflammatory pathways. Upside arrows indicate higher gene expression; downside arrows indicate lower gene expression. TEWL: transepidermal water loss; ROS: reactive oxygen species; MHC: major histocompatibility complex; APCs: antigen-presenting cells.

to the manifestation of skin diseases and may reflect differences in susceptibility to the effects of immunotoxicants. Although a correlation was conducted between genetic susceptibility and skin immunotoxicity, few studies demonstrated the influence of these genetic variants in immune responses following chemical exposure. Thus, large-scale analysis of gene-function (transcriptome) and proteomic investigations with these genetic variants may be relevant to identify their role in skin immunotoxicity. In addition, the limited sample size used by several published studies might affect statistical power and represents one of the limitations for genetics-immunotoxicity association. Importantly, allele and genotype frequencies might not be sufficient to explain immune disease rates; and in-depth investigations regarding epigenetics might be crucial to explain how environmental exposure is associated with rates of immune skin diseases in the human population. Genes and epigenetics modulate innate and adaptive immune cells (Lee and Lawrence 2018); and, investigating the mechanisms underlying this interplay is essential to understanding the distribution of health/disease phenomena and their conditioning and determining factors that may affect immune robustness and plasticity in the context of chemical exposure. Thus, validation of genetic markers of susceptibility is a critical challenge that needs to continue to be explored; however, it would be highly relevant to consider genetic factors in assessing immunotoxicity to minimize the risks of chemicals for humans.

In summary, this review article highlights the relevance of genetic background on skin immune responses by interconnecting molecular and cellular events related to development of skin disorders or immunotoxicity. Based upon the gathered information, there is a need for performing investigations on this matter to help understand better the functional role of these genetic variants on skin immune responses, and thus, providing knowledge that might be useful for reducing uncertainties in predicting chemical hazard/risks for human health.

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3.2. CAPÍTULO II: **The skin sensitization potential of 4-Octylphenol: an evaluation by integrated *in silico-in vitro* test strategy**

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Abstract

One of the major challenges in chemical toxicity testing is the need to protect human health against adverse effects with non-animal methods. In this article 4- Octylphenol (OP) is tested for skin sensitization and immunomodulatory effect by using integrated *in silico-in vitro* test approach. QSAR TOOLBOX 4.5, ToxTree and VEGA were used as *in silico* predictors, while *in vitro* tests include HaCaT cells (quantification of IL-6; IL-8; IL-1α and IL-18 by ELISA and expression of genes *TNF*, *IL1A* and *IL8* by RT- qPCR), RHE model (quantification of IL-6; IL-8; IL-1α and IL-18 by ELISA) and THP-1 activation assay (CD86/CD54 expression and IL-8 release). Additionally, the immunomodulatory effect of OP was tested by investigating lncRNAs *MALAT1* and *NEAT1* expression and LPS-induced THP-1 activation assay. The overall *in silico* results predicted OP as a sensitizer. OP also increased IL-6 expression (HaCaT cells); IL-18 and IL-8 expressions (RHE model); but also presented an irritant potential as defined by a great expression of IL-1α (RHE model); and increased expression of CD54 marker and IL-8 in THP-1 cells, being considered as a sensitizer by both *in silico* and *in vitro* methods. Immunomodulatory effects of OP are demonstrated by underexpression of the genes *NEAT1*, MALAT1 (epigenetic markers), and *IL8*; and an increase in LPS-induced CD54 and IL-8 expressions. Overall, this study suggests that OP is a sensitizer participating in three of the Key Events of the AOP for skin sensitization and present immunomodulatory effects.

Keywords: non-animal alternative methods, QSAR models; activation of keratinocytes;

THP-1 activation assay; long non-coding RNAs

1. Introduction

Consumers are often exposed to chemical substances in cosmetic and household products, most of which are marketed but not adequately tested for toxicity (Panico et al. 2019). 4-Octylphenol (OP) is a commercially produced alkyphenol, the largest nonionic surfactant group. It is mainly a chemical intermediate for producing phenolic resins and lacquers; however, also used in cleaning products, cosmetics and pesticides (Miller & Staples. 2005; Noorimotlagh et al., 2018; de Bruin et al., 2019). OP is slightly soluble in water (19 mg/L at 22°C) and is mainly released into surface waters primarily through the discharge of treated wastewater effluents (Miller & Staples, 2005). Although not persistent, the constant release of OP into the environment may confer risks to human health. For instance, one possible adverse effect in cases of dermal exposure is skin sensitization, for which allergic contact dermatitis (ACD) is the most common manifestation (Anderson et al.. 2014). To date, the ability of OP to affect immune responses and cause skin sensitization is not yet fully investigated and understood.

In the last decades, skin sensitization was exclusively evaluated by animal tests (Skin Sensitisation Guinea Pig Maximisation Test and Buehler Test – OECD Test Guideline (TG) 406; Local Lymph Node Assay (LLNA) in mouse – OECD TG 429); however, animal welfare and scientific concerns drive efforts to foster the development and use of non-animal methods, which have also gained regulatory acceptance. In this context, methods following Adverse Outcome Pathways (AOPs) of skin sensitization have been actively promoted by the Organisation for Economic Co-operation and Development (OECD). AOP is a concept applied to understand and organize key events (KE) within biological pathways that lead to adverse outcomes caused by chemical substances (Sakuratani et al. 2018). The AOP for skin sensitization starts with the covalent binding of electrophilic substances to nucleophilic sites in proteins in the epidermis (KE1). Then, inflammatory responses and changes in gene expression associated with specific cell signalling pathways in keratinocytes (KCs) are triggered and consist in key event 2 (KE2). Key event 3 (KE3) is marked by the activation of dendritic cells (DC), and key event 4 (KE4) by T-cell proliferation (OECD, 2021). Thus, mechanistically-based *in chemico* and *in vitro* test methods as well as *in silico* predictions are selected to support the application of the defined approaches for skin sensitization under the OECD guideline No. 497.

Despite skin sensitization, in the context of dermal exposure, chemical substances can aggravate the level of existing skin inflammation by exerting immunomodulatory effects (Corsini et al. 2011;2012). The immunomodulatory effect of chemicals may cause a reduction in immunity, which results in immunodeficiency and increased susceptibility to infectious microorganisms or enhancement of the immune response, which results in allergy (Chalubinski et al. 2006; Kuo et al. 2012). This effect deserves to be investigated when taking the rapid and dramatic increase in the prevalence of allergy over recent decades into consideration (Murrison et al. 2019).

In the present study, we aimed to fulfil knowledge gaps regarding the skin toxicity potential of the environmental contaminant OP. Thus, OP was studied regarding its skin sensitization potential and immunomodulatory effects in LPS-induced responses and lncRNAs associated with skin diseases and inflammatory cytokine expression. For that, only non-animal methods were considered in this study. For skin sensitization, an integrated *in silico-in vitro* test strategy covering different Kes of AOP for skin sensitization was used. Additionally, among the *in vitro* methods for skin sensitization, assays with immortalized human keratinocyte cell line (HaCaT cells), human leukemia monocytic cell line (THP-1 cells) and reconstructed human epidermis (RHE) model were used in this study to increase weight-of-evidence of *in vivo* responses to OP.

2. Material and methods

2.1. Test chemical

4-octyphenol (OP, CAS No. 1806-26-04, EC Number 2173025) at 99% purity was purchased from Sigma-Aldrich.

2.2. In silico data

Four *in silico* models, including the OECD QSAR Toolbox (version 4.5 at www.qsartoolbox.org), were used for the *in silico* predictions of the skin sensitization potential of OP.

The QSAR Toolbox uses an analogue-based read-across approach or structural alerts for protein binding identified by profilers to predict whether a chemical will be a sensitizer. It is part of one of the Integrated Testing Strategies (ITS) of the OECD Guideline No. 497:

Defined Approaches on Skin Sensitization, which consist of a fixed data interpretation procedure (*e.g.*, mathematical models, rule-based approaches) applied to *in silico*, *in chemico,* and/or *in vitro data* generated with a defined set of information to derive a prediction equivalent to that provided by animal studies without the need for expert judgment. The recent OECD QSAR Toolbox v.4.5 counts with an automated workflow for defined approaches for skin sensitization (DASS AW) to be used as the *in silico* source. The applicability domain of the QSAR Toolbox Skin sensitization predictions is based on the training set of 2268 substances, including experimental data of LLNA and/or GPMT. The test chemical (OP) was input using CAS No. (1806-26-4), and the option for automated workflow for "EC3 from LLNA or Skin sensitization from GPMT assays for defined approaches" was chosen in the "data gap filling module". This automated workflow is prepared to predict the substance as positive (sensitizer) or negative (non-sensitizer) using read-across or a customized profiler.

The second *in silico* prediction tool for skin sensitization alerts was the Toxtree v2.6.13 (freely available at www.toxtree.sourceforge.net), which is based on the identification of mechanisms of action for skin sensitization using a SMARTS pattern-based approach (Enoch et al. 2008).

The third and fourth prediction models were retrieved from the Virtual models for property Evaluation of chemicals within a Global Architecture (VEGA) platform (version 1.2.0; available at www.vegahub.eu): Skin sensitization model Caesar (version 2.1.7) and IRFMN/JRC model (1.0.1 from the Istituto di Ricerche Farmacologiche Mario Negri). Both models are based on the LLNA (Local Lymph Node Assay) in mice and on a data set that categorizes sensitizer compounds using the ranges established by the European Centre for Ecotoxicology and Toxicology of Chemicals *(*ECETOC*)*: extreme (EC3<0.1%), strong (0.1%<EC3<1%), moderate (1%<EC3<10%), weak (EC3>10%) and non-sensitizer.

The classification of OP into sensitizer or non-sensitizer was defined according to the results based on all the prediction models employed.

2.3. In vitro assays

2.3.1. In vitro models and biomarkers

Three *in vitro* models were used in this study: HaCaT cells (immortalized human keratinocyte cell line, Cell bank of Rio de Janeiro - BCRJ, Cat. No. 0341, Brazil); THP-1 cells (human leukemia monocytic cell line, Elabscience Biotechnology Inc., Cat. No. EP-CL-0233, 14,780 Memorial Drive, Suite 216, Houston, Texas, USA); and RHE model (EpiDerm™, MatTek Corporation, Ashland, Bratislava, Slovakia). HaCaT cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (both from Gibco, Life Technologies, USA) and addition of 100 IU/mL penicillin G, 100 mg/mL streptomycin, and 1 µg/mL amphotericin. THP-1 cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 10% heat-inactivated fetal calf serum (FCS) and addition of 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.01 ng/mL gentamycin (Iulini et al., 2022). Both cultures were maintained at 37°C, 5% CO_2, and 95% relative humidity. Subcultures were performed when cells reached approximately 80% confluency for HaCaT cells and every 3-4 days for THP-1 cells.

EpiDerm™ models were maintained according to the manufacturer's instructions in the DMEM-based tissue culture medium provided by the supplier at 37°C, 5% CO_2 and 95% relative humidity for 24 h (stabilization period before performing the experiments). These models were tested for several markers important in the KEs of the AOP for skin sensitization. Inflammatory responses as well as changes in gene expression (KE2) were covered by employing HaCaT cells and RHE. The expression of inflammatory cytokines (IL-6, IL-1α, IL-18 and IL-8) and expression of *IL6*, *IL8*, *IL1A*, *MALAT1*, *NEAT1* genes were investigated in HaCaT cells. RHE was used as a model to investigate the expression of the same cytokines investigated in HaCaT cells (IL-6, IL-1α, IL-18, IL-8).

The activation of dendritic cells (DC) (KE3) was typically assessed by the expression of specific cell surface markers CD86 and CD54 and chemokine IL-8 release in THP-1 cells.

2.3.2. Test concentrations and exposure conditions

HaCaT and TPH-1 cells were exposed to OP at 0.005, 0.05, 0.5, 5 and 50 µg/mL (sub-cytotoxic concentrations defined by cell viability assays) in the 2D culture assays. For gene expression assay by RT-qPCR, HaCaT cells were exposed to OP at 50 µg/mL. Test solutions were prepared using Dimethyl sulfoxide (DMSO) at the final concentration of

0.1%-v/v as a solvent. These cells were exposed to OP or controls directly through the culture medium and incubated for 24h or 48h.

RHE was exposed to OP at 50, 5 and 0.5 mg/mL in acetone olive oil (AOO) 4:1. For the exposure, Finn Chamber filter paper discs 7.5 (for epiCS®) or 11 mm (for VUMC- EE) (Epitest LTD Oy, Finland) were impregnated with 25 uL of chemicals or controls and applied topically to the RHE stratum corneum. Cultures were incubated for 24h. After incubation, filter paper discs were removed, and metabolic activity was determined immediately by the MTT assay and culture supernatants harvested and stored at -20°C for posterior interleukin quantification by ELISA. The experiments were carried out with negative (culture medium) and solvent controls (DMSO 0.1%-v/v: 2D cultures; AOO 1:4: RHE model). Positive control (PC) information is below described in each assay performed.

2.3.3. Cell viability assays

Different cell viability assays were used to select the tested sub-cytotoxic concentrations and to define positive responses in the RHE model assay. Thus, MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was used for HaCaT cells and RHE models, while THP-1 cell viability was evaluated by propidium iodide (PI, Sigma-Aldrich) using flow cytometry. Briefly, HaCaT cells ($5x10^4$ cells/well, 96-well plate) were exposed to OP (6 concentrations from 0.5 -1000 µg/mL) and incubated with MTT at 0.5 mg/mL for 3 h. After incubation, formazan crystals were dissolved with DMSO and the absorbance was read by the microplate reader Infinite 200™ (Tecan) (Mosmann, 1983). For the RHE, the exposed models were incubated with MTT at 5 mg/mL for 3 h. The tissues were then transferred to 24-well plates containing isopropanol. The plates were sealed with parafilm, protected from light, and incubated overnight at room temperature under an orbital shaker to extract the formazan crystals. The RHE models were discarded, and the absorbance of the extract solutions was taken at 540 and 570 nm. For THP-1 cells, $1x10^6$ cells/mL were exposed to OP (five concentrations from 0.005 to 50 µg/mL) and stained with PI (0.625 µg/mL), and the fluorescence intensity of labelled cells was acquired by flow cytometer (NovoCyte 3000, ACEA Biosciences, Inc). Triton X-100 1%-v/v was used as a PC for HaCaT cells and dinitrochlorobenzene (DNCB), which is a very strong contact allergen, was used as PC for RHE.

2.3.4. ELISA: quantification of cytokines

The cytokines IL-1α, IL-18, IL-6 and IL-8 were quantified in HaCaT ($2x10^5$ cells/well, 24-well plate) and RHE model assay, while the release of IL-8 was quantified in THP-1 cells. All cytokines were quantified in culture supernatants of RHE model and THP-1 cells. For HaCaT cells IL-6 and IL-8 were evaluated in the culture supernatants and IL-1α and IL-18 were evaluated regarding intracellular content. For quantifying intracellular cytokines, HaCaT cells were lysed by incubating the cells in EDTA 0.05% v/v for 5 min at 37°C in a 5% CO_2 in air atmosphere and then in Triton-X 100 0.5%-v/v for 15 min on ice. Cell lysates were harvested and stored at – 80°C till the analysis. In this case, total protein was also determined by the bicinchoninic acid method (BCA) to be later used in normalizing intracellular cytokines data (Corsini et al. 2013). P-phenylendiamine (PPD) 60 µg/mL was used as a PC for IL-18 quantification in HaCaT cells. DNCB was used as a positive control in RHE models.

The cytokines were quantified using commercial kits according to the instructions provided by the manufacturers. The following ELISA kits were used in the present study: Human IL-6 ImmunoTools sandwich ELISA (Cat. No. 31670069), Human IL-8 ImmunoTools sandwich ELISA (Cat. No. 31670089), Human IL-1α ELISA MAX™ Deluxe Set (Cat. No. 445804), and MBL (Nagoya, Japan). The absorbance of the microplates was read at 450 nm. Experiments were carried out in three technical replicates and 2-3 biological replicates.

2.3.5. Expression levels of inflammatory mediators by RT- qPCR

HaCaT cells were plated in a 6-well plate ($4x10^5$ cells/well) and let grow for 24 h for stabilization and then treated for 24 h. Subsequently, the RNA of HaCaT cells (treated and control cells) was extracted using the illustra™ RNAspin Mini SV Total RNA Isolation System (Cat. No. 25-0500-71; GE Healthcare), following the manufacturer's instructions. The extracted RNA was quantified using NanoDrop (Thermo Fisher Scientific), and the cDNA was obtained by High-Capacity cDNA Reverse Transcription kit (Cat. No. 4368814; Applied Biosystems). The expression of genes from inflammatory cytokines (*IL1A*, *IL6*, *IL8* and *TNF*) and long non-coding RNAs involved in inflammation (*NEAT1* and *MALAT1*) was quantified by quantitative reverse transcription polymerase chain reaction (RT-qPCR)

using Power SYBR® Green PCR Master Mix (Cat. No. 4367659; Applied Biosystems). The primer sequences are presented in Sumplementary Table 1.

STable 1. Primer sequences used in the RT-qPCR.

Genes	Direction	Sequence
GAPDH	F	5′-ACCCACTCCTCCACCTTTGA-3′
	R	5′-CTGTTGCTGTAGCCAAATTCGT-3′
TNF	F	5'-CCGAGGCAGTCAGATCATCTT-3'
	R	5'-AGCTGCCCCTCAGCTTGA-3'
IL6	F	5′-AGATTTGAGAGTAGTGAGGAACAAG-3′
	R	5′-CTGGCATTTGTGGTTGGGTC-3′
IL8	F	5′-ATACTCCAAACCTTTCCACCCC-3′
	R	5′-CCTCTGCACCCAGTTTTCCTTG-3′
IL1A	F	5′-CAGCCAGAGAGGGAGTCATT-3′
	R	5′-GGAGGAACTGTCTTCTTCATTTTCA-3′
NEAT1	F	5’-TCTCCATTTCCCCATCTGAG-3'
	R	5’-CAGCCACAGAAAAGGGAGAG-3’
MALAT1	F	5’-GGGTGTTTACGTAGACCAGAACC-3’
	R	5’-CTTCCAAAAGCCTTCTGCCTTAG-3’

F: forward; R: reverse.

2.3.6. THP-1 activation assay

The THP-1 activation assay is based on the upregulation of CD86 and CD54 surface membrane markers and the release of IL-8. The membrane markers CD83 and CD54 and IL-8 quantification were assessed according to Iulini et al. (2022). Briefly, the cells were exposed to OP at 0.005, 0.05, 0.5, 5 and 50 µg/mL for 24 h. After exposure, the suspension of cells was collected into cytometer tubes and centrifuged at 1200 rpm for 5 minutes. Supernatants were collected and stored at -20°C for posterior quantification of IL-8 (above described). Cells in the tubes were marked with FITC Mouse anti-human CD86 monoclonal antibody and PE Mouse anti-human CD54 monoclonal antibody and incubated for 30 minutes at 4°C. Each treatment had κ Isotype Control, marked with FITC Mouse IgG1 (for

CD86 monoclonal antibody) and PE Mouse IgG1 (for CD54 monoclonal antibody). The membrane markers CD83 and CD54 were also assessed by NovoCyte 3000 flow cytometer (ACEA Biosciences, Inc).

2.3.7. Immunomodulatory effect

Considering the significant results, 2 from the 5 tested concentrations were selected to verify the immunomodulatory properties of OP by its capacity to increase LPS-induced inflammation. In this case, cells were exposed to OP at 5 and 50 µg/mL for 24 h and LPS (1000 ng/mL to CD86 and 100 ng/mL to CD54) was added for an additional 24 h. Cells were then centrifuged and marked with antibodies for CD86 and CD54, as previously described. LPS plus DMSO were used as SC. Supernatants of cells exposed to LPS at 1000ng/mL were collected and stored at -20ºC for posterior IL-8 quantification. IL-8 was quantified using Human IL-8 ImmunoTools sandwich ELISA Cat. No. 31670089, according to the supplier's instructions. Experiments were performed in three technical replicates and three independent experiments to determine the expression of surface markers and the release of IL-8.

