

# An integrated transcriptomic- and proteomic-based approach to evaluate the human skin sensitization potential of glyphosate and its commercial agrochemical formulations



Tim Lindberg<sup>a</sup>, Renato Ivan de Ávila<sup>a,b,c</sup>, Kathrin S. Zeller<sup>a</sup>, Fredrik Levander<sup>a</sup>, Dennis Eriksson<sup>d</sup>, Aakash Chawade<sup>d</sup>, Malin Lindstedt<sup>a,\*</sup>

<sup>a</sup> Department of Immunotechnology, Lund University, Medicon Village, Lund, Sweden

<sup>b</sup> Laboratory of Education and Research in In Vitro Toxicology (Tox In), Faculty of Pharmacy, Universidade Federal de Goiás, Goiânia, GO, Brazil

<sup>c</sup> SenzaGen AB, Medicon Village, Lund, Sweden

<sup>d</sup> Swedish University of Agricultural Sciences, Alnarp, Sweden

## ARTICLE INFO

### Keywords:

Roundup  
Glyphosate  
POEA  
Allergic contact dermatitis  
Proteomics  
Transcriptomics

## ABSTRACT

We investigated the skin sensitization hazard of glyphosate, the surfactant polyethylenated tallow amine (POEA) and two commercial glyphosate-containing formulations using different omics-technologies based on a human dendritic cell (DC)-like cell line. First, the GARD™skin assay, investigating changes in the expression of 200 transcripts upon cell exposure to xenobiotics, was used for skin sensitization prediction. POEA and the formulations were classified as skin sensitizers while glyphosate alone was classified as a non-sensitizer. Interestingly, the mixture of POEA together with glyphosate displayed a similar sensitizing prediction as POEA alone, indicating that glyphosate likely does not increase the sensitizing capacity when associated with POEA. Moreover, mass spectrometry analysis identified differentially regulated protein groups and predicted molecular pathways based on a proteomic approach in response to cell exposures with glyphosate, POEA and the glyphosate-containing formulations. Based on the protein expression data, predicted pathways were linked to immunologically relevant events and regulated proteins further to cholesterol biosynthesis and homeostasis as well as to autophagy, identifying novel aspects of DC responses after exposure to xenobiotics. In summary, we here present an integrative analysis involving advanced technologies to elucidate the molecular mechanisms behind DC activation in the skin sensitization process triggered by the investigated agrochemical materials.

**Significance:** The use of glyphosate has increased worldwide, and much effort has been made to improve risk assessments and to further elucidate the mechanisms behind any potential human health hazard of this chemical and its agrochemical formulations. In this context, omics-based techniques can provide a multiparametric approach, including several biomarkers, to expand the mechanistic knowledge of xenobiotics-induced toxicity. Based on this, we performed the integration of GARD™skin and proteomic data to elucidate the skin sensitization hazard of POEA, glyphosate and its two commercial mixtures, and to investigate cellular responses more in detail on protein level. The proteomic data indicate the regulation of immune response-related pathways and proteins associated with cholesterol biosynthesis and homeostasis as well as to autophagy, identifying novel aspects of DC responses after exposure to xenobiotics. Therefore, our data show the applicability of a multiparametric integrated approach for the mechanism-based hazard evaluation of xenobiotics, eventually complementing decision making in the holistic risk assessment of chemicals regarding their allergenic potential in humans.

## 1. Introduction

Herbicides based on glyphosate [*N*-(phosphonomethyl) glycine] have been used for weed control in agricultural production and home gardens since the 1970s and the first formulation launched on the

market was Roundup® [1,2]. Today, more than 750 different products containing glyphosate as the main active ingredient have been catalogued [1]. Glyphosate exhibits its herbicidal activity by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase [3]. Since this enzyme is not present in humans, the impact of glyphosate on human health has

\* Corresponding author at: Lund University, Medicon Village (Building 406), 22381 Lund, Sweden.

E-mail address: [malin.lindstedt@immun.lth.se](mailto:malin.lindstedt@immun.lth.se) (M. Lindstedt).

<https://doi.org/10.1016/j.jprot.2020.103647>

Received 13 September 2019; Received in revised form 11 December 2019; Accepted 8 January 2020

Available online 30 January 2020

1874-3919/ © 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

been assumed to be minimal [4]. However, glyphosate and its formulations are currently under examination regarding their human toxicity [5], and their widespread and increasing use [6] warrants more scientific data to explain their effects on human immune cells.

Although several reports have investigated the potential toxicity of glyphosate and its commercial formulations, there is no consensus regarding their immunotoxic effects and other human health hazards. The European Food Safety Authority (EFSA) has reported that glyphosate is “unlikely to pose a hazard for humans” [7], while the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) has classified the chemical as “probably carcinogenic to humans” [1]. As herbicidal formulations are mixtures of several chemicals, it has been suggested that the other ingredients, which are commonly kept confidential and are misleadingly called “inert” by the manufactures, can give rise to toxicity apart from the active ingredient glyphosate [8]. A review of epidemiological studies of the relation between glyphosate and cancer indicates that it would be important to examine also the multi-component herbicide formulations [9], since they can show a different toxicity profile from the active ingredient alone and may therefore be classified differently by regulatory authorities [10]. Also, the finished product represents the “real-life” mixtures to which humans (e.g. workers and consumers) are exposed to.

Furthermore, combined effects (e.g. synergy, addition or potentiation) of the different components in a mixture, such as an herbicidal formulation, could influence the immunological response [11,12]. One group of commonly used co-formulants in herbicide formulations are surfactants, which improve the foliar uptake of the active ingredient [2,13]. Among them, polyethylated tallow amine (POEA) has been widely used and consists of a mixture of compounds containing different alkyl group substituents obtained from animal-derived fatty acids [13]. Findings have indicated the potential toxicity of this “inert” ingredient in glyphosate-based formulations [14]. Among the human health risks highlighted are, for example, carcinogenicity [1] and endocrine disruption, though the latter may rather be related to other co-formulants and/or a cocktail effect in the commercial formulations [15]. In addition, the toxicological assessments conducted by the EFSA has identified scientific evidence supporting a higher toxicity of POEA in several toxicological endpoints, including skin irritation and sensitization, when compared to the glyphosate alone [16]. In view of this, POEA has been prohibited in the European Union [17] and thus some manufacturers have replaced it with other surfactants, such as alkyl polyglycoside [2]. However, there is no evidence in humans if non-POEA surfactants provide safer formulations in comparison to the POEA-containing ones. Notably, in this context, knowledge about the cellular and molecular mechanisms underlying skin immunotoxicity induced by glyphosate and its commercial mixtures remains lacking.