2.3.8. Data analysis

For the cytotoxicity measured by MTT assay, 70% of cell viability of HaCaT cells was considered optimal to select the concentrations. For this analysis, cell viability of the treatments was defined by comparing with the viability of the SC (DMSO), which was established as 100%.

For RHE, between 5 to 50% of cell viability was considered optimal for identifying allergens (Gibbs et al. 2013). Cell viability of the vehicle control (AOO 4:1) was considered 100%. For cytotoxicity measured by PI-stained THP-1 cells, the concentration of the tested chemical resulting in up to 75% viability was considered not cytotoxic. Statistical significance among treated and control cells was determined by Kruskal-Wallis (nonparametric, one-way ANOVA) using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant when $p>0.05$.

The cell viability of HaCaT and THP-1 cells was determined by IC_{20}- IC_{25} (20-25% of cytotoxicity accepted) and CV75% (Cell Viability of 75%), respectively. For RHE models, the cell viability was expressed as a percentage relative to the absorbance value of the SC.

For interleukin quantification by ELISA, the results were calculated in pg/mL from a standard curve and then converted into Stimulation Index (SI). SI was obtained by dividing the concentration of interleukin in pg/mL of the samples and CP by the concentration of interleukin in pg/mL of the SC (fold-change). For intracellular interleukin quantification (IL-18 and IL-1α), the result was expressed as pg/mg of total cell protein as assessed by the BCA protein determination method, as showed by the example in the following equation:

$$\text{IL-18} = \frac{\text{IL-18 (pg/ml) in cell lysate}}{\text{Total protein content (mg/ml) in cell lysate}} = \text{pg/mg}$$

OP was considered a contact sensitizer when SI IL-6 or IL-1α ≥ 3; SI IL-18 ≥1.2 in HaCaT cells (Jeon et al. 2019; Corsini et al. 2013). For the RHE model, the significant SI values indicate sensitization when SI IL-18 ≥ 2 and SI IL-8 > IL-1α. But if SI IL-1α > IL-8 the chemical was considered an irritant (Galbiati et al. 2017; Coquette et al. 2003).

The results of the RT-qPCR were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test using ΔCT values of SC cells and ACH-treated cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control, and the expression levels of target genes were normalized relative to the control by the $2^{-\Delta\Delta CT}$ method (Leme et al. 2018). RT-qPCR was performed in technical and biological triplicates. The differences related to the SC were considered significant when fold-change >2 and $p< 0.05$.

The expression of CD86 and CD54 surface markers in THP-1 cells was analyzed by flow cytometry with the acquisition in channels FL-1 (FITC) and FL-3 (PE). A total of 10,000 events were acquired. Gate settings can be found in Iulini et al. (2022). By using the geometric mean fluorescence intensity (MFI), the relative fluorescence intensity (RFI) of markers in the SC cells and OP-treated cells were calculated according to the following equation:

$$RFI = \frac{\text{MFI of chemical treated cells} - \text{MFI of chemical treated isotype control cells}}{\text{MFI of vehicle treated control cells} - \text{MFI of vehicle treated isotype control cells}}.$$

For THP-1 cells, results indicate contact sensitization when SI CD86 and CD54 ≥ 1.5 and/or a significant increase in IL-8 is found for any tested concentrations (Iulini et al. 2022).

3. Results

3.1. In silico predictions

Results investigated through computational profilers are summarized in Table 2. Using the data gap filling method of QSAR Toolbox Automated workflow for EC3 from LLNA or Skin sensitization from GPMT assays for defined approaches (SS AW for DASS), OP was found to be positive (ECETOC Skin sensitization II) as a result of the profiler "Protein binding alerts for skin sensitization by OASIS with Skin metabolism simulator". This profiler identified an alert for Michael addition. No alerts were found without metabolism.

The ToxTree v2.6.13 available online has not identified mechanisms of toxic action for skin sensitization. Based on this platform OP does not present alerts such as: SNAr-nucleophilic aromatic substitution, Schiff base formation, Michael Acceptor, acyl transfer agent and SN2- nucleophilic aliphatic substitution.

The CAESAR model available at the VEGA platform predicted OP as a sensitizer, and this prediction was considered to be in the Applicability Domain (ADI=0.925). The IRFMN-JRC model showed the same result, although its prediction could be out of the Applicability Domain (ADI=0.661).

Table 2. Summary of *in silico* skin sensitization prediction for OP

Platform	Result	AD
QSAR TOOLBOX 4.5	Positive	Into AD
TOXTREE	Negative	ND
VEGA (CAESAR)	Positive	Into AD
VEGA (IRFMN-JRC)	Positive	May be out of AD

AD = Applicability Domain; ND = not defined.

3.2. In vitro assays

3.2.1. HaCaT assays

The viability of HaCaT cells was affected by OP (decreased), especially at the three highest tested concentrations (250-1000 µg/mL) (Fig. 1). Also, OP significantly increased the release of IL-6 in exposed HaCaT cells. This effect was verified with the magnitude of effect in the order of 2.9- (5 µg/mL) and 3.65- fold increase (50 µg/mL) (Fig. 2b). The levels of the

other cytokines (IL-8, IL-1α and IL-18) were not affected in HaCaT cells exposed to OP concentrations (Fig. 2). Regarding the gene expression analysis (RT-qPCR) from the inflammatory cytokine genes evaluated (*TNF*, *IL6*, *IL8*, *IL1A*), significant changes in the mRNA levels were only verified to *IL8*, which showed to be downregulated in HaCaT cells exposed to OP at 50 µg/mL. The expression of the lncRNAs *NEAT1* and *MALAT1* also showed significant downregulation in HaCaT cells after exposure to OP (Fig. 3).

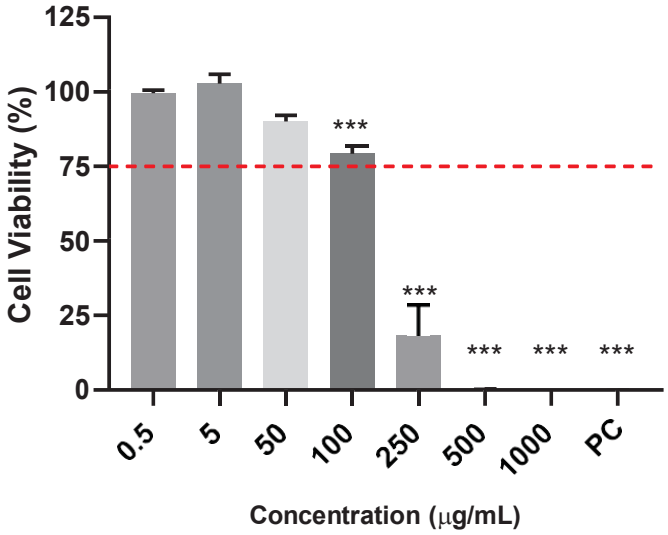

Figure 1. HaCaT cell viability after exposure (24 h) to octylphenol (OP) by the MTT assay. The red line represents the solvent control (100% of viability). PC: positive control (Triton X-100 1%-v/v). Data are expressed in mean ± SD and represent three independent experiments. ***$p < 0.001$.

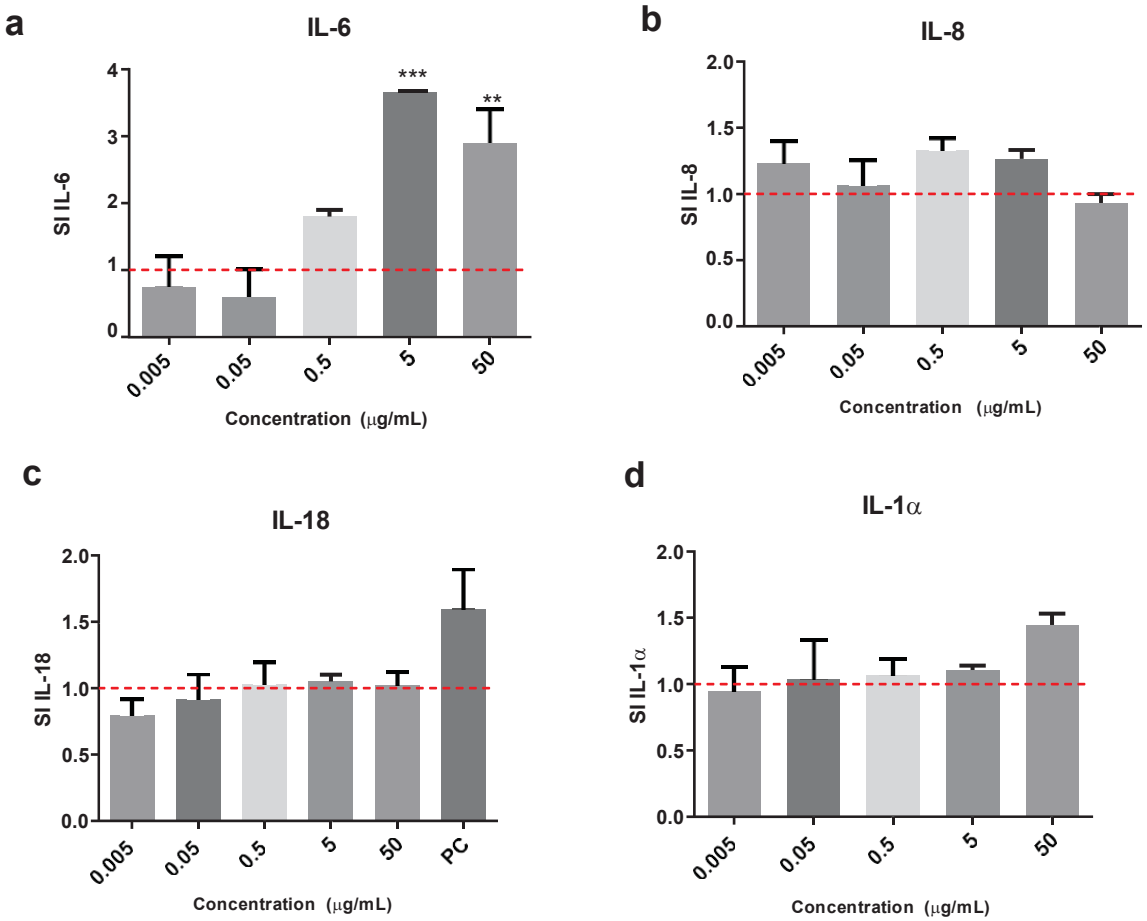

Figure 2. The evaluation of inflammatory cytokines in HaCaT cells after exposure (24 h) to octylphenol (OP) by ELISA assay. IL-8 and IL-6 were quantified in HaCaT culture supernatants, and IL-18 and IL-1α the quantification were performed in the intracellular content. The red line represents solvent control. PC: positive control (p-phenylendiamine). SI: stimulation index (fold-change over SC). The data is representative of three (IL-8 and IL-18) and two biological replicates (IL-1α and IL-6). Data are expressed in mean ± SEM and represent three independent experiments. **$p < 0.01$; ***$p < 0.001$.

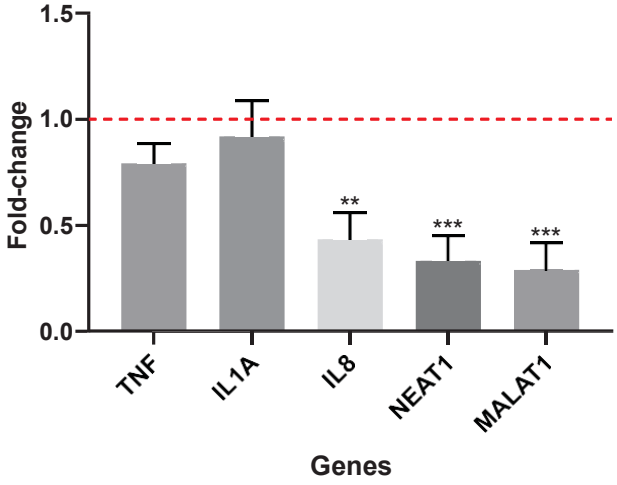

Figure 3. Fold-change in gene expression of inflammatory cytokine genes and long non-coding RNAs in HaCaT cells exposed (24 h) to octylphenol (OP) quantified by RT-qPCR. Results were normalized by the housekeeping gene GAPDH and expressions of the target genes in OP-treated cells relative to the solvent control (red line) are shown as mean ± SEM of three independent experiments. **$p < 0.01$; ***$p < 0.001$.

3.2.2. RHE model assays

RHE models exposed to OP were evaluated regarding the release of inflammatory cytokines relevant to skin sensitization and cell viability. The MTT assay shows that OP at 50 mg/mL decreased tissue viability; however, this was in the optimum viability range ($5 \leq 50\%$ viability) to obtain accurate responses for cytokine release and identify sensitizers (Gibbs et al. 2013). Thus, RHE models exposed to 50 mg/mL OP showed significant levels of IL-1α (52.40-fold), IL-18 (8.73-fold) and IL-8 (3.2-fold). OP at 5 mg/mL only affected the release of IL-8 (2.76-fold) in this model, and OP at 0.5 mg/mL (the lowest tested concentration in the RHE model assay) did not affect the release of any of the tested cytokines (Fig. 4).

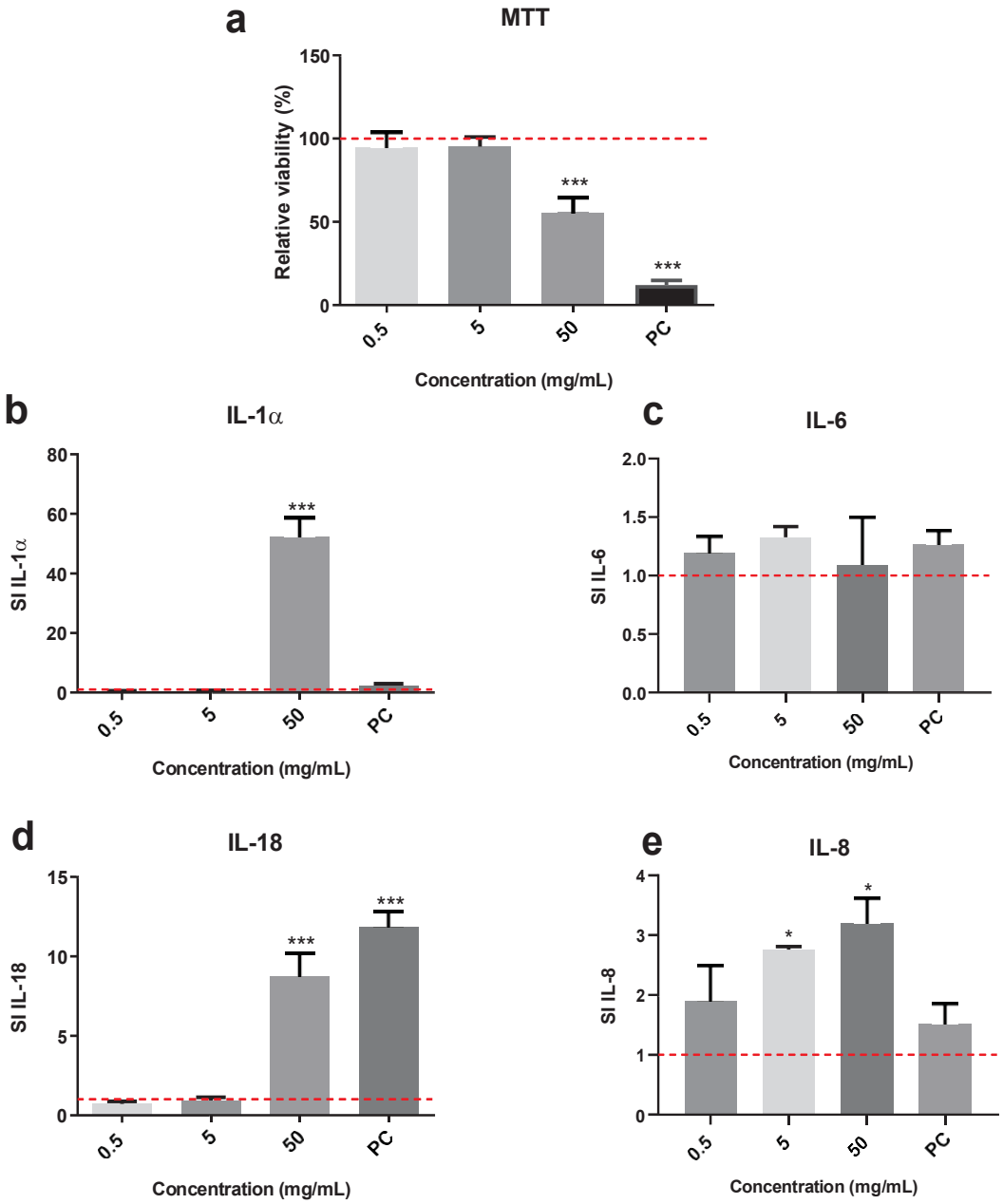

Figure 4. Cell viability (a) and release of inflammatory cytokines (b-e) in RHE models exposed (24 h) to octylphenol (OP). The red line represents solvent control. PC: positive control (dinitrochlorobenzene). SI: stimulation index (fold-change over SC). The data is representative of three (IL-18 and IL-6) or two biological replicates (IL-1α and IL-8). Data are expressed in mean ± SD. *$p < 0.05$; ***$p < 0.001$.

3.3.3. THP-1 assays

The PI quantification in THP-1 cells by flow cytometry showed that OP did not significantly affect the viability of this cell line (Fig. 5).

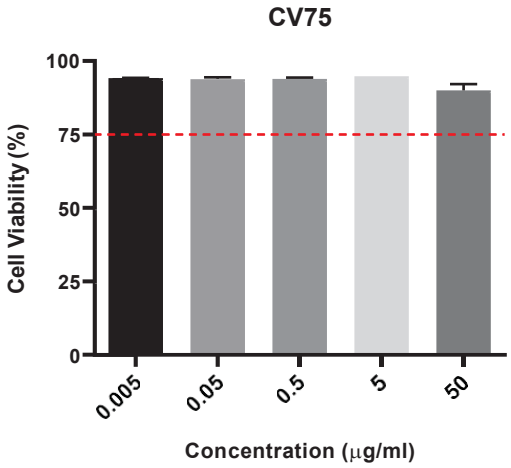

Figure 5. THP-1 cell viability after exposure (24 h) to octylphenol (OP) by PI staining quantified with flow cytometry. The red line represents the cut-off for cytotoxicity (≥ 75% of viability). Data are expressed in mean ± SD and represent three independent experiments.

The activation of THP-1 cells was evaluated by the expression of CD86 and CD54 and the production of IL-8. OP at 50 µg/mL significantly increased the release levels of IL-8 by 1.81-fold and the expression of the membrane markers CD86 and CD54 (1.14 and 2.14-fold increase, respectively) in the exposed THP-1 cells (Fig. 6). This positive result in THP-1 activation assay led us to test the potential of OP to act as an immunomodulatory substance in LPS- induced skin inflammation. This evaluation showed that OP could significantly enhance the production of IL-8 and the expression of CD54 at 50 µg/mL compared with the individual effect of LPS (Fig. 7).