While the exposure to glyphosate is mainly discussed in the context of the main users, i.e. farmers, traces of glyphosate are found in the urine of the general population not connected to agricultural occupations [18,19]. This further highlights the importance of performing risk assessment and making scientifically sound decisions on the restrictions or occupational care management of glyphosate-containing formulations. Historically, decisions for assessing the potential for xenobiotics to cause allergic contact dermatitis are derived from experiments on animals, e.g. the murine local lymph node assay (LLNA) [20]. However, scientific progress in the development of innovative *in vitro* predictive methods and several other factors, including economical, ethical and political concerns, have made animal testing increasingly unfavourable [21,22]. Associated with this, legislations, such as the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), state that animal testing for regulatory purposes should only be conducted as a last resort [23,24]. Moreover, the European Union has banned the use of animal testing in hazard and risk assessment of cosmetic materials as well as the sale of products undergone *in vivo* tests since 2009 and 2013, respectively [25].

As a consequence, the development of *in vitro* methods to identify

allergenic materials has been encouraged, as recently reviewed [26]. In fact, the mechanistic understanding of the chemical-induced skin sensitization process in humans, summarized in the adverse outcome pathway (AOP) for skin sensitization published by the Organisation for Economic Co-operation and Development (OECD) in 2012 [27], has driven such progress. Modern technology, such as omics-based techniques, can enhance the mechanistic knowledge of chemical toxicity through a multiparametric analysis of several biomarkers [28], which in turn can provide better prediction of chemical-induced adverse effects on human health. Thus, this mechanism-based approach contributes to the establishment of a holistic human health risk assessment of xenobiotics [29]. Among the *in vitro* techniques developed until now for skin sensitization prediction, several are based on human dendritic cell (DC) models [26], since DCs has a central role in the skin sensitization pathway [27]. In this regard, their gene and protein regulation pathways can reveal much about the underlying mechanisms and molecular events triggering allergic reactions towards chemicals and complex mixtures, such as glyphosate-based formulations.

In this study, we investigated the skin sensitization capacity of glyphosate, the surfactant POEA and two commercially available POEA-free glyphosate-based formulations using different omics-technologies and a human DC model. First, a transcriptomic-based analysis with the Genomic Allergen Rapid Detection (GARD™) assay for chemical skin sensitization prediction (GARD™skin) was performed. Thereafter, a proteomic-based approach was carried out by mass spectrometry (MS) analysis of purified protein extracts from chemical-exposed cells. Presented here are the protein groups differentially regulated in response to the glyphosate formulations and the surfactant POEA. Pathways associated with differentially regulated protein groups showed immunologically relevant events, which can lead to a better understanding of the molecular mechanisms induced by such materials. Furthermore, we present data from *in vitro* experiments indicating that glyphosate, in contrast to the surfactant POEA and the two commercially finished products tested, has no immunotoxic potential in the context of human skin sensitization.

## 2. Materials and methods

### 2.1. Chemicals and reagents

POEA was purchased from Crescent Chemical Company (Islandia, NY, USA). The following commercial formulations were obtained from a Swedish agricultural supplies retailer and received as gifts from the Swedish University of Agricultural Sciences (Alnarp, Sweden): Roundup® Flex (containing the surfactant alkyl polyglycoside and 588 g/L glyphosate; registration number of the Swedish Chemicals Agency: 5065) and Jablo (containing the surfactant ether amine ethoxylate and 441 g/L glyphosate; registration number of the Swedish Chemicals Agency: 4972). Dimethyl sulfoxide (DMSO), *p*-phenylenediamine (PPD), HyClone™ minimum essential medium alpha modification with L-glutamine, ribo- and deoxyribonucleosides (MEM- $\alpha$ ) and TRIZOL® were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was purchased from PeproTech (Rocky Hill, NJ, USA). Propidium iodide (PI), FITC-conjugated anti-human [CD86 (FUN-1), HLA-DR (L243), CD34 (581) and isotype control anti-IgG1 (MOPC-21)] and PE-conjugated anti-human [CD54 (HA58), CD80 (L307) and isotype control anti-IgG1 (MOPC-21)] antibodies were acquired from BD Biosciences (San Jose, CA, USA). FITC-conjugated anti-human CD11a (NA1/34) and PE-conjugated anti-human CD14 (TÜK4) antibodies were purchased from Dako (Santa Clara, CA, USA). Direct-zol™ RNA MiniPrep column purification kit and trypsin were acquired from Zymo Research (Irvine, CA, USA) and Promega Biotech AB (Madison, WI, USA), respectively. Hybridization buffer, reporter co-dset, capture probeset and nCounter® cartridges were purchased from NanoString® Technologies (Seattle, WA, USA). Protease inhibitor tables

EDTA-free and Silica C18 UltraMicro spin columns were acquired from Roche Diagnostics GmbH (Mannheim, Germany) and The Nest group (Southborough, MA, USA), respectively. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

## 2.2. Cell culture

MUTZ-3-derived cells (DSMZ, Braunschweig, Germany) were cultured in MEM- $\alpha$  supplemented with 20% (v/v) fetal bovine serum (FBS) and rhGM-CSF (40 ng/mL), and maintained in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37 °C. Cell viability was analyzed using LUNA™ automated cell counter (Logos Biosystems, Annandale, VA, USA), according to manufacturer's instructions. A cell viability value > 90% was considered satisfactory to carry out the experiments, which were performed using three independent batches of cells. Moreover, quality control analysis was performed for each cell batch using BD FACSCanto II flow cytometer (Biosciences, San Jose, CA, USA), in accordance with previously published protocols [30,31]. For that, the following antibodies were used: FITC-conjugated anti-human CD1a, CD34, CD86 or HLA-DR, and PE-conjugated anti-human CD14, CD54 or CD80; FITC- or PE-anti-IgG1 were used as isotype controls, while staining with PI (1  $\mu$ g/mL) was performed for cell viability analysis.

## 2.3. Cell exposure with the test materials

Cell exposures and selection of input non-cytotoxic concentration of each test material (e.g. test material concentration inducing 90% relative viability, when compared to unexposed cells, namely RV<sub>90</sub> value) were performed according to established protocols [31,32]. In short, test materials were diluted in appropriate vehicles (Table 1) and the cells (2  $\times$  10<sup>5</sup> cells/mL) were exposed to different concentrations for 24 h. After that, flow cytometer analysis was conducted to evaluate the cell viability using PI staining and then to obtain the RV<sub>90</sub> values. In the case of non-cytotoxic materials, an input concentration of 500  $\mu$ M was used. After that, cells were exposed to each test material at input concentration (Table 1) to carry out the flow cytometric analysis of CD86 expression and transcriptional analysis. Cell exposures were performed in triplicate cell batch reactions, i.e. a new cell batch was used each exposure round, resulting in a total of 18 samples. In addition, three replicate cell batches were exposed to POEA combined with glyphosate in their respective input concentrations as listed in Table 1 in order to investigate effects of this mixture. Total RNA was collected by lysing 2  $\times$  10<sup>5</sup> cells/exposure in TRIzol® and stored at -20 °C until RNA purification and further processing for transcriptional analysis and GARD™ skin predictions. Schematic view of experimental procedures can be seen in Fig. 1.

## 2.4. GARD™skin assay

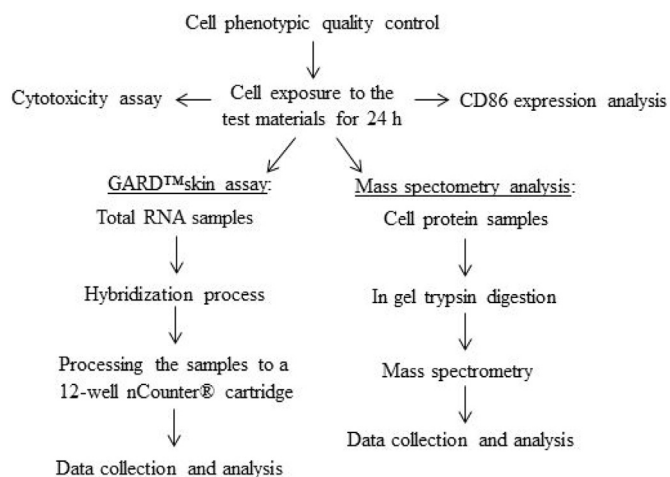
The *in vitro* assay GARD™skin (SenzaGen AB, Lund, Sweden) was used to predict the ability of chemical substances to induce skin sensitization. This technology was developed based on a transcriptomic approach and machine learning techniques, comprising a predictive

**Table 1**

Test materials used for cell exposures.

Material	Abbreviation	CAS no.	Vehicle	Classification	Input concentration
Double-distilled water	H <sub>2</sub> O	na	na	Unexposed	0.1% (v/v)
Dimethyl sulfoxide	DMSO	67-68-5	na	Vehicle control	0.1% (v/v)
<i>p</i> -Phenylenediamine	PPD	106-50-3	DMSO	Positive control	75 $\mu$ M
Glyphosate	GLY	1071-83-6	H <sub>2</sub> O	na	500 $\mu$ M
Polyethylated tallow amine	POEA	61791-26-2	H <sub>2</sub> O	na	0.001% (v/v)
Roundup® Flex	na	na	H <sub>2</sub> O	na	0.12% (v/v)
Jablo	na	na	H <sub>2</sub> O	na	0.008% (v/v)

na: not applicable.



**Fig. 1.** Schematic view of experimental procedures.

biomarker signature of 200 transcripts associated with the skin sensitization capacity of chemicals [30]. Also, GARD™skin has been demonstrated suitable for skin sensitization prediction of a wide variety of chemical classes and complex mixtures [12,31,33–36]. Total RNA was isolated from the cells lysed in TRIzol® reagent using the Direct-zol™ RNA MiniPrep column purification kit (Zymo Research), according to manufacturer' instructions. For each sample, concentration and RNA integrity was determined with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). The following gene expression analysis was performed on NanoString® GEN2 nCounter analysis system (NanoString® Technologies) using the GARD™skin assay. In brief, 5  $\mu$ L RNA sample were mixed with 8  $\mu$ L master mix, comprising hybridization buffer and reporter CodeSet, and 2  $\mu$ L Capture ProbeSet followed by 24 h hybridization at 65 °C using a 3Prime thermal cycler (Bibby Scientific, Staffordshire, UK). Samples were then processed and transferred to a 12-well nCounter® cartridge in the GEN2 Prep station 5 s set at high sensitivity followed by data collection on the Digital Analyzer 5 s with an imaging resolution of 555 fields of view. The Reporter Code Count files (RCC) files were then downloaded from the Digital Analyzer and processed using SenzaGen software version 1.0 (SenzaGen AB), which conducts both NanoString® quality control and data normalization. A support vector machine (SVM) algorithm was used to assign a so-called decision value (DV) to each test material replicate. A test material is classified as skin sensitizer when the SVM median output value is > 0 of the three independent replicates, while a median DV < 0 classifies the material as a non-sensitizer.

## 2.5. Proteomic analysis

### 2.5.1. Protein extraction and concentration determination

For proteomic analysis, cells were also exposed to each test material at input concentration (Table 1). After 24 h, cells were harvested and processed for MS analysis by pelleting the chemically exposed cells in a

maximum recovery Eppendorf tube followed by washing with phosphate buffered saline (PBS) before flash freezing of the pellet in liquid nitrogen. Cell pellets were stored at  $-80^{\circ}\text{C}$  before further MS processing. Each cell batch of chemical exposure was performed in duplicates of  $2 \times 10^6$  cells/exposure, and thereafter pooled to ensure a sufficient protein amount for MS analysis. In addition, triplicates of unexposed (water) samples were run resulting in a total of 21 samples for MS analysis. Following cellular exposure proteins were extracted from each sample by lysing the cells in 150  $\mu\text{L}$  lysis buffer (8 M urea, 39 mM Tris, 5 mM MgAc, 4% CHAPS, protease inhibitor tablet) through three freeze/thaw cycles and separating supernatant and cell debris by centrifugation at  $16000 \times g$  for 30 min. Protein extracts were stored in  $-80^{\circ}\text{C}$  until further processing. Protein concentration determination was performed using the Total Protein Micro-Lowry kit (Sigma-Aldrich) according to manufacturer's instructions. The concentration was determined by measuring the absorbance at 650 nm and 50  $\mu\text{g}$  per sample were used for further processing.

### 2.5.2. In gel trypsin digestion and mass spectrometry

Proteins were separated on an SDS-PAGE gel and digested into peptides using trypsin. Trypsinated peptides were then de-salted using UltraMicro spin columns (Silica C18, SUM SS18 V, Nest group) as described in Chawade et al. [37]. Peptides were thereafter resuspended in 0.1% formic acid (FA) and loaded into an EASY-nano liquid chromatography (LC) system 1200 (Thermo Fisher Scientific). Peptides were injected directly into the analytical column, a 15 cm long fused silica capillary (75  $\mu\text{m} \times 16$  cm Pico Tip Emitter, New Objective, Woburn, MA, USA) packed *in-house* with C18 material ReproSil-Pur 1.9  $\mu\text{m}$  (Dr Maisch GmbH, Germany). Peptides were separated using an 80 min gradient from 5% to 90% solvent B (80% acetonitrile, 0.1% FA, v/v) at a constant flow rate of 250 nL/min. The nLC system was coupled with a Q-Exactive™ HF-X (Hybrid Quadrupole-Orbitrap™) (Thermo Fisher Scientific) operated in a positive mode for data-dependent acquisition (DDA). The Orbitrap acquired the full MS scan with an automatic gain control (AGC) target value of  $3 \times 10^6$  ions and a maximum fill time of 50 ms. The 20 most abundant peptide ions were selected from the MS for higher energy collision-induced dissociation (HCD) fragmentation (collision energy: 40 V). The instrument was scanning at a target MS1 resolution of 120,000 between 375 and 1500  $m/z$  window with 15,000 MS/MS resolution for a target of  $1 \times 10^5$  and a maximum injection time of 20 ms using an isolation window of 1.2  $m/z$ .