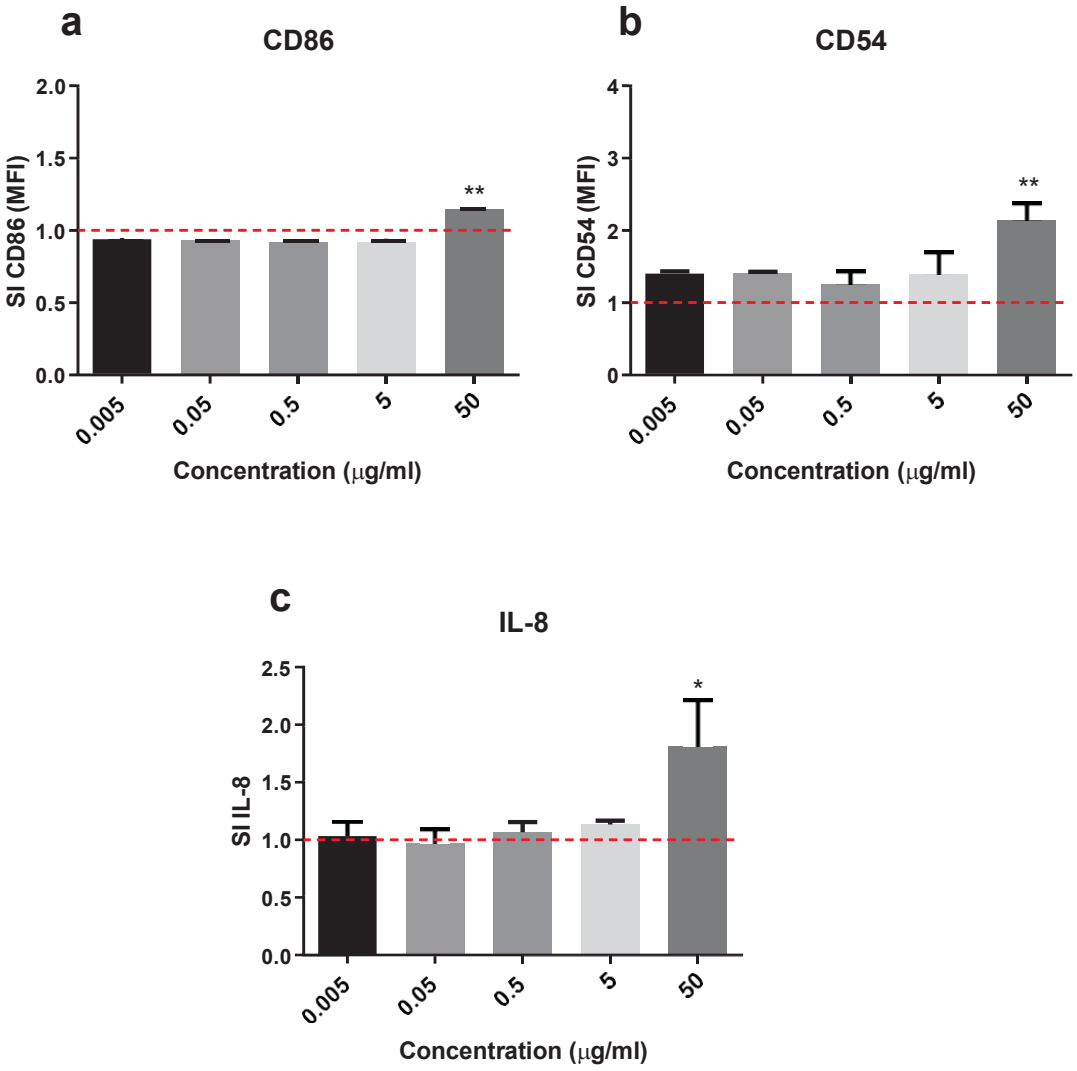

Figure 6. The activation of THP-1 cells after exposure (24 h) to octylphenol (OP). The red line represents solvent control. SI: stimulation index (fold-change over SC). Data are expressed in mean ± SD and represent three independent experiments. *$p < 0.05$; **$p < 0.01$.

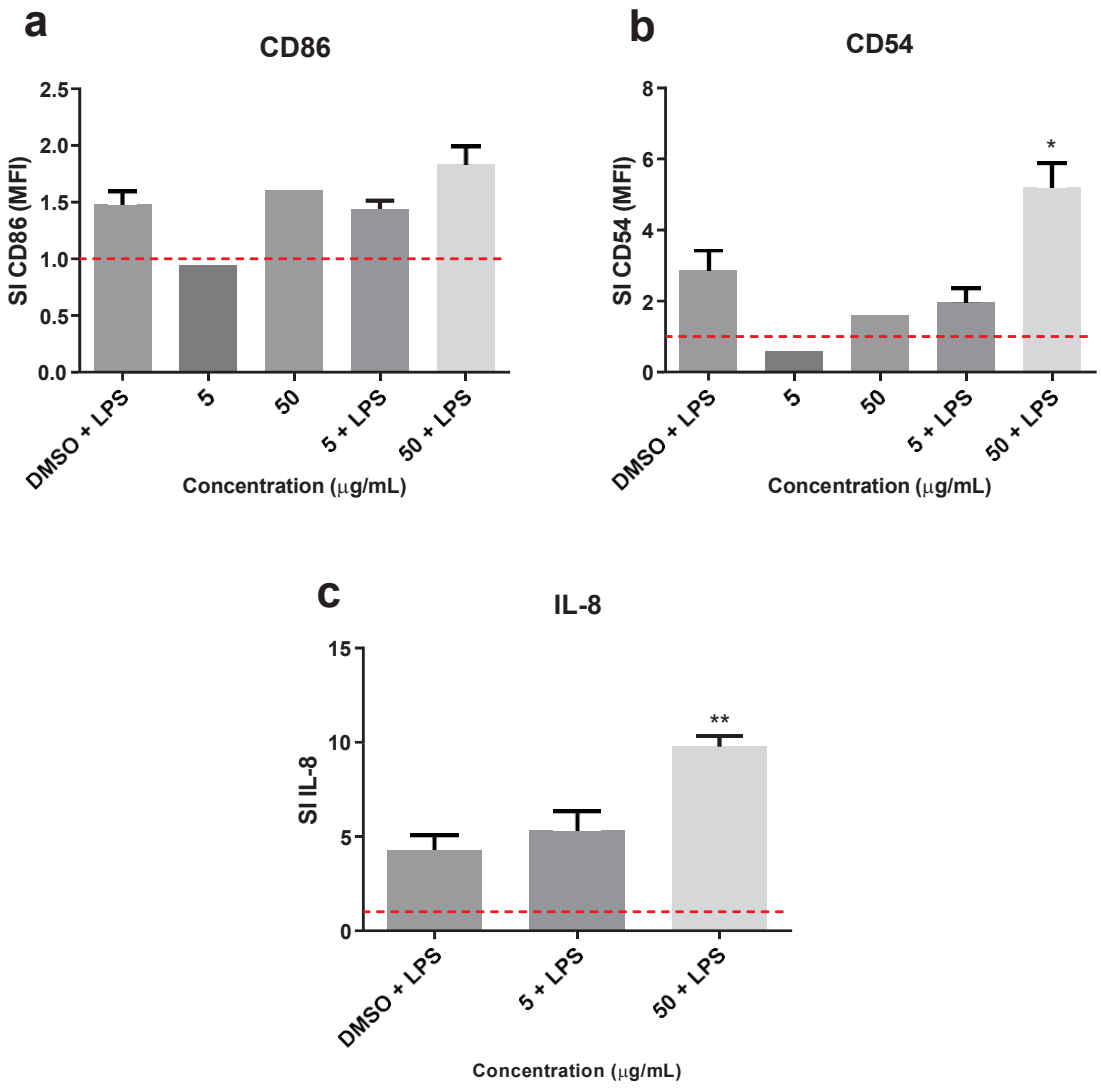

Figure 7. The evaluation of the potential of octylphenol (OP) in modulating LPS-induced THP-1 activation. The red line represents solvent control. SI: stimulation index (fold-change over DMSO+ LPS). Data are expressed in mean ± SD and represent three independent experiments. *$p < 0.05$; **$p < 0.01$.

3.3.4. Comparison of the responses obtained from the methods used in this study

Table 2 summarizes the *in silico* predictions and *in vitro* results regarding the skin sensitization potential and capacity to modulate skin inflammation of OP. The *in vitro* results regarding skin sensitization were organized based on the defined AOP.

Table 2. The overall results for the potential of octylphenol (OP) for skin sensitization and for modulation of skin inflammatory response by an integrated *in silico-in vitro* test strategy.

In silico: Skin sensitization				
Key events	*Model*	*Endpoint*	*Result*	*Conclusion*
1: Protein binding	QSAR TOOLBOX 4.5	Structure-based predictions for alerts related to protein binding	Michael addition alert	Sensitizer
	TOXTREE	Structure-based predictions for alerts related to protein binding	No alerts found	Non sensitizer
-	VEGA (CAESAR; IRFMN-JRC)	Structure-based predictions on the LLNA assay	Positive	Sensitizer
In vitro: Skin sensitization				
Key events	*Model*	*Endpoint*	*Result*	*Conclusion*
2: KC activation	HaCaT cells	Release of inflammatory cytokines (IL-6, IL-1α, IL-18, IL-8)	Increased IL-6 release (SI IL-6 > 3)	Sensitizer
	HaCaT cells	Expression of the genes *IL6*, *IL8 IL1A* and *TNF*	Downregulation of *IL8*	-

	RHE model	Release of inflammatory cytokines (IL-6, IL-1α, IL-18, IL-8)	Increased IL-18 release (SI IL-18 > 2)	Sensitizer
	RHE model	Comparison of the release levels of IL-8 and IL-1α	Increased IL-8 and IL-1α release (SI IL-1α > SI IL-8)	Irritant
3: Activation of DCs	THP-1 cells	THP-1 activation assay (CD86; CD54 and IL-8 expression)	Increased expression of CD54 (SI CD54 > 1.5); and significant release of IL-8	Sensitizer

Modulation of Skin Inflammatory Responses			
Model	*Endpoint*	*Results*	*Conclusion*
HaCaT cells	Expression of the lncRNAs genes *(MALAT1 and NEAT1)*	Downregulation of the lncRNA genes	May cause changes in skin homeostasis and regulation of inflammatory response
THP-1 cells activated by LPS	Expression of CD86 and CD54, and release of IL-8	Enhances LPS-induced CD54 (SI CD54 > 1.5) and IL-8 release.	Aggravates immune response, that may result in DC activation and allergy

LLNA, murine local lymph node assay; SI, stimulation index; lncRNAS, long noncoding RNAs.

Note: Although results are aligned with single KEs, the endpoints in the assays also recapitulate prior key events.

4. Discussion

Integrated *in silico-in vitro* test strategies for skin sensitization have been previously proposed, the most important being the recent OECD 497 (OECD, 2021), in which two defined approaches (DA) are described. The first one is the combination of test methods covering 2 out of 3 KEs of the AOP leading to skin sensitization (KE1 – *in chemico* method; KE2/KE3 – *in vitro* methods). The second strategy is based on *in chemico* (KE1) and/or *in vitro* data (KE3), completed with *in silico* predictions based on Derek Nexus or QSAR Toolbox 4.5.The validated OECD methods DPRA (TG 442C) (KE1), KeratinoSens™ (TG 442D) (KE2), and h-CLAT (TG 442E) (KE3) are considered for these DA.

In the present study, the skin sensitization potential of OP was evaluated by an integrated strategy based on a set of *in silico* models and *in vitro* methods covering protein binding (KE1), KC activation (KE2) and DC activation (KE3) – 3 out of 3 KEs of the AOP for skin sensitization. However, in this integrated strategy, models and biomarkers differ from those accepted by OECD due to the limited access of some of the *in vitro* models used in the OECD TG (*e.g.*, the KC cell line for KeratinoSens™ method), as well as to provide additional predictive evidence of KC activation response for humans (3D skin model).

Most *in silico* methods are based on assigning chemicals to electrophilic mechanisms according to the local lymph node assay (LLNA) dataset available in the literature. Chemicals elicit an immune response through their ability to react with nucleophilic reaction centers within skin proteins via one of five electrophilic–nucleophilic chemical reactions (Michael-type addition reactions, Schiff base formation, acylation, nucleophilic aromatic substitution (SNAr) and second order nucleophilic aliphatic substitution (SN2)) (Enoch et al., 2008). According to the OECD QSAR Toolbox protein binding profiler (by OASIS), a Michael addition alert was detected for OP after substance metabolization but not in the absence of metabolic transformation, indicating skin sensitization. Both VEGA models (Skin CAESAR Model and IRFMN/JRC Skin Sensitization model) confirmed this result and considered OP as a sensitizer. ToxTree, on the other hand, considered OP as a non-sensitizer, as no correlation for electrophilic reactivity (including Michael addition) was detected. When skin sensitization is evaluated by *in silico* tools, the applicability domain (AD) should be referred to. The AD of the QSAR Toolbox Skin sensitization is defined based on the training set of more than 2000 substances having experimental data. The presence of an alert (Michael addition in this case) is sufficient to consider the prediction within the mechanistic domain because the substance contains the toxicophore experimentally observed in skin sensitizers. AD in the VEGA

predictions were good enough to be acceptable, and ToxTree did not determine the AD.

Overall, considering that 3 out of 4 tested models supported the call for skin sensitization, OP was considered a sensitizer by *in silico* predictions (*i.e.*, by participating in covalent binding to nucleophilic centers in skin proteins – KE1). Although covalent protein binding is considered as a common property for skin sensitizing agents, not all protein binders are skin sensitizing agents *in vivo*. For instance, 2-hydroxypropyl methacrylate binds to proteins, but it is negative in the LLNA (Schultz et al. 2009). Thus, a comprehensive and mechanistic assessment for skin sensitization should include the evaluation of at least 2 out of 3 KEs described in the AOP. Therefore, to further investigate immune responses and enhance predictivity, we covered KE2 and KE3 of AOP for skin sensitization using two KC *in vitro* models (HaCaT cell line and RHE model) and the human promyelocytic cell line THP-1 as a surrogate for DCs.

KC activation is KE2 of the AOP for skin sensitization. Considering that KCs are the first cells to come into contact with chemicals and are involved in the early initiation phase of skin sensitization (Corsini et al., 2013b), several assays are actively used to investigate chemicals capable of inducing sensitization in KC cell lines (OECD, 2022). An example is the assays measuring Nrf2/ARE pathway activation in KCs (Natsch, 2010; Ramirez et al., 2014); however, as not all sensitizers activate the Nrf2/ARE pathway, these assays could result in high rates of false negatives (Chung et al., 2018). The false-negative rate can be reduced by directly measuring the secretion of inflammatory cytokines in KCs cell lines, such as NCTC-2544 (Corsini et al., 2013), HaCaT (Chung et al., 2017; Jeon et al. 2019).

In HaCaT cultures, studies have shown that IL-6 and IL-1α expression profile is the most accurate for identifying sensitizers since IL-8 expression are also produced by non-sensitizers (*e.g.*, irritants, such as sodium lauryl sulfate , lead to IL-18 and IL-8 release in HaCaT cells) (Jung et al., 2016; Jeon et al., 2019; Chung et al., 2017; Lee et al., 2015; Mohamadzadeh et al. 1994; White et al. 2015) and the evaluation of substances using IL-18 released by HaCaT cells is less feasible (Van Och et al. 2005). Thus, the HaCaT assay is a method where a chemical substance is a sensitizer when the level of IL-6 or IL-1α is greater than or equal to 3-fold (SI value); and a non-sensitizer if the level of IL-6 and IL-1α is lower than 3-fold (SI = 3) (Jeon et al. 2019). Although no difference in the levels of IL-1α at the intracellular content and supernatant was verified in this study (data not shown), HaCaT cells exposed to OP showed a significant increase in IL-6 level (3.65-fold increase related to control); thus, OP is considered as a sensitizer by this assay.

Although the HaCaT cell line shares similarities with normal human KCs (Colombo et al. 2017; Boukamp et al., 1988), phenotype differences may lead to uncertainties in responses to KC activation, and thus, the use of models that resemble in vivo human epidermis in a tiered test approach for KE2 can improve predictive capacity. The use of IL-18 biomarker in RHE model has been proposed as a test method to identify contact sensitizers (Galbiati et al. 2017). In this method, if IL-18 increases 2-fold or more in at least one of the tested concentrations compared to solvent control, the chemical is considered a contact sensitizer (Galbiati et al. 2017). IL-18 is a potent inducer of IFN-γ by activated T-cells (Okamura et al., 1995) and it plays a key proximal role in the induction of allergic contact sensitization (Lee et al. 2015). Our results showed that exposure to OP caused an increase in IL-18 release of 8.73-fold for OP at 50 mg/mL, indicating that OP is a contact sensitizer (Galbiati et al., 2017). Although the assessment of IL-18 in RHE, provides great predictivity of skin sensitization a combined evaluation of other cytokines may increase confidence in the final conclusion.

IL-1α is constitutively produced and retained in KCs; however, in response to physical or chemical stresses, this cytokine is released and participates in primary events of inflammation by stimulating the further release of secondary mediators, such as IL-8. IL-8 is a chemotactic cytokine that plays a role in the activation and migration of DCs and the recruitment of T lymphocytes and neutrophils (Coquette et al. 2003). The difference in production/release of IL-8 and IL-1α is a valuable parameter for screening chemicals regarding their potential as skin sensitizers and/or irritants, since sensitizers induce higher levels of IL-8 release, while irritants cause a substantial increase in the levels of IL-1α in RHE models (Coquette et al. 2003).Thus, positive responses to skin sensitization in RHE model assay can be confirmed by obtaining IL-8 > IL-1α (skin sensitizers); contrarily, IL-8 < IL-1α indicates skin irritation potential (Coquette et al. 2003). Non-sensitizers and non-irritants do not increase the release of any of these cytokines. In the present study, although RHE models exposed to OP had increased IL-18 release at the highest tested concentration, the fold increase of IL-1α was much higher than IL-8 in the same test concentration. Thus, the results of this assay classified OP as a skin irritant instead of a skin sensitizer, disagreeing with the HaCaT assay results and IL-18 assay in RHE model.

Although the RHE models reproduce *in vitro* key structural and functional features of the human epidermis, this *in vitro* model is constructed using a single cell type, the KC, and thus the model lacks important cell interactions, whichlimit the subset of skin immune response (Nguyen et al. 2019). DCs participate actively in skin sensitization after KC activation and DC

activation is considered the KE3. The THP-1 activation assay is an alternative method to evaluate the KE3. This method was developed based on the evaluation of the production of IL-8 and the expression of CD86 and CD54 in the human monocytic leukemia cell line THP-1 (Iulini et al., 2022). CD86 and CD54 are also markers used in the validated OECD test method h-CLAT; however, the expression profile of CD54 and CD86 may wrongly classify 30% of the overall test chemicals (Mitjans et al., 2008). The quantification of IL-8 production in THP-1 activation assay can increase the predictive capacity, showing advantages over h-CLAT (Iulini et al. 2022). Additionally, IL-8 is related to T-cell proliferation, and this biomarker may provide indirect information about possible participation in KE4 (Kienzl et al., 2019). THP-1 cells exposed to OP increased the release of IL-8 as well as showed SI $\geq$ 1.5 for CD86 and CD54, demonstrating skin sensitization potential of OP.

The overall results of the set of *in silico* and *in vitro* methods used to evaluate the skin sensitization potential of OP strongly suggest that this chemical substance is a skin sensitizer. However, OP can also cause skin irritation at high concentrations.

Despite the skin sensitization potential of OP, this study also comprises an investigation of other potentially harmful effects of OP within the context of dermal exposure. Chemical substances can modulate inflammatory skin responses by altering the expression of genes associated with skin diseases or enhancing existing skin inflammatory responses. In recent years, understanding the roles of long non-coding RNAs as important gene expression regulators has brought another layer of complexity to comprehending physiologic and pathologic cellular metabolism (Cipolla et al. 2018; Statello et al. 2021). Specifically, epigenetic changes, such as the expression modulation of lncRNAs, have shown a role in inflammatory skin diseases. In general, lncRNAs are described as poorly expressed and cell-specific molecules. However, some highly expressed lncRNAs, such as NEAT1 and MALAT1, occur in all skin cell types (Shefler et al. 2022). The nuclear-enriched abundant transcript 1 (NEAT1) and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) are involved in the regulation of a variety of cell signalling molecules (Tang et al. 2020). For instance, these lncRNAs were demonstrated as regulators of the expression of *TNF*, *IL6* and *IL8* (Li et al. 2020; Zhao et al. 2016; Zhu et al. 2021).