### 2.5.3. Downstream processing of peptide data

Raw data from Xcalibur software (Thermo Fisher Scientific) were converted to mzML format using ProteoWizard [38] and MGF peak list files were subsequently generated based on the DeMix cloning method [39] before further processing in the Proteios Software Environment (<http://www.proteios.org>) [40]. Peptide identification was performed using MS-GF+ [41] with a precursor tolerance of 10 ppm, fixed carbamidomethylation and variable methionine oxidation and protein N-terminal acetylation, with instrument set to QExactive against the THISP database level 2, version 2018-06-01 [42], containing 317,620 protein entries including decoys. Subsequent data processing for label free quantification (LFQ) was performed in Proteios using Dinosaur [43] feature detection with matching and alignment between runs as described by Sandin et al. [44]. Features were matched to MSMS identifications filtered at a peptide spectrum match false discovery rate (FDR) of 0.002 but maintaining MSMS identifications passing an FDR threshold of 0.1 when the same peptide sequence was passing the 0.002 FDR threshold elsewhere in the dataset. Peptide abundances extracted from MS1 intensities using the LFQ workflow were normalized using a retention-time-segmented LOESS approach in NormalizerDE [45]. In total, 65,717 peptide feature rows were rolled up to 6029 protein groups using an *in-house* python implementation (LK Möller, MSc Thesis) of reference peptide-based scaling [46]. The protein group table contained 15 decoy entries after the protein grouping, indicating a

protein level FDR  $< 0.003$ . The raw and processed MS data have been deposited to the ProteomeXchange Consortium via the PRIDE [47] partner repository with the dataset identifier PXD016859 and <https://doi.org/10.6019/PXD016859>.

For subsequent analysis, one sample of Jablo had to be removed due to a mix-up with another sample leaving a total of 20 samples for further analysis. Missing values in the dataset were put to 19.24, which were the lowest value in the data matrix and all values were log2 transformed. Visualization of the data was performed in QluCore Omics Explorer 3.5 (QluCore AB, Lund, Sweden). Subsequent principal component analysis (PCA) visualization of the data revealed batch effects, which were corrected with ComBat [48] provided by the *sva* package [49] in RStudio [50], before further analysis.

### 2.6. Statistical analysis

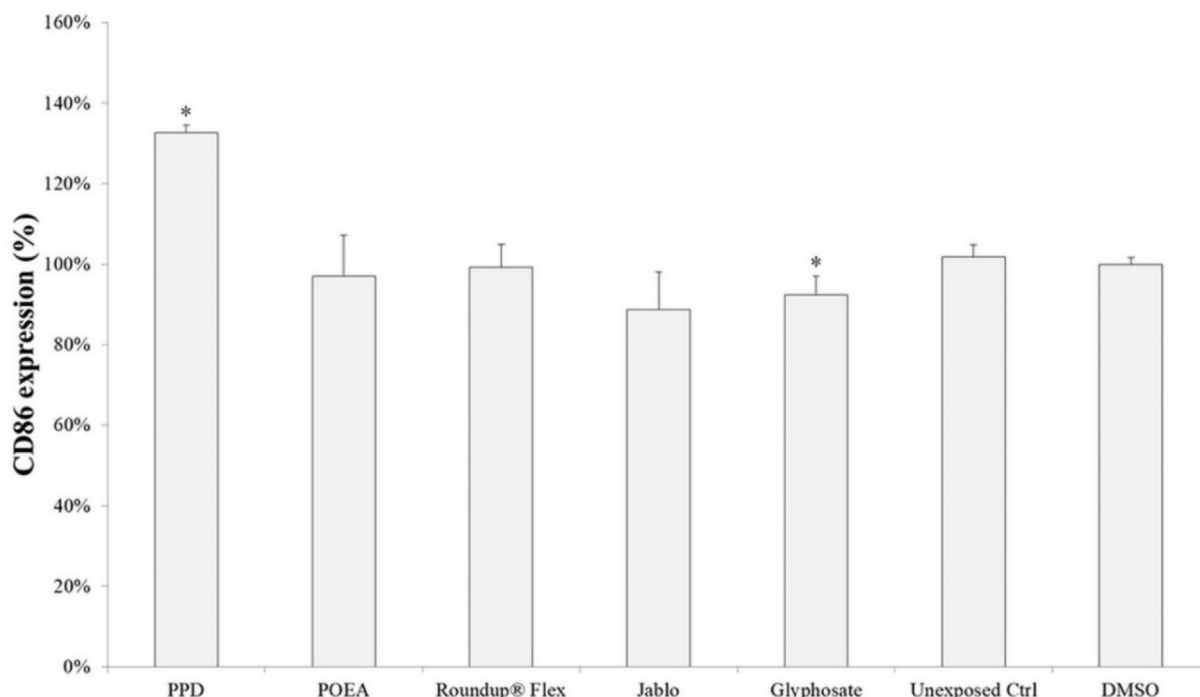
Data are expressed as mean or mean  $\pm$  SD of three independent assays. The inter group variation on CD86 expression assessment was measured by Student's *t*-test at  $p < .05$  established as statistical significance. For proteomic analysis, each group of proteins was associated with a corresponding set of gene(s) to identify predicted pathways enriched by differently regulated protein groups. Two-group comparisons (one-way ANOVA) were made between individual materials and DMSO and unexposed controls at a significance level of 0.05 (FDR) and an absolute fold change cut-off of 1.3. Subsequent fold changes and *p*-values were used as input for the Key Pathway Advisor (KPA) tool (version 17.4) [51], which associates the input genes with upstream and downstream processes to allow biological interpretation. Default settings were used and Key Hubs Calculation Algorithm was set to "Casual Reasoning Analysis". Differentially regulated protein groups were visualized in volcano plots using RStudio.