Additionally, in a study using HaCaT cells, *MALAT1* has been associated with wound healing, specifically attenuating TGF-β1, which is related to temporal changes in the morphology of KCs at the wound edge and psoriasis by regulating the release of inflammatory cytokines (Zhang et al. 2021; Zhou et al. 2022). The lower expression of *NEAT1* is associated

with over proliferation and migration of psoriatic HaCaT cells (Wang et al. 2022). In the present study, HaCaT cells exposed to OP showed downregulation of *MALAT1* and *NEAT1*. *IL8* gene was also significantly downregulated. Studies suggest that *NEAT1* expression modulates *IL8* expression by targeting miR-139 (Zhu et al. 2021) or by binding to proline/glutamine-rich splicing factor SFPQ and preventing the suppression of several immune-related genes, including *IL8* (Zhu et al. 2021; Wu et al. 2015). Nevertheless, the ELISA assay did not show any alteration in IL-8 release in HaCaT cells after exposure to OP, showing that *IL8* downregulation also needs further investigation.

The potential of exacerbating the inflammatory response in the skin was also verified in this study by an assay based on the increase in LPS-induced CD86 and CD54 expression and IL-8 release (THP-1 activation assay). Of note, after OP exposure, the increase in the expression of these parameters was much higher, demonstrating that OP can modulate and strengthen skin inflammatory responses caused by other agents.

5. Conclusions

The overall results from *in silico* and *in vitro* methods demonstrated that OP is a skin sensitizer, providing evidence from three KEs of the AOP for skin sensitization. Also, OP can be harmful to human health by inducing other undesirable skin responses, *i.e.*, modulating the expression of lncRNAs associated with skin diseases, as well as acting as an enhancer of skin inflammatory response. Although additional investigations are needed to understand the epigenetic changes caused by OP on keratinocytes, the findings suggest that dermal exposure to OP may pose risks to human health. With respect to the integrated test strategy used in this study, we could demonstrate its applicability in predicting chemical skin sensitization.

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Disclosure statement

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CAPÍTULO 3: **An integrated *in silico-in vitro* test approach to evaluate effects of endocrine-disrupting chemicals on human skin: a case study of the Diisopentyl phthalate**

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Abstract

Several marketed products are untested for several endpoints of toxicicity. New Approach Methodologies (NAMs) have arisen to help fulfil the knowledge gap on the safety of chemical substances. In this article new insights into DiPeP sensitization mechanism are verified by an *in silico-in vitro* integrated approach based on the AOP for skin sensitization. QSAR TOOLBOX 4.5, ToxTree and VEGA defined DiPeP as a non-sensitizer. *In vitro* tests with HaCaT cells (quantification of IL-6; IL-8; IL-1α, and IL-18 by ELISA and expression of genes *TNF*, *IL1A* and *IL8* by RT- qPCR), RHE model (quantification of IL-6; IL-8; IL-1α and IL-18 by ELISA) and THP-1 activation assay (CD86/CD54 expression and IL-8 release) were performed to investigate the participation in KE1, KE2 and KE3 of the AOP for skin sensitization. Evaluation of lncRNAs *MALAT1* and *NEAT1* expression and LPS-induced THP-1 activation assay was also performed to verify the immunomodulatory effect of DiPeP. In this *in vitro* approach, DiPeP was verified to participate in KE2 by increasing IL-6 (2.15-fold), IL-8 (8.85-fold) and IL-1α (22.09-fold) expression and *ILA* gene expression in HaCaT cells. IL-6 release also significantly increased in RHE model; An increase in CD54 (2.15-fold), IL-8 release (2.8-fold) and TNF-α release (1.26-fold) in THP-1 cells, determined the participation of DiPeP in the KE3 . Immunomodulatory effects of DiPeP are demonstrated by an increase in LPS-induced CD54 expression (4.2-fold). DiPeP also suppresses and modulates CD86 expression. Overall, this study suggests that the DAs used provided interesting insights into DiPeP sensitization.

Keywords: non-animal alternative methods, QSAR models; activation of keratinocytes; THP-1 activation assay; long non-coding RNAs

1. Introduction

The latest chemical management policies require toxicological evaluation of marketed but untested chemicals (Kostal et al. 2020). Nevertheless, hazard and risk assessments of thousands of existing chemicals are challenging for regulatory bodies worldwide (Kavlock et al. 2018). New Approach Methodologies (NAMs) have arisen to help fulfil the knowledge gap on the safety of chemical substances in a fast and reliable prediction manner (Kavlock et al. 2018; Kostal et al. 2020). NAMs refer to any technology, methodology, approach, or combination that can provide information on the chemicals' impacts on human and ecological health by non-animal methods (*i.e.*, *in chemico* and *in vitro* assays and *in silico* models) (US EPA, 2018). The successful adoption and use of NAMs depend upon the development of new methods and/or approaches that provide equivalent or better scientific quality and relevance than existing approaches.

Skin sensitization is a process in which a substance will lead to an allergic response following repeated skin contact (Basketter et al. 2008). The key biological events underlying skin sensitization are known, and this scientific knowledge made possible the development of an Adverse Outcome Pathway (AOP) for skin sensitization (Sakuratani et al. 2018). With the skin sensitization AOP knowledgebase, approaches can be defined to predict skin sensitization hazards by sequential testing using non-animal assays, and recently two defined approaches (DAs) were accepted by Organisation for Economic Co-operation and Development (OECD 497). The DAs for skin sensitization under this OECD guideline cover Key Events (KE) of the skin sensitization (OECD, 2021).

The first KE (KE1) is a molecular initiating event, in which is verified the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. After KE1, the response progress to keratinocyte (KC) activation by inducing an inflammatory response and changing the expression of genes associated with specific cell signalling pathways, which, in turn, will lead to the activation of dendritic cells (KE3). Ultimately, the proliferation of T-cells is stimulated (KE4), and the adverse outcome is verified as allergic contact dermatitis (OECD, 2021). Alternative validated OECD test methods exist for each of the first three KEs (OECD, 2021).; however, each information source presents limitations that keep the perspective for improvements on

chemical sensitization predictions. Additionally, limited access to *in vitro* models for some of the validated test methods (*e.g.*, KeratinoSens™ assay) impairs their international adoption in skin sensitization studies, especially in developing countries where the acquisition of unique cell lines is subject to several difficulties imposed by customs.

Besides skin sensitization, chemical substances can cause other effects on human skin, leading to a diverse set of undesirable skin manifestations. Long non-coding RNAs (lncRNA) have been associated with epigenetic changes and chronic inflammatory skin diseases, including atopic dermatitis (Mervis et al. 2020; Wang et al. 2018). Given that, evaluating lncRNAs is important to seek a better understanding of skin sensitization processes. Additionally, chemical substances have been shown to not only induce but also enhance immune response, modulating allergic inflammation by causing a reduction in immunity or enhancement of the immune response (Corsini et al. 2011;2012; Kuo et al. 2012). This immunomodulatory effect may influence the skin sensitization process (Chalubinski et al. 2006; Kuo et al. 2012).

Diisopentyl phthalate (DiPeP), also known as diisoamyl phthalate, is a substance with five carbon atoms in the alkyl side chain, and different isomers of this substance are found on the market. DiPeP can be used as a plasticizer in PVC plastics as well as it can have many other applications in industry; however, the exposure sources of DiPeP to humans are largely unknown (ECHA, 2012). DiPeP is toxic for reproduction category 1B (presumed human reproductive toxicity) (EU regulation (EC) No 1272/2008), and the exposure of pregnant women to DiPeP (urine samples) in the south of Brazil was recently reported (Bertoncello et al., 2018). The registration dossier of DiPeP at ECHA (European Chemicals Agency) presents knowledge gaps for several toxicity endpoints; however, it brings the information that DiPeP is classified as a potential skin sensitizer (mouse local lymph node assay – LLNA) and not a skin irritant (ECHA, 2022). Thus, this chemical substance can be a useful case study to address the applicability of proposals for integrated *in silico-in vitro* test strategies to evaluate skin sensitization.

For this study, DiPeP was used as a chemical substance model to verify the applicability of an integrated *in silico-in vitro* test strategy to evaluate skin sensitization potential of endocrine-disrupting compounds (EDCs). The integrated test strategy consisted of *in silico* structure-based predictions (QSARs) and cell- and artificial tissue-

based assays, covering 3 out of 4 KEs of skin sensitization (KE1, KE2 and KE3). Through this case study, we illustrated the use of NAMs in a weight-of-evidence context, information is accrued, leading to decreased uncertainty relative to a result and increased confidence to answer questions about the skin sensitization hazard of EDCs. Also, this study investigated other potential effects of DiPeP on human skin, such as its capacity to act as an enhancer of skin inflammation and modulate lncRNAs associated with inflammatory skin diseases.

2. Material and methods

2.1. Tested chemical

Diisopentyl phthalate (DiPeP; CAS 84777-06-0) with a purity of 99% was provided by PETROM (Petroquímica Mogi das Cruzes – Mogi das Cruzes, SP, Brazil).

2.2. In silico data

Four *in silico* models were used to evaluate the skin sensitization potential of DiPeP, as described below.

The OECD QSAR Toolbox (version 4.5 at www.qsartoolbox.org) is part of one of the integrated testing strategies (ITS) of the OECD Guideline No. 497: Defined Approaches (DAs) on Skin Sensitization and is an important profiler applied (Hoffman et al. 2022). The DAs consist of a fixed data interpretation procedure (*e.g.,* mathematical models, rule-based approaches) applied to *in silico*, *in chemico,* and/or *in vitro* data generated with a defined set of information to derive a prediction equivalent to that provided by animal studies without the need for expert judgment. The integrated testing strategies (ITS) of the defined approaches includes an *in silico* information that can be sourced by the OECD QSAR Toolbox v4.5 – a computational tool which uses an analogue based read-across approach or structural alerts for protein binding identified by profilers to predict whether a chemical will be a sensitizer.

The recent OECD QSAR Toolbox v.4.5 counts with an automated workflow for defined approaches for skin sensitization (DASS AW) to be used as the *in silico* information source for the defined approaches. The applicability domain of the QSAR Toolbox Skin sensitization predictions for use in the ITS defined approach approaches automated workflow (DASS AW) is based on the training set substances of 2268 substances having LLNA and/or GPMT skin sensitization experimental data. The

chemical was inserted by using its CAS number (84777-06-0) and the option for automated workflow for "EC3 from LLNA or Skin sensitization from GPMT assays for defined approaches" was chosen in the "data gap filling module". This automated workflow predicted the substance as positive (sensitizer) or negative (non-sensitizer) using the read-across or a customized profiler.

Other employed tool for sensitization alerts was the ToxTree v2.6.13 (freely available at www.toxtree.sourceforge.net) which is based on the identification of mechanisms of toxic action for skin sensitization using a SMARTS pattern-based approach (Enoch et al. 2008).

The VEGA platform (version 1.2.0; available at www.vegahub.eu) was assessed through two models: Skin sensitization model Caesar (version 2.1.7) and IRFMN/JRC model (1.0.1 from the Istituto di Ricerche Farmacologiche Mario Negri). Both models are based on the LLNA (Local Lymph Node Assay) in mice and on a data set that categorizes sensitizer compounds using the ranges established by the *European Centre for Ecotoxicology and Toxicology of Chemicals* (*ECETOC*): extreme (EC3<0.1%), strong (0.1%<EC3<1%), moderate (1%<EC3<10%), weak (EC3>10%) and non-sensitizer.

Classification of DiPeP into sensitizer or non-sensitizer was defined according to the results based on all the models employed.

2.3. In vitro assays

2.3.1. In vitro models and biomarkers

Three *in vitro* models were used in this study: HaCaT cells (immortalized human keratinocyte cell line, Cell bank of Rio de Janeiro - BCRJ, Cat. No. 0341, Brazil); THP-1 cells (human leukemia monocytic cell line, Elabscience Biotechnology Inc., Cat. No. EP-CL-0233, 14,780 Memorial Drive, Suite 216, Houston, Texas, USA); and RHE model (EpiDerm™, MatTek Corporation, Ashland, Bratislava, Slovakia).

These models were used to evaluate the skin sensitization potential and other toxic effects of DiPeP; thus, they were combined with a variety of biomarkers. For skin sensitization, KC activation (KE2) was evaluated by quantifying IL-6, IL-1α, IL-18 and IL-8 markers in HaCaT cells. This KE was also verified in RHE models using the same biomarkers. LncRNAs in HaCaT cells were used to verify the capacity of DiPeP in

participating in epigenetics, and combined exposure of DiPeP with LPS in THP-1 cells was used to verify the immunomodulatory effect of DiPeP in skin inflammation.

These models were tested for several markers important in the KEs of the AOP for skin sensitization. Inflammatory responses and changes in gene expression (KE2) were covered by employing HaCaT cells and RHE. The expression of inflammatory cytokines (IL-6, IL-1α, IL-18 and IL-8) and expression of *IL6*, *IL8*, *IL1A*, *MALAT1*, *NEAT1* genes were investigated in HaCaT cells. RHE was used as a model to investigate the expression of the same cytokines investigated in HaCaT cells (IL-6, IL-1α, IL-18, IL-8).

The activation of dendritic cells (DC) (KE3) was typically assessed by expression of specific cell surface markers CD86 and CD54 and chemokine IL-8 release in THP-1 cells. The expression of the membrane marker HLA-DR was also investigated to better understand the mechanisms of DC activation in THP-1 cells. Protocols used for each of the assays are described in the following subsections.

HaCaT cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (both from Gibco, Life Technologies, USA) and addition of 100 IU/mL penicillin G, 100 mg/mL streptomycin, and 1 µg/mL amphotericin. THP-1 cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 10% heat-inactivated fetal calf serum (FCS) and addition of 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.01 ng/mL gentamycin (Iulini et al., 2022). Both cultures were maintained at 37°C, 5% CO_2, and 95% relative humidity. Subcultures were performed when cells reached approximately 80% confluency for HaCaT cells and every 3-4 days for THP-1 cells.

EpiDerm™ models were maintained according to the manufacturer's instructions in the DMEM-based tissue culture medium provided by the supplier at 37°C, 5% CO_2 and 95% relative humidity for 24 h (stabilization period before performing the experiments).

2.3.2. Test concentrations and exposure conditions

HaCaT and TPH-1 cells were exposed to DiPeP at 0.01, 0.1, 1, 10 and 100 µg/mL (sub-cytotoxic concentrations defined by cell viability assays). For gene expression assay by RT-qPCR, HaCaT cells were exposed to DiPeP at 100 µg/mL.

Test solutions were prepared using dimethyl sulfoxide (DMSO), and a final concentration of 0.1%-v/v were considered for the exposure. The exposure of 2D culture systems was carried out directly in the culture medium, and the exposure time was 24 h for most of the assays. Exposure of DiPeP with LPS in immunomodulatory assays was also in exposure times of 48 h, and DiPeP was exposed for 48 and 72 h in THP-1 cells to verify HLA-DR expression (these exceptions are indicated in the description of the methods).

RHE was exposed to DiPeP at 10 and 100 mg/mL in acetone olive oil (AOO) 4:1, according to Gibbs et al. (2013). Cultures were incubated for 24h.

The experiments were carried out with negative (culture medium) and solvent controls (DMSO 0.1%-v/v: 2D cultures; AOO 1:4: RHE model). Positive control (PC) information is below described in each assay performed.

2.3.3. Cell viability assays

Different cell viability assays were used to select the tested sub-cytotoxic concentrations and to define positive responses in the RhE model. Thus, MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was used for HaCaT cells and RHE models, while THP-1 cell viability was evaluated by propidium iodide (PI, Sigma-Aldrich) using flow cytometry. Briefly, HaCaT cells ($5x10^4$ cells/well, 96-well plate) were exposed to DiPeP and incubated with MTT at 0.5 mg/mL for 3 h. After incubation, formazan crystals were dissolved with DMSO, and the absorbance was read by the microplate reader Infinite 200™ (Tecan) (Mosmann, 1983). For the RHE, the exposed epidermis was incubated with MTT at 5 mg/mL for 3 h. The tissues were then transferred to 24-well plates containing isopropanol. The plates were sealed with parafilm, protected from light, and incubated overnight at room temperature under an orbital shaker to extract the formazan crystals. The RhE models were discarded, and the absorbance of the extract solutions was taken at 540 and 570 nm. For THP-1 cells, $1x10^6$ cells/mL were exposed to DiPeP concentrations and stained

with PI (0.625 µg/mL), and the fluorescence intensity of labelled cells was acquired by flow cytometer (NovoCyte 3000, ACEA Biosciences, Inc). Triton X-100 1%-v/v was used as a PC for HaCaT cells and dinitrochlorobenzene (*DNCB*), which is a very strong contact allergen, was used as PC for RHE.

2.3.4. ELISA: quantification of cytokines

The cytokines IL-1α, IL-18, IL-6 and IL-8 were quantified in HaCaT ($2x10^5$ cells/well, 24-well plate) and RHE model, while the release of IL-8 and TNF-α was quantified in THP-1 cells. All the cytokines evaluated in RHE, and THP-1 cells were quantified in culture supernatants (release). For HaCaT cells IL-6 and IL-8 were evaluated in the culture supernatants, while IL-1α and IL-18 were evaluated regarding intracellular content. For quantifying intracellular cytokines, HaCaT cells were lysed by incubating the cells in EDTA 0.05% v/v for 5 min at 37°C in a 5% CO_2 in air atmosphere and then in Triton-X 100 0.5%-v/v for 15 min on ice. Cell lysates were harvested and stored at -80°C till the analysis. In this case, total protein was also determined by the bicinchoninic acid method (BCA) to be later used in normalizing intracellular cytokines data (Corsini et al. 2013). P-phenylendiamine (PPD) 60 µg/mL was used as a PC for IL-18 quantification in HaCaT cells. DNCB was used as a positive control in RHE model.

The cytokines were quantified using commercial kits according to the instructions provided by the manufacturers. The following ELISA kits were used in the present study: Human IL-6 ImmunoTools sandwich ELISA (Cat. No. 31670069), Human IL-8 ImmunoTools sandwich ELISA (Cat. No. 31670089), Human IL-1α ELISA MAX™ Deluxe Set (Cat. No. 445804), Human IL-18 ELISA kit MBL (Cat. No. 7620; Nagoya, Japan) and DuoSet® ELISA Development System Human TNF-α (R&D Systems - Cat. No. DY210-05). The absorbance of the microplates was read at 450 nm. Experiments were carried out in three technical replicates and 2-3 biological replicates.

2.3.5. Expression levels of inflammatory mediators by RT- qPCR

HaCaT cells were plated in a 6-well plate ($4x10^5$ cells/well) and let grow for 24h for stabilization, and then cells were treated for 24h. Subsequently, the RNA of HaCaT

cells (treated and control cells) was extracted using the illustra™ RNAspin Mini SV Total RNA Isolation System (Cat. No. 25-0500-71; GE Healthcare), following the manufacturer's instructions. The extracted RNA was quantified using NanoDrop (Thermo Fisher Scientific), and the cDNA was obtained by High-Capacity cDNA Reverse Transcription kit (Cat. No. 4368814; Applied Biosystems). The expression of genes from inflammatory cytokines (*IL1A*, *IL6*, *IL8* and *TNF*) and long non-coding RNAs involved in inflammation (*NEAT1* and *MALAT1*) was quantified by quantitative reverse transcription polymerase chain reaction (RT-qPCR) using Power SYBR® Green PCR Master Mix (Cat. No. 4367659; Applied Biosystems). RT-qPCR was performed in technical and biological triplicates. The primer sequences are presented in Table S1.

Table S1. Primer sequences used in the RT-qPCR.

Genes	Direction	Sequence
GAPDH	F	5′-ACCCACTCCTCCACCTTTGA-3′
	R	5′-CTGTTGCTGTAGCCAAATTCGT-3′
TNF	F	5'-CCGAGGCAGTCAGATCATCTT-3'
	R	5'-AGCTGCCCCTCAGCTTGA-3'
IL6	F	5′-AGATTTGAGAGTAGTGAGGAACAAG-3′
	R	5′-CTGGCATTTGTGGTTGGGTC-3′
IL8	F	5′-ATACTCCAAACCTTTCCACCCC-3′
	R	5′-CCTCTGCACCCAGTTTTCCTTG-3′
IL1A	F	5′-CAGCCAGAGAGGGAGTCATT-3′
	R	5′-GGAGGAACTGTCTTCTTCATTTTCA-3′
NEAT1	F	5’-TCTCCATTTCCCCATCTGAG-3'
	R	5’-CAGCCACAGAAAAGGGAGAG-3’
MALAT1	F	5’-GGGTGTTTACGTAGACCAGAACC-3’
	R	5’-CTTCCAAAAGCCTTCTGCCTTAG-3’

F: forward; R: reverse.

2.3.6. Quantification of CD86, CD54 and HLA-DR in THP-1

The THP-1 activation assay is based on the upregulation of CD86 and CD54 surface membrane markers and the release of IL-8. The membrane markers CD83 and CD54 and IL-8 quantification were assessed according to Iulini et al. (2022). Briefly, the cells were exposed to DiPeP concentrations (0.01 – 100 µg/mL) for 24 h. After exposure, the suspension of cells was collected into cytometer tubes and centrifuged at 1200 rpm for 5 minutes. Supernatants were collected and stored at − 20°C for posterior quantification of IL-8 and TNF-α (above described). Cells in the tubes were marked with FITC Mouse anti-human CD86 monoclonal antibody, PE Mouse anti-human CD54 monoclonal antibody, PE Mouse anti-human HLA-DR monoclonal antibody and incubated for 30 minutes at 4 °C. Each treatment had κ Isotype Control, marked with FITC Mouse IgG1 (for CD86 monoclonal antibody) and PE Mouse IgG1 (for CD54 and HLA-DR monoclonal antibodies). Each treatment had κ Isotype Control, marked with FITC Mouse IgG1 (for CD86 monoclonal antibody) and PE Mouse IgG1 (for CD54 and HLA-DR monoclonal antibodies). Quantification of these membrane markers was assessed by NovoCyte 3000 flow cytometer (ACEA Biosciences, Inc).

2.3.7. Immunomodulatory effect

LPS-induced inflammation model in THP-1 was used to evaluate immunomodulatory effect of DiPeP. In this case, cells were exposed to DiPeP at 10 and 100 µg/mL for 24h and LPS (1000 ng/mL to CD86 and 100 ng/mL to CD54) was added for an additional 24 h. Cells were then centrifuged and marked with antibodies for CD86 and CD54, as previously described. LPS plus DMSO were used as SC. Supernatants of cells exposed to LPS at 1000ng/mL were collected and stored at – 20º C for posterior IL-8 quantification. IL-8 was quantified using the Human IL-8 ELISA kit described before, according to the supplier's instructions. Experiments were performed in three technical replicates and three independent experiments to determine the expression of surface markers and the release of IL-8.

2.3.8. Data analysis

For the cytotoxicity measured by MTT assay, 70% of cell viability of HaCaT cells was considered optimal to select the concentrations. For this analysis, cell viability of

the treatments was defined by comparing with the viability of the SC (DMSO), which was established as 100%.

For RhE, between 5 and 50% of cell viability was considered optimal for identifying allergens (Gibbs et al. 2013). Cell viability of the vehicle control (AOO 4:1) was considered 100%. For cytotoxicity measured by PI-stained THP-1 cells, the concentration of the tested chemical resulting in up to 75% viability was considered not cytotoxic. Statistical significance among treated and control cells was determined by Kruskal-Wallis (nonparametric, one-way ANOVA) using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant when $p>0.05$. The cell viability of HaCaT and THP-1 cells was determined by IC_{20}- IC_{25} (20-25% of cytotoxicity accepted) and CV75% (Cell Viability of 75%), respectively. For RhE models, the cell viability was *expressed as a* percentage relative to the absorbance value of the SC.

For interleukin quantification by ELISA, the results were calculated in pg/mL from a standard curve and then converted into Stimulation Index (SI). SI was obtained by dividing the concentration of interleukin in pg/mL of the samples and CP by the concentration of interleukin in pg/mL of the SC (fold-change). For intracellular interleukin quantification (IL-18 and IL-1α), the result was expressed as pg/mg of total cell protein as assessed by the BCA protein determination method, as showed by the example in the following equation:

$$\text{IL-18} = \frac{\text{IL-18 (pg/ml) in cell lysate}}{\text{Total protein content (mg/ml) in cell lysate}} = \text{pg/mg}$$

DiPeP was considered a contact sensitizer when SI IL-6 or IL-1α ≥ 3; SI IL-18 ≥1.2 (Jeon et al. 2019; Corsini et al. 2013), and IL-8 increase was significant in HaCaT cells. For the RHE model, the significant SI values indicate sensitization when SI IL-18 ≥ 2 and SI IL-8 > IL-1α. But if SI IL-1α > IL-8 the chemical was considered as an irritant (Galbiati et al. 2018; Coquette et al. 2003).

The results of the RT-qPCR were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test using ΔCT values of SC cells and DiPeP-treated cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous

control, and the expression levels of target genes were normalized relative to the control by the $2^{-\Delta\Delta CT}$ method (Leme et al. 2018). The differences related to the SC were considered significant when fold-change >2 and $p< 0.05$.

The expression of CD86, CD54 and HLA-DR surface markers in THP-1 cells was analyzed by flow cytometry with the acquisition in channels FL-1 (FITC) and FL-3 (PE). A total of 10,000 events were acquired. Gate settings can be found in Iulini et al. (2022). By using the geometric mean fluorescence intensity (MFI), the relative fluorescence intensity (RFI) of markers in the SC cells and DiPeP-treated cells were calculated according to the following equation:

$$RFI = \frac{\text{MFI of chemical treated cells} - \text{MFI of chemical treated isotype control cells}}{\text{MFI of vehicle treated control cells} - \text{MFI of vehicle treated isotype control cells}}.$$

For THP-1 cells, results indicate contact sensitization when SI CD86 and CD54 ≥ 1.5 and/or a significant increase in IL-8 is found for any of the tested concentrations (Iulini et al. 2022).

3. Results

3.1. In silico predictions

Results investigated through computational profilers are summarized in Table 1. Using the data gap filling method of QSAR Toolbox Automated workflow for EC3 from LLNA or skin sensitization from GPMT assays for defined approaches (SS AW for DASS), no alerts were found for DiPeP with and without metabolism.

The ToxTree v2.6.13 has not identified mechanisms of toxic action for skin sensitization. Based on this platform DiPeP does not present alerts such as: SNAr-nucleophilic aromatic substitution, Schiff base formation, Michael Acceptor, acyl transfer agent and SN2- nucleophilic aliphatic substitution.

The CAESAR model available at the VEGA platform predicted DiPeP as a non-sensitizer, although this prediction may be out of the Applicability Domain (ADI=0.778). In contrast, IRFMN-JRC model showed a positive result, but out of AD (ADI=0).

Table 1. Summary of *in silico* skin sensitization predictions for Diisopentyl phthalate (DiPeP).

Platform	Result	AD
QSAR TOOLBOX 4.5	Negative	Into AD
TOXTREE	Negative	ND
VEGA (CAESAR)	Negative	May be out of AD
VEGA (IRFMN-JRC)	Positive	Out of AD

AD = Applicability Domain; ND = not defined.

3.2. In vitro assays

3.2.1. HaCaT assays

A decrease in cell viability was not verified in HaCaT cells exposed to DiPeP in any tested concentrations (Fig. 1).

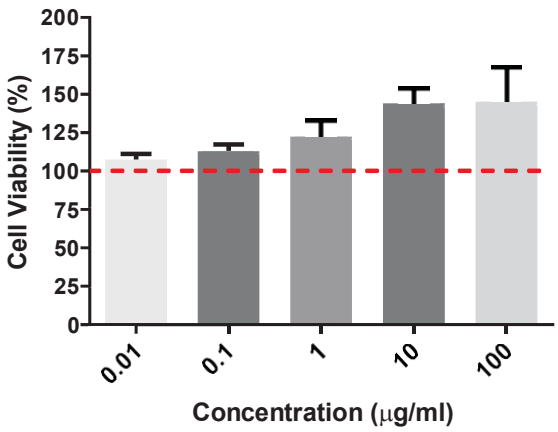

Figure 1. HaCaT cell viability after exposure (24 h) to diisopentyl phthalate (DiPeP) by the MTT assay. The red line represents the solvent control (100% of viability). PC: positive control (Triton X-100 1%-v/v). Data are expressed in mean ± SD and represent three independent experiments.

The quantification of pro-inflammatory cytokines by ELISA assay (IL-6, IL-8, IL-18 and IL-1α) demonstrated that DiPeP at 100 µg/mL significantly increased the expression of IL-8 (2.15-fold), IL-6 (8.85-fold) and IL-1α (22.09-fold) in HaCaT

cells (Fig. 2). Increased mRNA levels of *IL1A* was also verified in HaCaT cells exposed to DiPeP at 100 µg/mL DiPeP (Fig. 3). Significant changes in the mRNA levels of the other tested cytokine genes and the lncRNAs (*NEAT1* and *MALAT1*) were not verified in exposed HaCaT cells (Fig. 3).

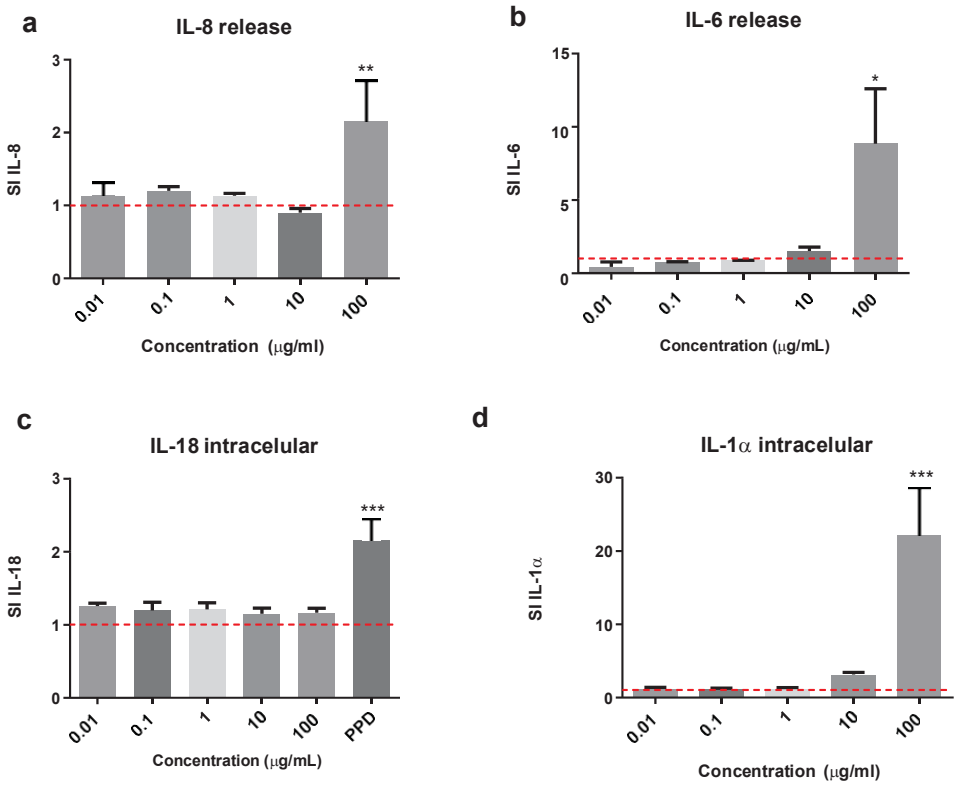

Figure 2. The evaluation of inflammatory cytokines in HaCaT cells after exposure (24 h) to diisopentyl phtalate (DiPeP) by ELISA assay. IL-8 and IL-6 were quantified in HaCaT culture supernatants, and IL-18 and IL-1α the quantification were performed in the intracellular content. The red line represents solvent control (SC). PC: positive control (p-phenylendiamine). SI: stimulation index (fold-change over SC). The data is representative of three (IL-8 and IL-18) and two biological replicates (IL-1α and IL-6). Data are expressed in mean ± SEM and represent three independent experiments. *p < 0.05 **p < 0.01; ***p < 0.001

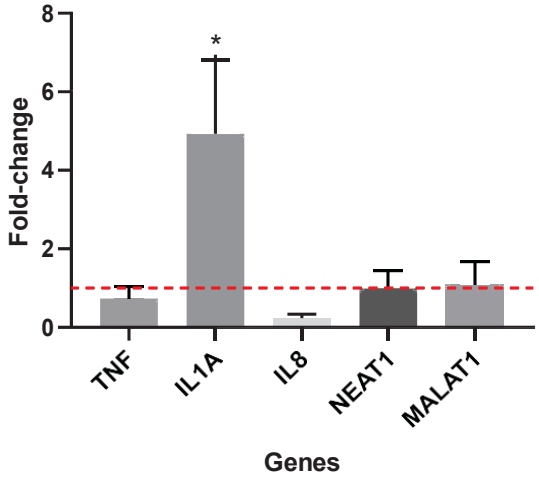

Figure 3. Fold-change in gene expression of inflammatory cytokine genes and long non-coding RNAs in HaCaT cells exposed (24 h) to diisopentyl phtalate (DiPeP) quantified by RT-qPCR. Results were normalized by the housekeeping gene GAPDH and expressions of the target genes in DiPeP-treated cells relative to the solvent control (red line) are shown as mean ± SEM of three independent experiments. *$p < 0.05$.

The cell viability of RHE tissues was quantified by the MTT assay and results showed that the tissues presented viability higher than 50% after the exposure to DiPeP (10 and 100 mg/mL). Among the evaluated cytokines (IL-1α, IL-6, IL-8, IL-18), increased release was only verified to IL-6 at the concentration of 100 mg/mL of DiPeP (1.24-fold) (Fig. 4).

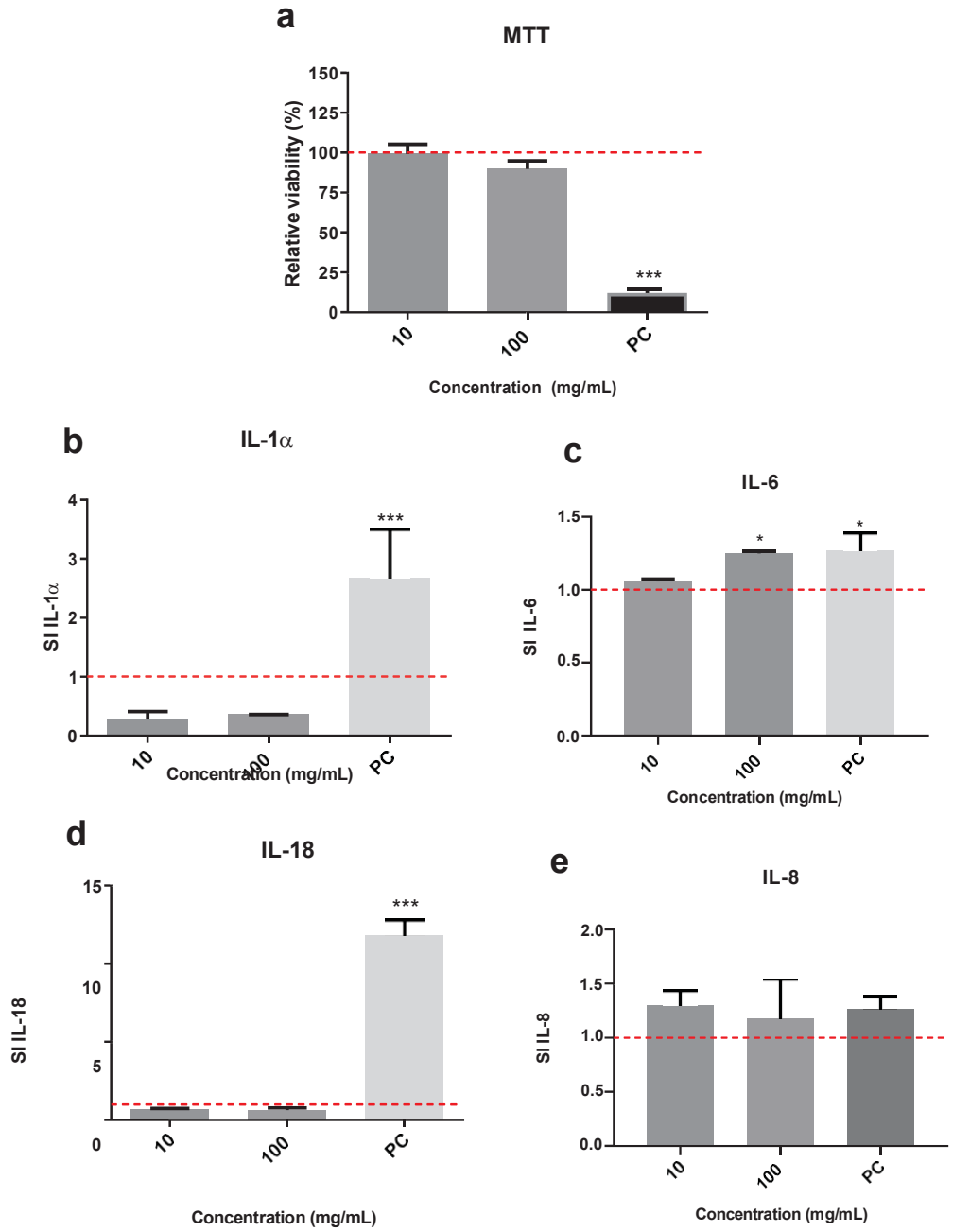

Figure 4. Cell viability (a) and release of inflammatory cytokines (b-e) in RHE models exposed (24 h) to diisopentyl phtalate (DiPeP). The red line represents solvent control. PC: positive control (dinitrochlorobenzene). SI: stimulation index (fold-change over SC). The data is representative of three (IL-18 and IL-6) or two biological replicates (IL-1α and IL-8). Data are expressed in mean ± SD. *$p < 0.05$; ***$p < 0.001$.

3.2.2. THP-1 assays

Similarly to other cell models, DiPeP did not affect the cell viability of THP-1 cells (Fig. 5).

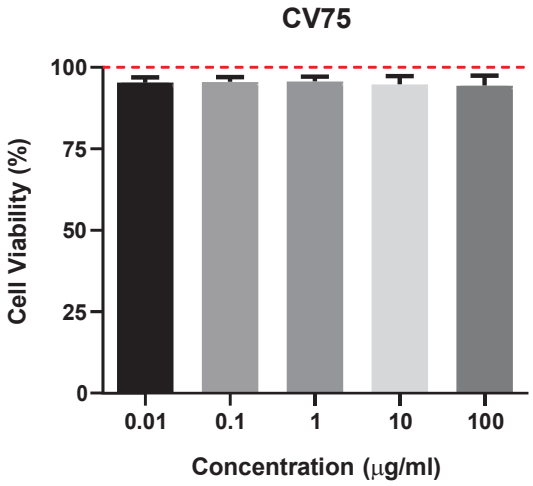

Figure 5. THP-1 cell viability after exposure (24 h) to diisopentyl phtalate (DiPeP) by PI staining quantified with flow cytometry. The red line represents the cut-off for cytotoxicity (≥ 75% of viability). Data are expressed in mean ± SD and represent three independent experiments.

Results from the THP-1 activation assay showed that DiPeP at the two highest tested concentrations (10 and 100 µg/mL) significantly increased the expression of CD54 (1.42 and 2.15-fold, respectively) (Fig. 6a). However, a significant decrease in the expression of CD86 was observed at 10 and 100 µg/mL DiPeP (Fig. 6b). The inflammatory cytokine IL-8 (Fig. 6c) and TNF-α (Fig. 6d) were also significantly increased in THP-1 cells exposed to 100 µg/mL DiPeP (2.8-fold and 1.26-fold increase, respectively).