## 3. Results

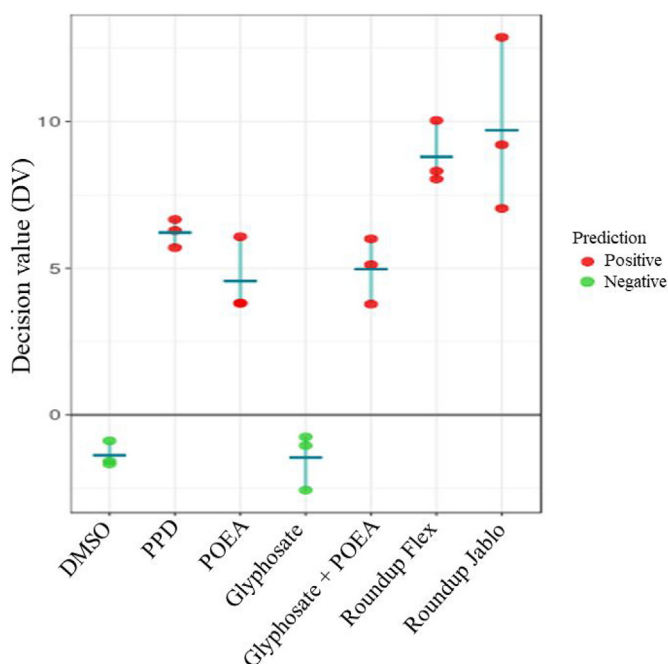
### 3.1. Glyphosate-containing formulations and POEA are predicted as sensitizing by the GARD™skin assay

Following 24 h of exposure with different concentrations of glyphosate-containing formulations (Roundup® Flex and Jablo) as well as the surfactant POEA and pure glyphosate, cytotoxicity analysis was performed using PI staining to define the input concentration for each test material (Table 1). Cells were then exposed to the test materials at input concentrations for 24 h and the maturity state of the cells was assessed by measuring levels of cell surface expression of the co-stimulatory marker CD86 (Fig. 2). Only the positive control PPD induced a significant upregulation of CD86 expression ( $p < .05$ ) in comparison to unexposed control, while Roundup® Flex, Jablo and POEA did not induce any upregulation. Cell exposure with pure glyphosate resulted in a modest downregulation compared to control ( $p < .05$ ). No significant difference between DMSO and unexposed controls was observed.

Furthermore, skin sensitization hazard predictions were performed with the GARD™skin assay. Transcriptomic analysis of the 200 biomarkers from the GARD™skin prediction signature was carried out using the NanoString® nCounter™ System. The skin sensitizing hazard was predicted using an SVM algorithm where each test material replicate was given a DV (Fig. 3). As expected, PPD and DMSO were correctly classified as skin sensitizer and non-sensitizer, respectively. Also, glyphosate was classified as a non-sensitizer with a mean SVM DV of  $-1.45$ . Although, POEA showed a mean DV of 4.56, which classified it as skin sensitizer. Interestingly, the mixture of POEA and glyphosate displayed a similar profile (mean DV = 4.96), indicating that these materials have no combined effect as judged by the GARD™ DV. Moreover, both glyphosate-containing formulations, Roundup® Flex (mean DV = 8.80) and Jablo (mean DV = 9.70), were classified as skin sensitizers.



**Fig. 2.** Analysis of CD86 expression induced by the test materials. Cells were exposed to the test materials at non-cytotoxic input concentrations for 24 h. After that, CD86 expression was determined by flow cytometry. The values were normalized to vehicle control. Error bars indicate the mean standard deviation in percentage out of three biological replicates for each exposure. (\* $p < .05$  vs. control).



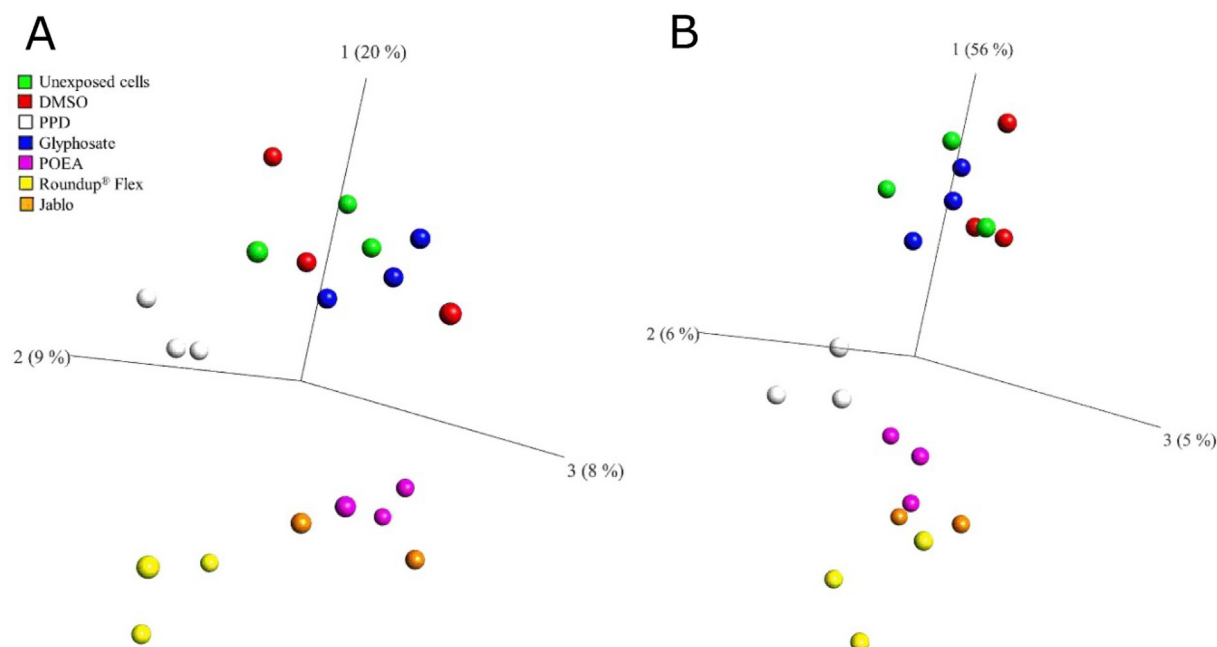
**Fig. 3.** Transcriptomic-based evaluation using GARD™skin assay. Cells were exposed to the test materials at non-cytotoxic input concentrations for 24 h. Total RNA samples were then obtained to perform transcriptomic-based evaluation. Transcript analysis of the 200 genomic biomarkers from the GARD™skin prediction signature was carried using a NanoString® GEN2 nCounter analysis system. A test material was classified as a skin sensitizer when the support vector machine median output value of the three independent replicates  $> 0$ .