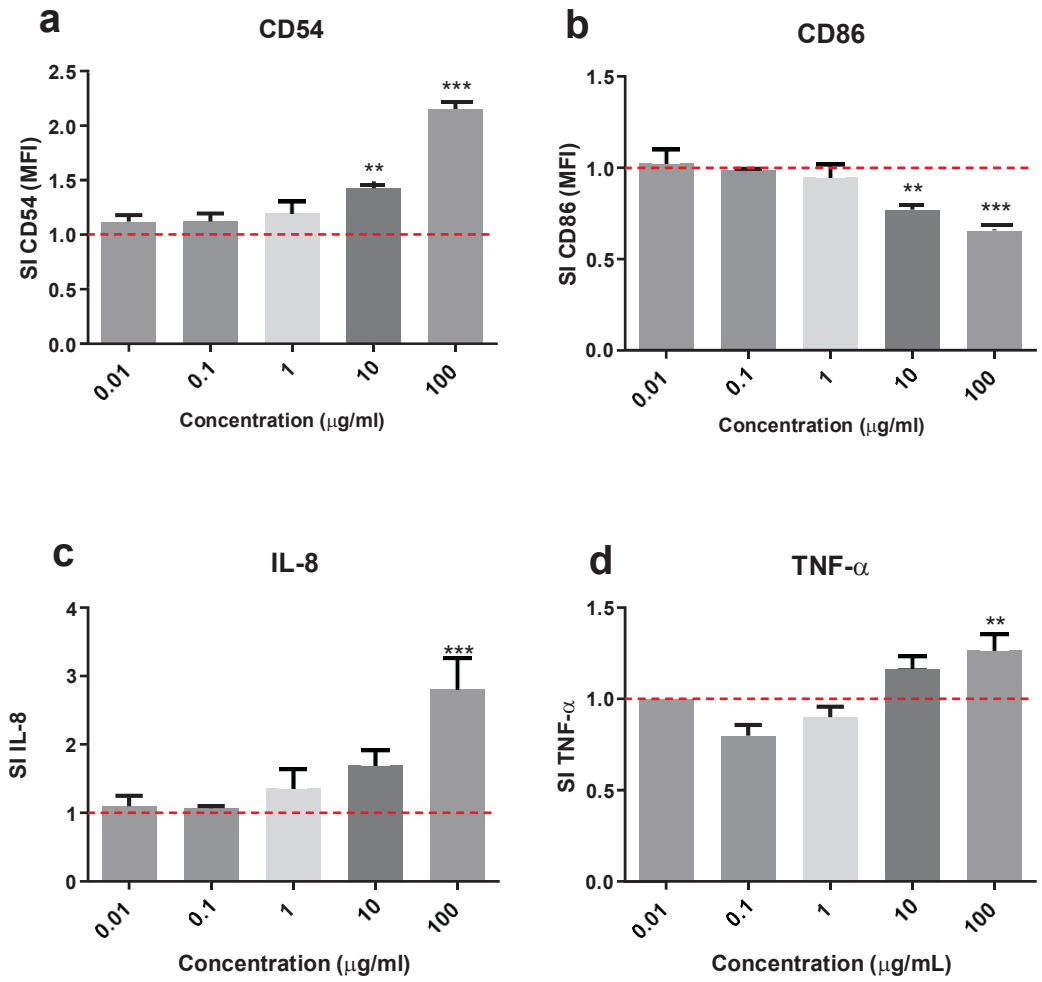

Figure 6. The expression of CD54 (a), CD86 (b), IL-8 (c) and TNF-α (d) in THP-1 cells after 24 h of exposure to diisopentyl phtalate (DiPeP). The red line represents solvent control. SI: stimulation index (fold-change over SC). Data are expressed in mean ± SD and represent three independent experiments. **$p < 0.01$; ***$p < 0.001$.

Regarding DiPeP potential to act as an immunomodulator in LPS-induced skin inflammation, results showed that 100 µg/mL DiPeP increased 4.2-fold the expression of CD54 in LPS-treated cells.(Fig. 7a). For CD86, the subsequent exposure of DiPeP and LPS resulted in a reduction in CD86 expression at the concentrations of 10 and 100 µg/mL (Fig. 7b). Significant increase in CD54 and decrease in CD86 of DiPeP compared to SC was also verified, showing individual effects of DiPeP in 48 h of exposure (Fig. 7).

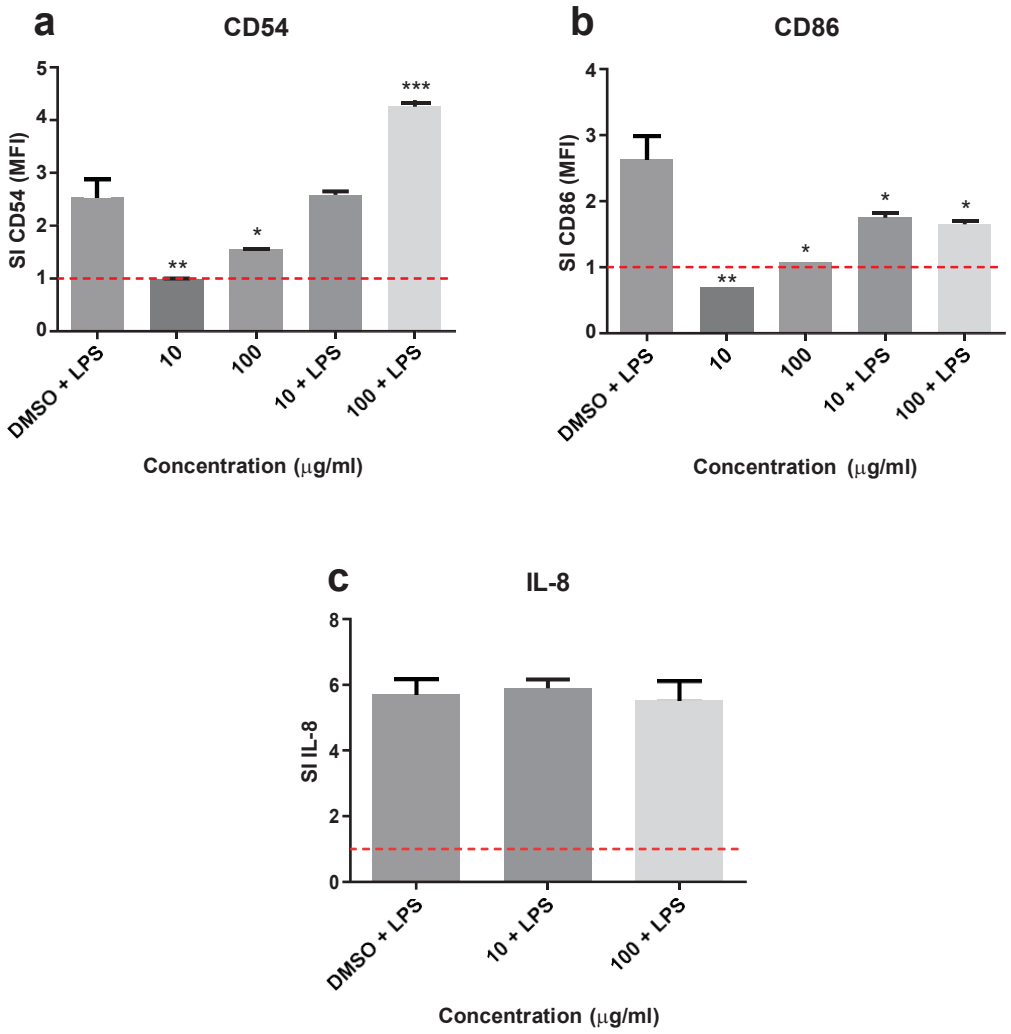

Figure 7. The potential of diisopentyl phtalate (DiPeP) in modulating LPS-induced THP-1 activation. The red line represents solvent control. SI: stimulation index (fold-change over DMSO+ LPS). Data are expressed in mean ± SD and represent three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

In order to understand the mechanisms underlying the reduction of CD86 expression, the membrane marker HLA-DR was analyzed in THP-1 cells. The HLA-DR expression was not affected by DiPeP in any of the exposure times, 24 h or 72 h (Fig. 8a-b). For this analysis, CD86 was evaluated at the time point 72 h, and the reduced expression of this membrane marker after DiPeP exposure was once again verified (Fig. 8c).

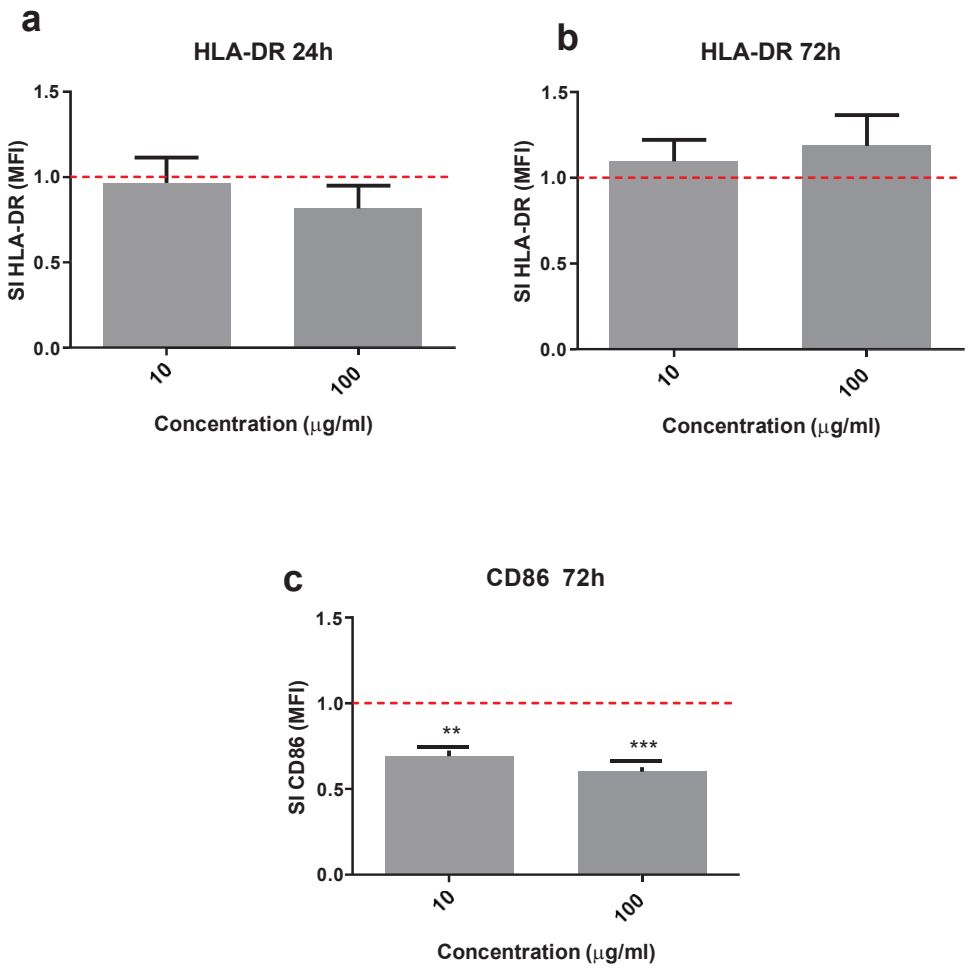

Figure 8. Association of CD86 under expression with HLA-DR expression after exposure (24 and 72 h) to diisopentyl phthalate (DiPeP) (a-b) and expression of CD86 membrane markers (72 h) (c). SI: stimulation index (fold-change over solvent control).The red line represents the SC (DMSO 0.1% v/v). Data are expressed in mean ± SD and represent three independent experiments. ***$p < 0.001$; **$p < 0.01$.

3.2.3. Overall in silico and in vitro data for DiPeP

Table 2 summarizes the main findings obtained for DiPeP with the proposed integrated *in silico-in vitro* approach regarding conclusions on skin sensitization potential, as well as other possible skin toxicity effects of this endocrine disruptor.

Table 2. The overall results for the potential of diisopentyl phtalate (DiPeP) for skin sensitization and for immunodulatory effect by an integrated *in silico-in vitro* test strategy.

In silico: Skin sensitization				
Key events	*Model*	*Endpoint*	*Result*	*Conclusion*
1: Protein binding	QSAR TOOLBOX 4.5	Structure-based predictions for alerts related to protein binding	No alerts found	Non sensitizer
	TOXTREE	Structure-based predictions for alerts related to protein binding	No alerts found	Non sensitizer
-	VEGA (CAESAR; IRFMN-JRC)	Structure-based predictions on the LLNA assay	Negative (CAESAR); Positive (IRFMN-JRC - may be out of AD)	Non sensitizer
In vitro: Skin sensitization				
Key events	*Model*	*Endpoint*	*Result*	*Conclusion*
2: KC activation	HaCaT cells	Release of inflammatory cytokines (IL-6, IL-1α, IL-18, IL-8)	Increased IL-6 and IL-1α release (SI > 3); and IL-8.	Sensitizer
	HaCaT cells	Expression of the genes *IL8, IL1A* and *TNF*	Overexpression of *IL1A*	-

	RHE model	Release of inflammatory cytokines (IL-6, IL-1α, IL-18, IL-8)	Increased IL-6 release	Inflammation
3: Activation of DCs	THP-1 cells	THP-1 activation assay (CD86; CD54 and IL-8 expression)	Increased CD54 expression (SI CD54 > 1.5); and release of IL-8	Sensitizer
		Release of TNF-α	Increased release of TNF-α	Inflammation
		CD86 expression	Decreased the expression of CD86	Immunesupressor of this marker

Modulation of Skin Immune Responses			
Model	*Endpoint*	*Results*	*Conclusion*
THP-1 cells activated by LPS	Expression of CD86 and CD54, and release of IL-8	Enhances LPS- induced CD54 expression and IL-8 release (SI CD54 > 1.5)	Aggravates immune response, which may result in DC activation and allergy

LLNA, murine local lymph node assay; SI, stimulation index; lncRNAS, long noncoding RNAs.

Note: Although results are aligned with single KEs, the endpoints in the assays also recapitulate prior key events.

4. Discussion

Mechanistically-based *in chemico* and *in vitro* test methods have been developed to address events of skin sensitization; however, although adopted by OECD, these methods individually cannot conclude on the skin sensitization potential of chemicals or respond to the regulatory needs. DAs have been proposed as a basis for integrating data from various non-animal information sources in a specific combination and using fixed data interpretation procedures to derive a prediction of *in vivo* response (Casati et al., 2022). Although DAs for skin sensitization were already proposed and recently documented by OECD under guideline No. 497 (OECD, 2021), actions are currently seen to identify limitations and increase the level of confidence in these predictions (Casati et al., 2022). Lesser confidence in such predictions can be associated with limitations from individual methods (information sources) and the chemical group, which can be out of the applicability domain of the non-animals methods due to their chemical nature (Roberts et al. 2018). In this study, a proposal of an integrated *in silico-in vitro* test approach had its performance in evaluating the skin sensitization potential of EDCs verified by the case study of DiPeP.

In silico predictions are mostly based on non-quantitative and/or quantitative structure-activity relationship models ((Q)SAR models), and for skin sensitization, KE1 is addressed by searching for a number of structural alerts that can indicate the ability of the chemical substance to react with the nucleophilic reaction centres of skin proteins (Enoch et al., 2008; OECD, 2021). Based on the *in silico* prediction from OECD QSAR Toolbox, DiPeP elicited a negative read-across, indicating DiPeP as a non-sensitizer. The result was in the AD. The AD of QSAR Toolbox skin sensitization is defined based on the training set of 2268 substances having LLNA and/or GPMT experimental data. When getting negative predictions by read-across, as in the case of DiPeP, the tool ensures the similarity with substances in its training set to be selected as suitable analogues for read-across; thus, ensuring that the target substance is in its mechanistic domain (OECD, 2021).

ToxTree prediction considered DiPeP as a non-sensitizer, confirming the first result, as well as *in silico* results from Skin CAESAR in the VEGA platform, although CAESAR prediction could be out of the AD (ADI=0.778). IRFMN/JRC Skin Sensitization model considered DiPeP as a sensitizer, but this result was totally out of AD. In a previous study done by JRC in collaboration with Istituto Mario Negri this

profiler was the best compared with other profilers for skin sensitization present within the OECD QSAR Toolbox (Internal data). These joined lines of evidence support the call for lack of skin sensitization when it comes from *in silico* predictions. However, most *in silico* models cover the KE1, correlating covalent interaction with skin proteins and KE4 by comparing the substance with LLNA *in vivo* data. According to the OECD 497, a comprehensive and mechanistic assessment for skin sensitization includes the evaluation of 2 out of 3 KEs described in the AOP, since a chemical's ability to induce each KE is a critical information for the assessment of skin sensitization (OECD, 2022).

Thus, to cover 2 out of 3 KEs of the AOP leading to skin sensitization, *in vitro* test methods for KC activation (KE2) and activation of DCs (KE3) were employed in this study. KE2 was covered by using two different KC *in vitro* models (HaCaT cell line and RHE model) associated with inflammatory cytokine markers (IL-6; IL-1α; IL-18; IL-8). KE3 was covered by using the THP-1 activation assay to investigate the release of the cytokine IL-8 and the expression of CD86 and CD54 membrane markers.

HaCaT cells present the advantages of being similar to normal human KCs, exhibiting normal morphogenesis and expressing surface markers and functional activities of isolated KCs (Colombo et al. 2017), are easy to cultivate, present less variation than primary cells and actively demonstrate clinical relevance for skin sensitization (Chung et al. 2017; Jung et al. 2015; Van der veen et al. 2015). Additionally, the HaCaT assay presents predictive capacity similar to KeratinoSens™ and LuSens assays (a conclusion from a study with 22 reference chemicals) (Jeon et al., 2019). RHE model is a 3D culture tissue reproducing *in vitro* key structures and features of the human epidermis (Arnette et al., 2016). The use of this model to evaluate KE2 of skin sensitization can increase the confidence levels of the prediction – due to its high similarity with the *in vivo* condition regarding tissue architecture (human epidermis) and exposure condition (topical exposure) (Moniz et al. 2020; Bergal et al. 2020).

An increase of the pro-inflammatory cytokines IL-8 (2.15-fold), IL-6 (8.85-fold) and intracellular IL-1α (22.09-fold) was verified in HaCaT cells exposed to DiPeP. The exposed HaCaT cells also presented increased IL-1α mRNA level; and*IL8* gene was not upregulated. However, in the RHE model, IL-6 was the only cytokine significantly produced (1.24-fold).

The IL-1α release is the major event of the skin defence after damage to KCs, and this cytokine has a role in activating several effectors related to the skin inflammatory response. L-1α stimulates the further release of secondary mediators, such as IL-6 and IL-8 (Di Paolo et al. 2016). In KC, IL-6 predominantly exerts its inflammatory property and activates the immune system by recruiting monocytes to the inflamed area and playing a crucial role in T differentiation and proliferation (Kaplanski et al. 2003; Akira et al. 1990; Scheller et al. 2011; Kang et al. 2020). The chemokine IL-8 is associated with the activation and migration of dendritic cells (DCs) and also the recruitment of T lymphocytes and neutrophils (Coquette et al. 2003). IL-18 is a potent inducer of IFN-γ by activated T-cells (Okamura et al. 1995) and it plays a key proximal role in the induction of allergic contact sensitization by stimulating Th2 and Th1 responses (Lee et al. 2015). Based on the role of these cytokines and results obtained in HaCaT cells (with increase inIL-6, IL-8 and IL-1α expression) and in RHE model (with increase in IL-6 release), KC activation was obtained; thus, DiPeP can participate in the KE2, activating the immune system and causing skin inflammation.