### 3.2. Profiling of agrochemical-induced changes in the cellular proteome

Initially, peptide data, generated from the trypsin-digested cells exposed with test materials for 24 h were normalized and assembled to protein groups. In total, this resulted in 6029 protein groups. PCA evaluation was used to identify patterns, i.e. which samples exhibit a similar protein expression profile. As a first step, unsupervised clustering of all protein groups was performed (Fig. 4A). A clear separation between sensitizers (PPD, POEA, Roundup® Flex and Jablo) and non-sensitizers (unexposed, DMSO, glyphosate) is observed. Separating the sensitizers from the non-sensitizers based on a one-way ANOVA (FDR = 0.05) resulted in 1726 differentially expressed protein groups (data not shown). Additionally, by applying a multi-group comparison (FDR = 0.05) based on all the treatments, i.e. clustering of the individual treatments most alike based on their protein expression, a similar pattern was observed as when unsupervised clustering was applied (Fig. 4B). Interestingly, samples representing exposures with POEA and Jablo clustered together.

### 3.3. Differentially regulated protein groups are linked to genes associated with immune-related pathways

Protein groups, assembled from the peptide data, were investigated by KPA to identify enriched predicted pathways induced by exposure with the glyphosate-based formulations. The input data for the KPA analysis were gene names associated to the differentially regulated protein groups identified by comparing each formulation to the control group at a specific cut-off level. At a significance level of 0.05 (FDR) and a fold change cut-off of 1.3, the number of unique protein groups that were identified as differentially expressed, as compared to controls DMSO and unexposed, were 365 for Roundup® Flex exposure (Fig. 5A), 140 for Jablo exposure (Fig. 5B) and 124 for POEA exposure (Fig. 5C). A total of 80 protein groups were commonly regulated by Jablo and Roundup® Flex (Fig. 5D). With the same comparison and at the same fold change cut-off and significance level, glyphosate exposure of cells resulted in only 3 regulated protein groups (data not shown).



**Fig. 4.** PCA plots of protein groups. PCA plots based on the expression profile of the 6029 protein groups of the unexposed cells (green) and those one exposed to DMSO (red), PPD (white), POEA (pink), Glyphosate (blue), Roundup® Flex (yellow) or Jablo (orange) for 24 h. (A) Unsupervised clustering with no statistical analysis applied. (B) Multi-group comparison (FDR = 0.05) based on treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Among the 47 proteins commonly regulated by Roundup® Flex, Jablo, and POEA (Supplementary Table S1), three enzymes appear that are all also part of the GARD™skin prediction signature, namely 7-dehydrocholesterol reductase (DHCR7), 24-dehydrocholesterol reductase (DHCR24) and lanosterol-14a-demethylase, the product of CYP51A1 [52]. Other proteins differentially expressed in response to POEA and the two formulations with highest fold changes are more enzymes and proteins involved in cholesterol synthesis and homeostasis, such as farnesyl-diphosphate farnesyltransferase 1 (FDFT1) and apolipoprotein B (APOB) [52]. Further, thymidylate synthase (TYMS) [53], involved in DNA repair, and p62/SQSTM1, a multifunctional ubiquitin-binding protein [54], are also among the differentially regulated proteins.

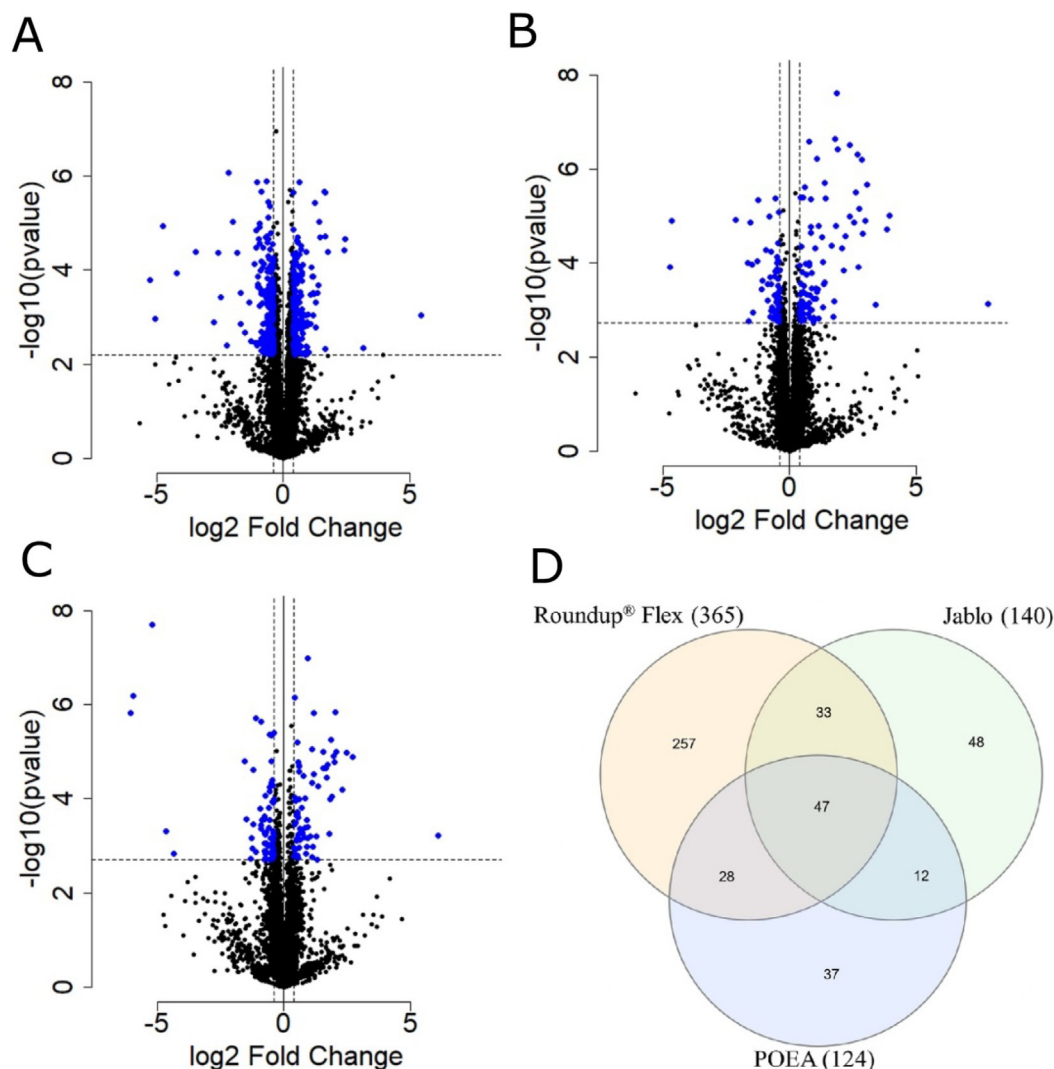
Furthermore, KPA associates the genes connected to the protein groups with pathways and key hubs, a molecule that is predicted to be upstream of the input transcripts in the KPA analysis, to allow for biological interpretation of the data. The KPA analysis revealed several predicted pathways enriched in the exposures by the different glyphosate formulations (Fig. 6). A cut-off of  $-\log(p\text{-value}) \geq 3.88$ , corresponding to a p-value of  $\leq 0.000132$ , was chosen to include all pathways predicted in response to exposure with POEA and accordingly all pathways (13 and 25 pathways for Roundup® Flex and Jablo, respectively), at the same cut-off. The total number of enriched pathways was 25 for Roundup® Flex, 51 for Jablo and 5 for POEA. Furthermore, three pathways were predicted to be enriched in cell exposures with all three substances, namely, “Sirtuin6 regulation and functions”, “Role of ER stress in obesity and type 2 diabetes” and “mTORC1 downstream signaling”.