As mentioned above, the THP-1 activation assay (Iulini et al. 2022) was performed. This method associates the quantification of the surface markers CD54 and CD86 with the release of IL-8 in THP-1 cells (Mitjans et al., 2008;Sakaguchi et al. 2009). CD86 and CD54 are co-stimulatory molecules of major histocompatibility complex (MHC) class II, mainly expressed on DCs. CD86 on DCs interact with CD28 on T cells, which provides T cells with co-stimulatory signals to be activated (Baravalle et al. 2011), while CD54 is related to activating and priming T cells by strengthening synapse among DCs and T cells (Sheikh et al. 2008). CD54 and CD86 are also biomarkers in the h-CLAT assay; a validated OECD test method (OECD TG 442E) and a method accepted in the recently launched 2o3 DA for skin sensitization (OECD 497) (OECD, 2021). However, the evaluation of CD54 and CD86 alone is less sensitive than in combination with IL-8, demonstrating a rate of about 30% failure in hazard identification (Iulini et al. 2022). IL-8 is associated with T cells recruitment and activation (Taub et al. 1996). Thus, the THP-1 activation assay, by combining the three biomarkers in one assay (CD54, CD86 and IL-8), has the purpose of increasing the confidence level of the predictions (Iulini et al. 2022). The assay results showed that DiPeP can activate DCs by significantly increasing the release of IL-8 and expression of CD54; thus, participating in the KE3 of the AOP for skin sensitization.

Besides using DiPeP as a model substance to verify the performance of an integrated test approach based on NAMs to evaluate the skin sensitization potential of EDCs, this study also investigated other potential toxic effects of DiPeP on skin. Thus, changes in the expression of lncRNAs associated with skin diseases and the potential to act as an enhancer of skin inflammatory response were investigated.

Epigenetics has been discussed as a potential factor influencing the pathophysiology and severity of inflammatory skin diseases, and environmental factors can contribute to disease by epigenetic mechanisms (Möbus et al. 2019). However, within the context of chemical exposure, there is a considerable knowledge gap in the potential of chemicals to cause epigenetic changes. LncRNA is a class of molecules greater than 200 nucleotides (nt) in length, often forming relatively stable secondary and higher structures that can participate in cellular organization, regulation, and epigenetic process (Wang et al. 2017). Particularly to *MALAT1,* it has a role in KC proliferation, inflammation, apoptosis, morphology and removal of UVB-induced DNA damage, participating in psoriasis, wound healing and vitiligo (Zhang et al. 2021; Zhou et al. 2021; Brahmbhatt et al. 2020). *NEAT1* is involved with immune regulation, proliferation and migration of immune cells and KCs, ROS production and inflammation by increasing INFγ, associated with psoriasis (Wang et al. 2022). Although these lncRNAs have shown relevance for inflammatory skin diseases, these genes were not significantly altered by exposure to DiPeP.

With regard to DiPeP immunomodulatory effect, results showed that this substance could increase LPS-induced CD54 and IL-8 expressions in THP-1 cells, thus enhancing the immune response to LPS.

Surprisingly, DiPeP suppressed LPS-induced CD86 expression and also suppressed the expression of CD86 in cells not exposed to LPS. DCs require co-stimulatory molecules, such as CD86, to efficiently activate T cells and induce adaptive immunity. However, we did not consider DiPeP as an anti-inflammatory substance by suppressing CD86 expression for two main reasons: (1) CD86 suppression can not affect DCs migration and motility (Bechetoille et al. 2010). Thus, DCs with DiPeP-induced CD54 overexpression would reach linfnodes independent of this effect and can activate T cells; (2) Although CD86 bond to CD28 dominantly contributes to T-cell activation, CD80 – another DC co-stimulatory molecule known for its role in T cells activation – presents a high affinity to the same opposing receptors of CD86 (CD28 and CD151) (Coyle et al. 2001). Since these markers share the same opposing receptors on

T cells, CD80 can compensate for the function of CD86 in conditions where CD86 is lacking (Borriello et al. 1997). Thus, suppression in the CD86 pathway alone may not be sufficient to impair T cell activation by DCs (Bechetoille et al. 2010; Borriello et al. 1997; Coyle et al. 2001).

Additionally, a 1.26-fold increase in the release of TNF-α was verified in THP-1 cells exposed to DiPeP. TNF-α is a powerful inflammatory cytokine that can significantly stimulate LC activation, motility, and antigen presentation to T cells (Clayton et al. 2017). Importantly, Miyazawa et al. (2008) observed that TNF-α augments CD54 in a dose-dependent manner but does not increase CD86 expression after exposure to tested sensitizers. The upregulation of CD54, IL-8 and TNF-α on THP-1 cells demonstrates that DiPeP exerts a predominant inflammatory effect and may activate DCs (KE3).

Regarding mechanisms of CD86 suppression, protein degradation by ubiquitination is the major mechanism that controls the surface expression of CD86 (Baravalle et al. 2011). MARCH1 E3 ubiquitin ligase is responsible for inducing CD86 intracellular degradation via the transmembrane domains, and this mechanism is not associated with CD54 expression (Baravalle et al., 2011; Zhu et al. 2020). MARCH1 also lead to MHC-II degradation in the lysosomes (Zhu et al. 2020). Thus, to understand if the decreased CD86 expression after DiPeP exposure is influenced by MARCH1, the expression of HLA-DR (MHC-II molecule) was also evaluated. As HLA-DR required more time to be expressed at a detectable level, an additional time point of DiPeP exposure (72 h) was included. Interestingly, HLA-DR expression did not alter in THP-1 cells exposed to DiPeP in both time points (24 h and 72 h), suggesting that MARCH1 is not related to the reduced CD86 expression found. Thus, additional investigations are needed to elucidate the mechanisms underlying the decrease in CD86 expression caused by the tested phthalate.

5. Conclusion

The present article highlights new frameworks and initiatives applied to assess evidence from multiple models to predict skin sensitization. By using *in silico-in vitro* approaches, several KEs in AOP for skin sensitization could be investigated and give valuable information about chemical sensitizers such as DiPeP. We also provided new

insights into DiPeP sensitization mechanisms by assessing immunomodulatory effect, changes in membrane expression (CD86 supression), and expression of lncRNAs.

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CAPÍTULO 4: **DNA damage and epigenetic effects of 4- Octylphenol evaluated by new approach methodologies (NAMs)**

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Abstract

Most chemicals were evaluated for carcinogenicity before new data on their epigenetic effects became available. 4-Octylphenol (OP) is OP is an endocrine disruptor with estrogenic activity. In this study, epigenetic changes caused by OP are used to better understand its chemical association with genotoxicity and carcinogenicity. For that, *in silico* methods with QSAR TOOLBOX 4.5, ToxTree and VEGA were used. These tools predicted OP as non genotoxic, mutagenic or carcinogenic but presenting an alert for DNA binding for being an Michael acceptor. For *in vitro* approach, OP at 0.5-50 μg/mL was tested using H2AX phosphorilation (γ-H2AX) to detect double strand breaks (DSBs) and using 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) to determine DNA epigenetics. Additionally, experiments of ROS, using H2DCFDA probe evaluated oxidative stress. The results showed that the lowest tested concentration of OP significantly increase γ-H2AX, 5mC and intracellular ROS formation in HaCaT cells. These results show that OP can bind to DNA, cause DSBs and cause DNA metylation, which means OP is genotoxic and brings concerns regarding possible non-genotoxic carcinogeniticy.

Keywords: human keratinocyte cell line; γ-H2AX; 5-methylcytosine; 5-hydroxymethylcytosine; reactive oxygen species.

1. Introduction

4-Octylphenol (OP) belongs to the alkylphenol ethoxylates group, the largest nonionic surfactant group, and is used in cleaning products, cosmetics, and pesticides. OP is also recognized as an emerging contaminant and is majorly released into the environment by pesticide application, sludge application to agriculture sites and discharge of inappropriate treated industrial effluents and domestic sewages into water bodies. OP is not a persistent chemical substance; however, its constant release into the environment may confer risks to human health (Kim et al. 2019). The hazardous effects of OP on human health are primarily related to its endocrine-disrupting effects, and there is a gap in knowledge regarding other undesirable effects of OP on human health, such as changes in DNA.

OP is an endocrine disruptor with estrogenic activity (Hernández-Rodríguez et al. 2007; Bendsen et al. 2001; Bian et al. 2006; Blake et al. 2004; Singh et al. 2007; de Bruin et al. 2019; Xie et al. 2019). Several environmental estrogens are considered genotoxic; thus, their interaction with the DNA has recently attracted extensive attention (Li et al. 2020). The capacity of OP to induce point mutation has been previously shown by the AMES test (TA98 and TA100 strains) (Boyacıoğlu et al. 2007), and several environmental studies showed that OP is genotoxic for freshwater fish (Sreedevi et al. 2014), earthworm and monocotyledonous plants (Oliveira et al. 2021). Additionally, it can increase the frequency of MN in RTG-2 rainbow trout permanent cell line (Llorentea et al. 2012) However, the mechanism of genotoxicity OP is still unclear.

Epigenetic endpoints can be used to better understand the potential association of chemicals with genotoxic endpoints (Chappell et al. 2016; Goodman et al. 2021). Changes in epigenetic marks, such as DNA methylation, histones/chromatin

structure, nucleosome positioning, and expression of non-coding RNAs may occur as a consequence of exposure to environmental chemicals (Chappell et al. 2016; Goodman et al. 2021). These changes are recognized as key characteristics of carcinogens and play a role in the etiology of several other human diseases (Casey et al. 2016; Oh & Petronis 2021). Within the context of dermal exposure, there is great interest in exploring epigenetics in skin cells, since it has been shown that epigenetics is involved in the control of epidermal development and terminal keratinocyte differentiation and may influence skin cancer and inflammatory skin diseases (Botchkarev et al. 2012; Andersen et al. 2021).

Despite that, it is recognized that most chemicals are evaluated for toxicity without data on their epigenetic effects. The current OECD guidelines comprise 20 genotoxicity/mutagenicity procedures (OECD, 2017), which are used in the European Union (EU) for routine testing of chemicals, but none of these validated methods comprises an evaluation of epigenetic changes (Misik et al. 2022; Chappell et al. 2016). The United States Environmental Protection Agency (US EPA) has been promoting several actions to push forward the evaluation of chemicals regarding potential epigenetic effects. (http://cfpub.epa.gov/ ncea/cfm/recordisplay.cfm?deid=308271). Moreover, the International Agency for the Research on Cancer (IARC) Monographs Programme recommends looking for epigenetic effects during carcinogenicity studies (Chappell et al. 2016). There are no reports regarding the OP effects on DNA methylation to date.

In this context, we aimed to evaluate DNA damage and epigenetic alterations induced by OP exposure in an immortalized human keratinocyte cell line (HaCaT). Thus, we used *in silico* tools and *in vitro* biomarkers to predict potential genotoxic and DNA methylation effects of OP on HaCaT cells. Additionally, we incorporate time-

dependent evaluation of intracellular reactive oxygen species (ROS) to understand the association of oxidative stress with OP effects on DNA. Recently, the use of New Approach Methodologies (NAMs) to evaluate changes in DNA caused by chemicals has been growing exponentially. Thus, the evaluation of the DNA damage potential of chemicals has been made by the growing amount of reliable *in vitro* data, as well as the attempts to understand the pathways and the development of computer-based prediction systems demonstrating high predictivity (Wichard et al. 2017; Glück et al. 2018; Beneveti et al. 2022).

2. Material and methods

2.1. Test chemical

4-octyphenol (OP, CAS No. 1806-26-04, EC Number 2173025) with 99% purity was purchased from Sigma-Aldrich.

2.2. In silico predictions

Genotoxicity of OP was evaluated by different *in silico* models, as follow. ToxTree v2.6.13, freely available at www.toxtree.sourceforge.net, ran predictions for structural alerts for Ames test, *in vivo* Micronucleus (MN) assay in rodents, DNA binding (*e.g.*, Michael Acceptor), and carcinogenicity and mutagenicity based on Benigni/Bossa rulebase. *In silico* predictions for genotoxicity of OP were also run in different models retrieved from the Virtual models for property Evaluation of chemicals within a Global Architecture (VEGA) platform (version 1.2.0; available at www.vegahub.eu): Caesar, ISS, SarPy and KNN models as well as IRFMN-VERMEER and IRFMN models for *in vitro* and *in vivo* MN activity, respectively. The OECD QSAR Toolbox (version 4.5 at www.qsartoolbox.org) based on the presence or absence of structural alerts for DNA binding and different endpoints of genotoxicity and carcinogenicity (gene mutation, chromosome aberration, MN) to predict the genotoxic potential of a chemical substance. The classification of OP into genotoxic and non-genotoxic was defined according to the results based on all the prediction models employed.

2.3. In vitro assays

2.3.1. Cell culture

Human immortalized keratinocyte cell line, HaCaT, (immortalized human keratinocyte cell line, Cell bank of Rio de Janeiro - BCRJ, Cat. No. 0341, Brazil), was the *in vitro* model used in this study. HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and addition of 100 IU/mL penicillin G, 100 mg/mL streptomycin, and 1 µg/mL amphotericin (all from Gibco, Thermo Fisher Scientific, USA), at 37°C, in a humidified atmosphere containing 5% CO_2. Subcultures were performed when cells reached approximately 80% confluency.

2.3.2. MTT assay and the selection of the tested concentrations

.The MTT assay was used to define the subcytotoxic concentrations to investigate the potential DNA damage and epigenetic effects of OP on HaCaT cells. The MTT assay was performed as described by Mosmann (1983), with modifications (Thá et al., 2022). Briefly, $5x10^4$ cells/wells were seeded in 96-well plates and incubated for 48 h for stabilization. Cells were exposed to seven test concentrations of OP for 24 h (1000; 500; 250; 100; 50; 5 and 0.5 µg/mL). For negative (NC) and positive controls (PC), cells were exposed to culture media and Triton X-100 1%-v/v, respectively. After treatments, cells were incubated with 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) for 3 h. Dimethylsulfoxide (DMSO) was used to dissolve formazan crystals. Spectrophotometric measurements were carried out in the microplate reader Infinite 200™ (Tecan) at 570 nm. IC_{50} value was calculated by non-linear regression analysis using GraphPad Prism 8, and 3 sub-cytotoxic concentrations were defined for the subsequent tests.

Thus, the concentrations of OP 0.5, 5 and 50 µg/mL were used for the subsequential assays. The solutions for these tested concentrations were prepared in dimethyl sulfoxide (DMSO), and a DMSO at 0.1%-v/v was the final concentration used in the exposure culture medium.

2.3.3. Determination of intracellular reactive oxygen species (ROS)

The production of intracellular ROS was evaluated by the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Cat. # D399, Invitrogen) probe using flow cytometry (Ameziane-El-Hassani and Dupuy, 2013; Leme et al., 2018). HaCaT cells ($2x10^5$ cells/well, 24-well plates) exposed for 24 h to OP or DMSO (solvent control – SC) or non-exposed (negative control – NC) were incubated with H_2DCFDA (1 μM in PBS) for 30 min at 37°C. After that, cells were harvested, centrifuged, and resuspended in PBS. The fluorescence was acquired on BD FACSMeody™ flow cytometer, excitation was performed at 488 nm, and fluorescence emission was detected with 527/32 bandpass filter (dichlorofluorescein, DCF). Data were expressed in mean fluorescence intensity (MFI) and analyzed using FlowJo Software.

ROS was also determined in a kinetic approach, according to Corsini et al. (1999) and Galbiati et al. (2014). HaCaT cells were plated as described above, and after 24 h of stabilization, cells were incubated with H_2DCFDA for 30 minutes, and the fluorescence was acquired by flow cytometry at different time points, as follows: 0 (without OP exposure) and 5, 15, 30 and 60 min after exposure to OP.

2.3.4. Evaluation of DNA damage

The potential of OP to cause DNA damage was investigated using the marker phospho-histone - H2AX (γ-H2AX). H2AX is a marker of DNA double-strand breaks (DSBs) and plays an essential role in the recruitment and accumulation of DNA repair proteins to sites of DSB damage (Fernandez-Capetillo et al. 2003; Fillingham et al. 2006; Paull et al. 2000; Furuta et al. 2003). Briefly, HaCaT cells ($5x10^5$ cells/well, in a 6 well-plate) exposed to OP or DMSO (solvent control – SC) or non-exposed (negative control – NC) for 4 h. Phospho-histone H2AX (γ-H2AX) was quantified in exposed cells and NC cells using FlowCellect™ Histone H2A.X Phosphorylation Assay Kit (MilliporeSigma), according to the manufacturer's instructions. HaCaT cells were washed with PBS, harvested by trypsinization, and added to polystyrene tubes. Cells were fixed (fixation buffer, Part No. CS202122) and permeabilized (permeabilization buffer, Part No. CS203284), both for 20 min at 4 °C. Immunostaining was performed with anti-phospho-Histone H2A.X (Ser139) direct conjugate to Alexa Fluor ® 488 (Part No. CS206387, clone JBW301) antibody at 1:100 in assay buffer, in the dark for 30 min at 4 °C. Cells were kept in the dark and at 4 °C until flow cytometry analysis.

2.3.5. Evaluation of epigenetic effects

Changes in DNA methylation in HaCaT cells exposed to OP were evaluated based on quantifying the markers 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) according to Tha et al. (2022). Briefly, HaCaT cells ($5x10^5$ cells/well, in a 6- well plate), prior stabilized for 24 h, and exposed to OP or DMSO (solvent control – SC) or non-exposed (negative control – NC) for 24 h, were harvested, washed with PBS and fixed in 4%-w/v paraformaldehyde (PFA, Electron Microscopy Sciences) for 30 min at room temperature (RT). After fixation, cells were centrifuged (700 x g for 7 min), and permeabilized with 0.5%-v/v Tween-20 (Hexapur) in PBST (PBS with 0.05%-v/v Tween-20 and 1 mM EDTA) for 1 h at room temperature (RT). Cells were subsequently submitted to an acid treatment with 4 N hydrochloric acid (HCl, Merck) for 10 min at RT, for chromatin denaturation. After acid removal by centrifugation and wash steps, cells were incubated with 0.25% trypsin-EDTA (Gibco) for 1 min at 37 °C, for antigen retrieval, and DMEM medium with 10% FBS was used to inactive trypsin. To block non-specific antibody binding, cells were incubated with 3% bovine serum albumin (BSA, Sigma-Aldrich) for 1 h at RT. After the blocking step, cells were counted and labelled with primary anti-5-methylcytosine (clone 33D3, Cat. # MABE146, Sigma-Aldrich) and anti-5-hydroxymethylcytosine (clone RM236, Cat. # MA5-24695, Thermo Fisher Scientific). For that, 100 µL of antibody solutions at 1:500 dilution in 1% BSA/PBST were added for every $4x10^5$ cells. Cells were incubated overnight at 4 °C to allow specific binding, and on the next day, cells were incubated for 1 h with goat anti-Mouse IgG1 Alexa Fluor® 488 conjugated secondary antibody (1:400 in PBST, Cat. # A-21121, Invitrogen) to label anti-5-mC primary antibody, or with goat anti-Rabbit IgG (H+L) Alexa Fluor® 488 conjugated secondary antibody (1:400 in PBST, Cat. # A-11034, Invitrogen) to label anti-5-hmC, at RT and in the dark. After labelling, cells were thoroughly washed, centrifuged, and resuspended in PBS and kept in the dark at 4 °C until flow cytometry analysis.

2.3.6. Data analysis

Data obtained in the flow cytometric assays (intracellular ROS, γ-H2AX, 5-mC, and 5-hmC) were analyzed using the software FlowJo Software. Data were normalized by dividing the values of each treatment by the value corresponding to the SC, since there was no significant difference between the SC and the NC in any assay carried out.

Normalized values were analyzed by ANOVA followed by Dunnett's multiple comparisons test, considering $p<0.05$ as a significant difference related to the SC. For intrecellular ROS evaluated in different time-points statistical analysis was performed with analysis of variance followed by Bonferroni post-tests.Flow cytometry experiments assays were performed in biological triplicates.

3. Results

3.1. In silico prediction of genotoxicity

Overall, the results from the *in silico* predictions classify OP as a non-genotoxicant (Table 1). In ToxTree, positive predictions based on structure alerts were only verified for DNA binding, in which an alert for Michael Acceptor was identified. No alerts for the MN assay in rodents and no alerts for mutagenicity and carcinogenicity by Benigni/Bossa rule base were verified.This means that OP is negative for genotoxic carcinogenicity; and nongenotoxic carcinogenicity.