#### 4. Discussion

Recently, much effort has been invested in elucidating the toxicity of glyphosate and its commercial “real-life” agrochemical formulations. Controversies related to what actually induces toxic reactions from a formulation have led to different regulations being implemented around the world and no consensus has been reached [5]. As glyphosate-containing formulations are among the most used herbicides worldwide [6], it is essential to increase our knowledge about their

impact on human health. In the case of skin sensitization assessment, there is no global harmonization of needed data, and testing requirements among the regulatory agencies from different regions vary. However, human-relevant *in vitro* approaches have recently started to receive regulatory acceptance [55]. Decision making regarding the toxicity testing of pesticides is mainly performed on pure substances by some authorities (e.g. EFSA), i.e. ingredients to be used in various products are tested one by one. However, we are exposed to the final product, which represents a complex mixture of different chemicals. As there are examples where formulation ingredients were assessed as safe yet allergic reaction occurred to the product, also complete formulations should be thoroughly investigated regarding their human health hazard [56]. For instance, specific mixtures of “inactive” ingredients, namely excipients, used in drug products to provide the desired physical properties have been associated with allergy cases in humans even though the active pharmaceutical ingredient does not show allergenic potential [57]. Similarly, the combination of chemicals, as found in an herbicidal mixture, can show a different toxicological profile compared to the one of the main active ingredient alone. In view of this, some authorities, such as the US regulatory agency [58], make the testing of both pure substance and final product mandatory for skin sensitization hazard assessment. In fact, some reports have shown that glyphosate tested alone had a reduced toxicity as compared to the commercial glyphosate-based formulations [13].

Most of the knowledge regarding the toxicological effects of xenobiotics has traditionally been generated by animal experimentation, which has been criticized. One reason is that these experiments suffer from poor human toxicological utility, especially for predicting immunological effects [59]. In view of this, human-relevant *in vitro* methods have been developed to carry out the hazard identification for contact allergens. In fact, some of these *in vitro* methods for skin sensitization prediction have shown better performance than animal assays [26]. The objective of this study was to perform *in vitro* sensitization testing using agrochemicals with a parallel MS-based proteomic approach to investigate the underlying cellular mechanisms induced by agrochemicals and their formulations. The experimental setup of the *in vitro* assay GARD™skin was used to stimulate cells of a human DC-like



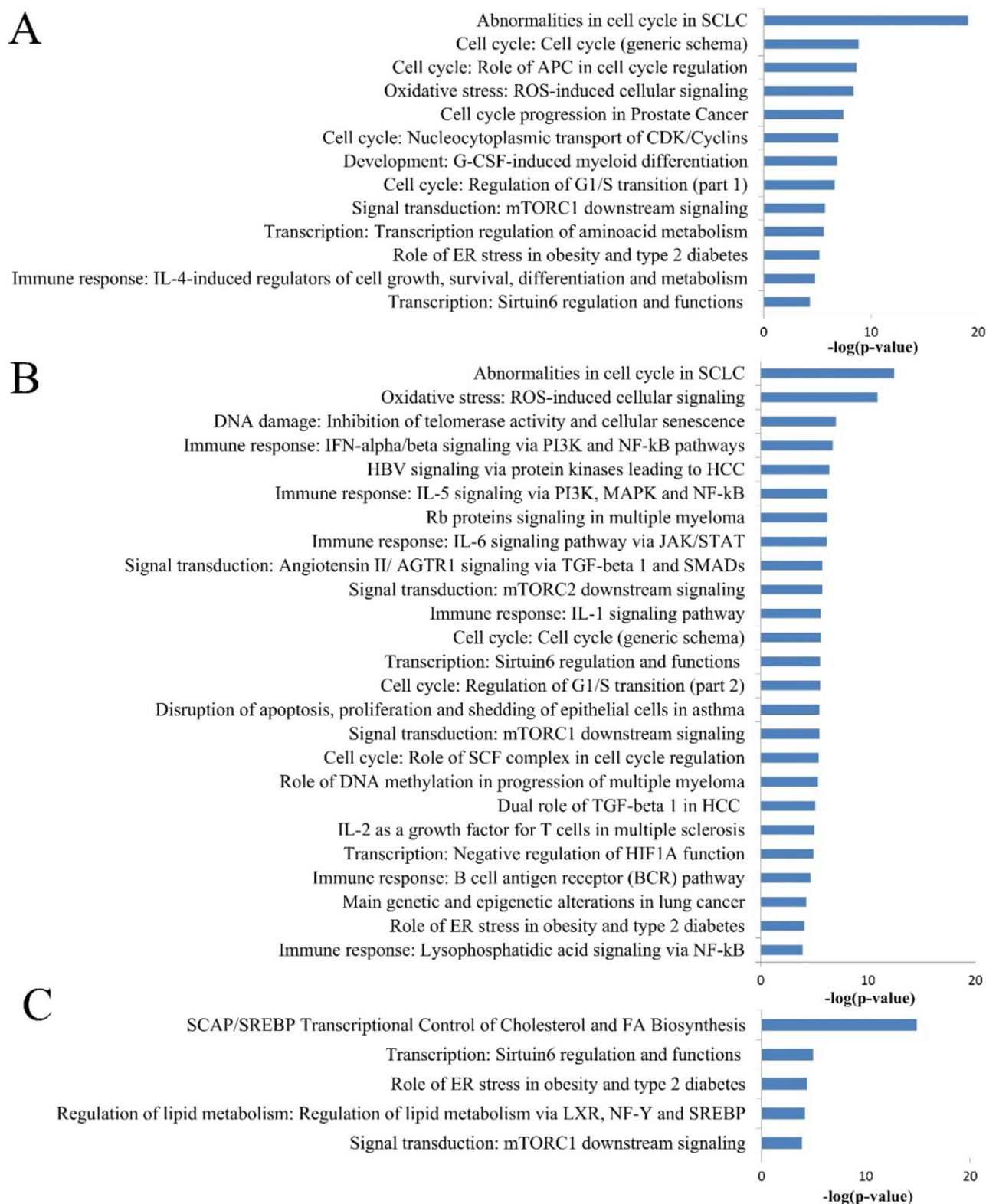
**Fig. 5.** Differentially regulated protein groups. Volcano plots with differentially regulated ( $FDR \leq 0.05$ , fold change  $> 1.3$ ) protein groups marked in blue in exposures with Roundup® Flex (A), Jablo (B) and POEA (C). Horizontal dashed line represents  $FDR = 0.05$  while the two vertical dashed lines represent fold change = 1.3. Venn diagram (D) displaying overlapping differentially regulated protein groups for the two herbicidal formulations Jablo and Roundup® Flex, and the surfactant POEA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cell line with different materials, including glyphosate, the surfactant POEA, their mixture and two commercially available POEA-free glyphosate-based formulations. As a first step, the DC maturation marker CD86 was investigated (Fig. 2). While the positive control PPD led to a significant upregulation of CD86, none of the formulations or POEA did compared to control. However, glyphosate induced a significant downregulation of CD86. Furthermore, the skin sensitizing capacity of the test materials was determined (Fig. 3). Glyphosate was predicted as a non-sensitizer and the two glyphosate-containing formulations and POEA were labelled as sensitizers. Corroborating these findings, POEA and five out of six glyphosate-containing formulations were previously identified to be potentially skin (photo)sensitizing using *in chemico* analyses [10]. In contrast, glyphosate tested alone showed negative results in the same study. Moreover, in the here presented study, glyphosate together with POEA resulted in a similar GARD™ DV when compared to POEA alone, indicating that glyphosate likely does not increase the sensitizing capacity when co-administrated with POEA. Even though the two formulations do not contain POEA as surfactant (but alkyl poly glucoside and ether amine ethoxylate in Roundup® Flex and Jablo, respectively), we observed a higher GARD™ DV for these formulations when compared to POEA. This illustrates that “real-life” formulations can show a hazard profile different from that observed for