None of the predictions from VEGA platform identified mutagenicity for OP, and all predictions were in the Applicability Domain (AD). *In vitro* MN activity of IRFMN-VERMEER also indicated inactivity, although it could be out of the AD. Predictions from QSAR ToolBox agreed with the findings from ToxTree and models from VEGA platform that OP is not genotoxic; although, an alert was found to DNA binding caused by Michael addition, caused by the formation of alkyl phenols and P450 metabolism to quinones or quinone type-chemicals, in accordance with the results of ToxTree for the same endpoint. Regarding mutagenicity, carcinogenicity, and genotoxicity, the QSAR ToolBox predicted OP to be negative, searching alerts of several endpoints: carcinogenicity (genotoxic and non-genotoxic) by ISS; protein biding alerts for chromosomal aberration (CA) by OASIS; *in vivo* mutageniticy (MN) by ISS; *in vitro* mutagenicity (Ames test) by ISS; and DNA alerts for Ames, CA, and MN Test by OASIS.

Table 2. Summary of *in silico* predictions of octylphenol (OP) for genotoxicity.

Platform	Result	AD
TOXTREE	Negative for mutageniticity, carcinogenicity, genotoxicity; positive for DNA biding (alert for Michael acceptor).	ND
VEGA Mutagenicity (IRFMN)	Negative	Into AD
VEGA (IRFMN-VERMEER)	Negative	May be out of AD
QSAR TOOLBOX 4.5	Negative for mutagenicity, carcinogenicity, genotoxicity; positive for DNA binding (alert for Michael acceptor).	ND

AD = Applicability Domain; ND = not defined.

3.2. In vitro assays

3.2.1. Oxidative Stress

The production of the intracellular ROS was evaluated in two different conditions: in a single time point (after 24 h of exposure) and in different time points from 0 to 60 min (kinetic experiment). Thus, for HaCaT cells exposed to OP for 24 h, a significant increase in ROS level was verified only for the lowest tested concentration (0.5 µg/mL) (Fig. 2). In the kinetic experiment, a significant time-dependent increase in ROS levels was not verified to any of the tested concentrations (Fig.3).

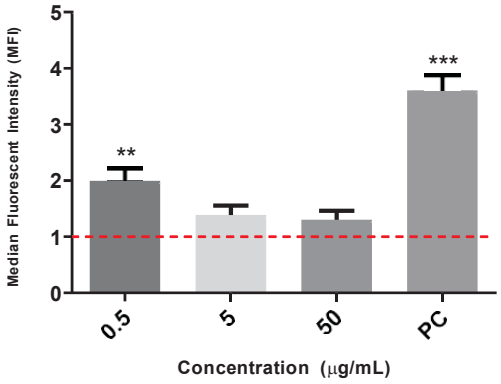

Figure 2. Quantification of intracellular ROS in HaCaT cells exposed to octylphenol (OP) (0.5, 5 and 50 ug/mL) for 24 h, using H_2DCFDA probe. Data are expressed in mean ± SD of the normalized geometric mean fluorescence intensity (gMFI) of three independent experiments. H_2O_2 at 100 µM was used as the PC. ** $p < 0.01$, *** $p < 0.001$. PC: positive control.

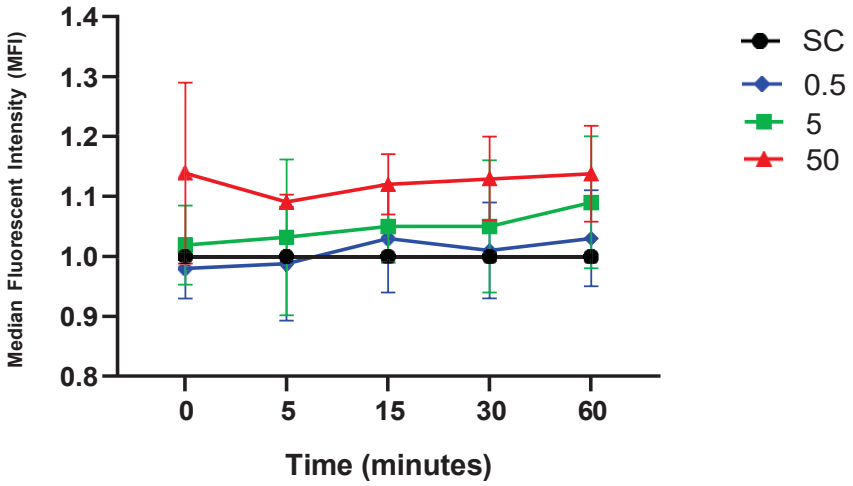

Figure 3. Quantification of intracellular ROS in HaCaT cells exposed to octylphenol (OP) (0.5, 5 and 50 ug/mL) at different time points, using H_2DCFDA probe. Data are expressed in mean ± SD of the normalized geometric mean fluorescence intensity (gMFI) of three independent experiments. Results are expressed as mean±SD, of three biological replicates. Statistical analysis was performed with analysis of variance followed by Bonferroni post-tests, with *$p<0.05$ versus solvent control cells.

3.2.2. DNA damage

In addition, OP at 0.5 ug/mL could induce DNA damage which could be verified by phosphorylation of histone variant H2AX. OP at 0.5 ug/mL enhanced the levels of H2AX compared to untreated cells, showing OP can cause DNA damage to HaCaT cells (Fig. 3).

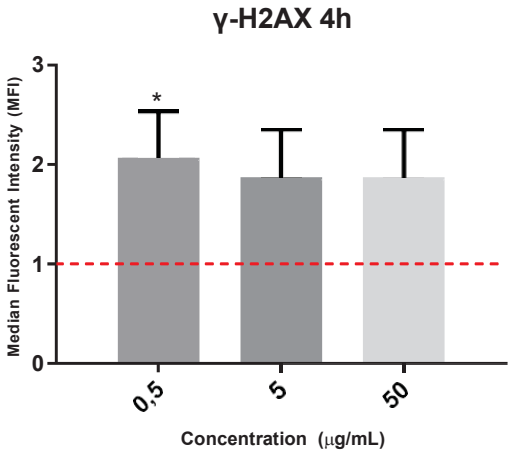

Figure 3. Gamma H2AX (γ-H2AX) levels in the DNA of HaCaT cells after exposure to OP (0.5, 5 and 50 ug/mL) for 4 h. Data are expressed in mean ± SD of the normalized median fluorescence intensity (MFI) of Alexa Fluor 488® of three independent experiments. The red line represents the SC. *$p < 0.05$.

3.2.3. Epigenetic effects

DNA levels of 5mC showed OP at 0.5 ug/mL can present an effect on global methylation pattern of HaCaT cells. 5hmC appeared to be unaffected by the exposure of any tested concentration of OP (Fig.4).

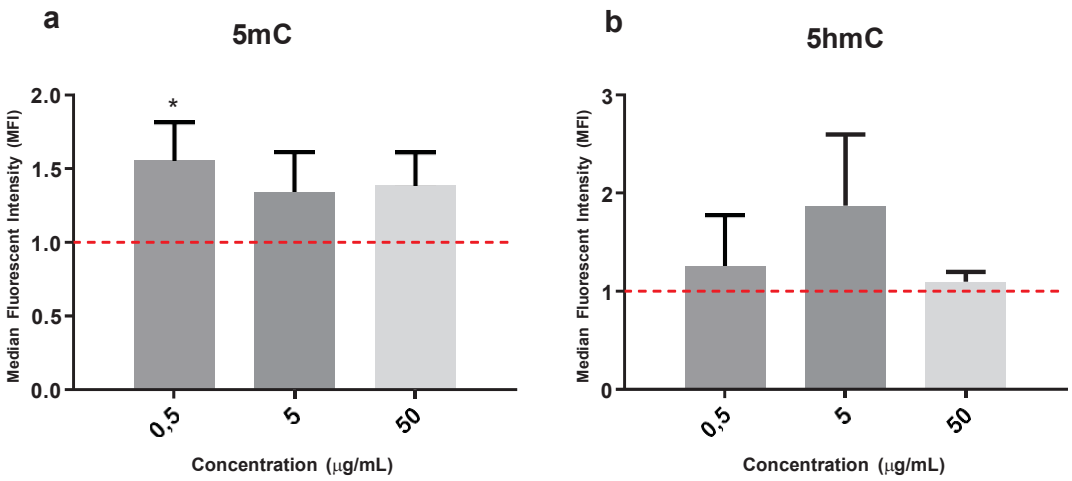

Figure 4. 5-methylcytosine (5mC) (a) and 5-hydroxymethylcytosine (5hmC) (b) levels in the DNA of HaCaT cells after exposure toOP (0.5, 5 and 50 ug/mL) for 24 h. Data are expressed in mean ± SD of the normalized median fluorescence intensity (MFI) of Alexa Fluor 488® of three independent experiments. Ascorbic acid (AA) at 50 µM was used as PC of 5mC and Hydrogen peroxide (H_2O_2) at 600 µM was used as the PC of H2AX. The red line represents the SC. *$p < 0.05$.

4. Discussion

In silico tools are important for evaluating genotoxicity because they can predict the outcome of the Ames test and MN and CA assays based on well-understood structure-activity relationships. Although these models do not use any biological system, they are generally built based on biological data, providing mechanisms insights into the genotoxic activity of chemicals and limiting laboratory experimentation by assuming that similar substances will exhibit similar biological activity in general (Wichard et al. 2017; Worth et al. 2010; ECHA, 2008; OECD, 2007).

In this study, QSAR ToolBox and ToxTree found a structural alert for DNA binding, which means that an atom or group of adjacently connected atoms in the OP molecule is associated with DNA binding. Both tools predicted OP as positive for DNA binding by presenting an alert for Michael acceptor. According to QSAR Toolbox, OP, as an alkyl phenol, can be oxidated by cytochrome P450 to quinones and quinone-type chemicals. Quinones are Michael acceptors (Bolton et al. 2000) and thus participate in Michael addition reactions. Michael addition is a special type of 1,4-addition (or

conjugate addition) that can form a carbon-carbon bond, an important reaction for organic synthesis (*i.e.,* converts a carbon-carbon double bond into two carbon-carbon single bonds). The conjugate addition of thiols and amines from DNA polymerases to Michael acceptors may inactivate DNA-repair mechanisms; thus, Michael acceptors may be carcinogenic agents (Kazlauskas et al. 2012). Chemical oxidation by cytochrome P450 to a quinone methide followed by Michael addition has been suggested to be the primary route of DNA binding (Bolton et al. 2014)). Michael acceptors can be further detoxified by the tripeptide glutathione, which reacts with them by the conjugate addition of a glutathione thiol group or causes several DNA damages (Kazlauskas et al. 2012). Recently, a study demonstrated the ability of OP to bind to the DNA of calf thymus (ctDNA). OP is mainly bound to the T–C rich region in the minor groove of DNA, and the OP−ctDNA complex was formed by hydrogen bonds and van der Waal's forces (Li et al. 2020). This finding may support the *in silico* positive prediction for DNA binding to OP.

Additionally, some quinones are also potent redox-active compounds that can undergo enzymatic (by P450) or non-enzymatic redox cycling with their corresponding semiquinone radical, resulting in the production of superoxide anion radicals (Bolton et al. 2000). Superoxide anions can participate in the formation of hydrogen peroxide (H_2O_2). H_2O_2 molecules can cause oxidative DNA damage by generating hydroxyl-free radicals (OH) – powerful oxidizing agents that damage macromolecules. OH modifies purines and pyrimidines to their hydroxyl derivatives, such as 8-oxoGua and can also generate DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) (Rodrigues-Souza et al. 2022; Bolton et al. 2000; Li et al. 2021). Thus, the presence of a quinone group in the chemical structure of a compound can result in the production of DNA strand breaks induced by oxidative DNA damage (Begleiter & Blair, 1984). Experiments investigating ROS formation showed that OP at 0.5 µg/mL can cause oxidative stress following 24 h of exposure in HaCaT cells, which may be a consequence of the formation of quinones by alkylphenols. Additionally, although studies regarding OP-induced oxidative stress are scarce, some studies report a decrease in the activity of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)) in fish and frog hepatocytes (Kaptaner et al. 2016; Li et al. 2018), causing oxidative damage. ROS was also evaluated following different time-

points (0-60 minutes) after exposure to OP to understand the kinetics of OP-induced oxidative stress; however, significant results were not found.

Regarding DNA damage, γH2AX histone phosphorylation increased after OP exposure (0.5 µg/mL, 4 h). Following DBS, ATM and/ or DNA-PK phosphorylate histone H2AX at Ser139 to form γH2AX, which is the earliest and critical event in the DNA damage response (DDR) pathway. H2AX not only serves to indicate the localization of DNA lesions but its phosphorylation and subsequent ubiquitylation by the RNF8 ubiquitin ligase are required for DNA damage signal amplification and the accumulation of numerous DDR proteins at the sites of DSBs (Kang et al. 2012; Paquin et al. 2018). Thus, an increase in γH2AX abundance suggests induction of DBS as it provides sites for recruiting DNA repair enzymes (Desaulniers et al. 2021). The DNA repair may be successful or not, and the cell may enter senescence, leading to apoptosis, as opposed to carcinogenesis (Kang et al. 2012; Desaulniers et al. 2021). H2AX marker was just present after 4 h of exposure and not 24 h (data not shown), suggesting that cell repair mechanisms have already acted after 24 h. Of note, H2AX marker and ROS production increased at the same OP test concentration. Although, the DSBs detected by the H2AX marker may be a result of oxidative stress, ROS can also be significantly increased by γH2AX overexpression. After chemical-induced DNA damage, H2AX may increase ROS by activating Nox1. Nox1 is a NAD(P)H oxidase expressed in epithelial cells and non-phagocytes and generates ROS through aerobic metabolism (Kang et al. 2012). H2AX increases Nox1 activity after exposure to genotoxicants because it binds to 14-3- 3zeta in Rac1, and thus releases NoxA1 from Rac1. Thus, NoxA1 becomes available to bind to Nox1, activating this enzyme by the Nox1/NoxA1 complex, thus increasing ROS formation (Kang et al. 2012). Although, more investigation is needed to understand the relation between γH2AX and ROS overexpression after OP exposure, the genotoxic potential of OP could be verified in HaCaT cells.

Regarding other epigenetic markers, DNA methylation (DNAm) is also significantly increased in HaCaT cells after OP exposure (0.5 µg/mL), while no changes in hypomethylation were observed (no increased levels of 5hmC). DNAm involves the addition of a methyl group by DNA methyltransferases (DNMTs), primarily to the carbon-5 of cytosine within cytosine–phosphate–guanine dinucleotides

(CpGs) to produce 5mC, which is the most studied epigenetic marker for environmental contaminants (Desaulniers et al. 2021; Martin et al. 2018). The disruption of methylation patterns is frequently observed in cancer (in CpG island hypermethylation), which is associated with silencing tumour suppressor genes (Dawson et al. 2012; Desaulniers et al. 2021). Changes in DNAm can also disturb the normal pattern of gene expression and imprinting, alternative splicing, X chromosome inactivation and can affect the maintenance of genome stability (Klose et al. 2006; Tomkova et al. 2018). With respect to mutation induction, 5mC can suffer spontaneous hydrolytic deamination, converting cytosine into timine (Lindahl et al. 1974; Cooper et al. 2010), which, if not correctly repaired, will result in a C > T mutation in the next replication cycle. The resulting C to T transitions in CpG sites (CpG > TpG) is the most common type of mutations observed in cancer, but also in normal somatic cells, as well as in the germ line (Alexandrov et al. 2013; Rahbari et al. 2015).

Importantly to skin cells, 5mC can increase UV light-induced DNA damage, by increasing cis-syn cyclobutane pyrimidine dimers (CPD) formation by sunlight and UVB exposure, but not UVC exposure (Tommasi et al. 1997; Mitchell et al. 2007; Rochette et al. 2009). CPD are DNA lesions mainly formed by photochemical reactions between adjacent pyrimidine bases, which can be repaired by nucleotide excision repair (NER). Most of the observed UV-induced mutations in human skin cells are C > T and CC > TT transitions due to CPDs (Brash et al. 2015). It is suggested that 5mC may induce damages for having fivefold higher molar absorption coefficient of methylated compared with unmethylated cytosine at 290 nm, but more similar values (1.3-fold lower for 5mC versus C) at 254 nm (UVC) (Rochette et al. 2009; Sharonov et al. 2003). Additionally, cytosines in CPDs are unstable and spontaneously deaminate within hours to days due to the loss of aromatic stabilization (Cannistraro et al. 2009), which could lead to C > T mutation. However, deamination of 5mC-containing CPDs may be strongly affected by the sequence context, with TCG exhibiting an approximately 50 times faster rate than CCG (Cannistraro et al. 2009). These effects can be observed in the mutational signature characteristic of melanoma patients, which consists almost exclusively of C > T mutations, mostly in a CC or TC sequence context, with the highest mutation frequency in a TCG context (Alexandrov et al. 2013). Additionally, the deamination rate of 5mC inside CPDs depends also on the rotational positioning of DNA around the nucleosome, with 42-fold higher deamination rate when positioning

away from the nucleosome surface compared with positioning against the histone core surface (Mao et al. 2016).

Regarding how OP can induce DNAm, it has been proposed that EDCs can cause these alterations by changing the expression of DNMTs (Desaulniers et al. 2021). However, how this specificitis is controlled remains unclear. To date, oxidative stress has been associated with 5hmC but not with 5mC changes (Chia et al. 2011; Zhao et al. 2014). While more experimental work is needed to understand DNAm mechanisms of EDCs, it is nevertheless of interest that OP is described to change epigenetics in human keratinocytes (HaCaT) for the first time.

5. Conclusions

In the present study, the ability of OP to bind to DNA, cause DNA damage and increase DNAm levels in human keratinocytes was demonstrated using NAMs (*in silico* and *in vitro* methods). Changes in DNAm show that OP may present properties of non-genotoxic carcinogens, while the histone modifications (H2AX) showed that OP is genotoxic by causing DSBs. DNA damage may be a consequence of the oxidative stress caused by OP. However, the mechanisms underlying the positive effects verified in this study still need further investigations to better elucidate. In summary, the present finding showed concerns regarding the toxic effects of OP on the skin.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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5. CONCLUSÕES GERAIS

Soluções devem ser continuamente criadas e testadas em contextos diversos, no intuito de garantir a saúde humana. Este artigo traz a revisão acerca das variantes genéticas que podem influenciar a susceptibilidade individual a respostas inflamatórias e imunológicas na pele, que podem servir de base na criação e implantação de testes toxicológicos futuros. Além disso, o presente artigo destaca como novas estruturas e iniciativas podem ser aplicadas para avaliar as melhores evidências disponíveis de vários modelos para prever a sensibilização da pele e promover a segurança e a confiança para avaliar os potenciais efeitos adversos de produtos químicos industriais relacionados à saúde humana. A integração de métodos *in silico* e *in vitro* foi usada com eficiência para definir o potential de sensibilização cutânea do OP e do DiPeP e também o potencial genotóxico do OP. O OP pode atuar na sensibilização cutânea através da ligação a proteínas da pele (KE1), ativação de queratinócitos (KE2) e células dentríticas (KE3). O DiPeP por sua vez pode atuar no KE2 e K3. Adicionalmente, este estudo também traz informações sobre o efeito imunomodulatório das duas substâncias (aumentando a inflamação causada por outros agentes inflamatórios). A investigação de novos marcadores, trouxe informações sobre a capacidade das substâncias testadas de atuar na epigenética, mecanismo que carece de maiores informações dentro dos endpoints investigados. Desta forma, definimos que o OP pode alterar marcas epigenéticas atuando na metilação do DNA e reduzindo a expressão de RNAs longos não condificantes associados à homeostase cutânea. Para obter aceitação e implementação direta dos métodos usados para a avaliação da toxicicidade de produtos químicos para aplicação em contextos regulatórios ou outros contextos de tomada de decisão sistemática, há uma necessidade contínua de demonstrar relevância biológica dessas abordagens em estudos mais profundos sobre os marcadores e técnicas utilizadas. No geral, o presente trabalho propõe alternativas e destaca como novas abordagens de teste podem ser aplicadas para predizer a sensibilização da pele e promover a segurança à saúde humana.

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