pure ingredients such as the surfactants and the active ingredient glyphosate. However, as the surfactants used in Roundup® Flex and Jablo were not tested in this study, we cannot make any conclusions about their skin sensitizing characteristics.

Furthermore, the transcriptomic data were complemented with a proteomic analysis to better understand the toxicological mechanisms involved in the skin sensitization process triggered by POEA and the formulations tested. The assembled protein groups, deduced from the peptides obtained from the MS analysis, revealed several patterns in response to the formulations. At first, the analysis of all protein groups ( $n = 6029$ ) resulted in a clear separation between sensitizers and non-sensitizers (Fig. 4A). Further, by investigating the similarities between the expressed protein groups using a multigroup ANOVA ( $FDR = 0.05$ ) (Fig. 4B), four clusters were seen. Interestingly, Jablo clustered with POEA, indicating similarities in their protein regulation, even though Jablo is a POEA-free formulation.

To assess the cellular processes and enriched pathways involved in the response towards these agrochemical materials, we further analyzed the differentially regulated ( $FDR = 0.05$ ) protein groups, identified by comparing each glyphosate formulation and POEA to the control group (Fig. 5). Investigating the commonly regulated proteins more in detail, we observed three proteins, namely DHCR7, DHCR24,



**Fig. 6.** Enriched pathways predicted to be activated by exposure with the herbicidal formulations and surfactant POEA. Pathway enrichment hits predicted in exposures with (A) Roundup® Flex (365 protein groups), (B) Jablo (140 protein groups) and (C) POEA (124 protein groups) using KPA. Significance level for input variables into KPA was FDR = 0.05 and a fold change cut-off of 1.3. Only the pathways with a  $-\log(p\text{-value}) > 3.88$  are shown, Roundup® Flex ( $n = 13$ ), Jablo ( $n = 25$ ) and POEA ( $n = 5$ ).

CYP51A1, which are also represented by transcripts of the 200 biomarkers in the GARD™skin prediction signature. These enzymes are involved in cholesterol biosynthesis, where more than 20 enzymes are required in total; DHCR7 converts dehydrodesmosterol to desmosterol

while DHCR24, in turn, reduces it to cholesterol as final steps [60]. Interestingly, DHCR24 is further included in the 52 transcripts identified by Zeller et al. to predict skin sensitizer potency using GARD™ technology [33].



Naturally, lipids of the membrane bilayer are crucial for the dynamics of virtually all cell-environment interactions due to involvement in processes such as internalization, transport and signalling. Indeed, several reports have been published indicating that cholesterol, oxysterols and proteins regulating sterol traffic can influence differentiation, maturation and migration of DCs [reviewed in [61]] in the context of allergy, asthma, and autoimmune diseases. However, studies investigating the role of cholesterol and associated processes specifically for DC activation in skin sensitization are to our knowledge not available. Interestingly, a link between innate immune responses and cholesterol homeostasis has previously been established for macrophage function [62]. It has further been reported that Toll-like receptor (TLR) 4 activation in macrophages causes lanosterol, the first sterol intermediate in the cholesterol biosynthetic pathway, to accumulate by down-regulation of CYP51A1. In turn, lanosterol accumulation increases both membrane fluidity and ROS production, which increases phagocytosis and the ability to kill bacteria [63]. Another study connects sterol regulatory element binding protein-1a (SREBP-1a) to activation of lipogenesis genes in macrophages and of a core inflammasome component, NOD-like receptor (NLR) family pyrin domain containing 1 (Nlrp1a), thus linking lipid metabolism to the innate immune response [62]. The authors further hypothesize that SREBP-1a “evolved to regulate cellular reactions to external challenges that range from nutrient limitation and hypoxia to toxins and pathogens”. Notably, cells under stress can release damage-associated molecular patterns, which are recognized by pattern recognition receptors (PRRs) including families of TLRs and NLRs [64]. More recently, PRRs, especially TLRs, have been shown to regulate DNA repair by modulating the expression of several enzymes involved in this response, among them TYMS [65].

Each protein group was further associated with gene(s) and used as input in the KPA analysis. This revealed several pathways (Fig. 6), which seem biologically relevant in the context of skin sensitization, for example “IL-4-induced regulators of cell growth, survival, differentiation and metabolism” and “ROS-induced cellular signalling”, both linked to immune responses [66,67]. Furthermore, two of the predicted pathways found in response to chemical exposures of POEA, Roundup® Flex and Jablo, namely, “Sirtuin6 regulation and functions” and “mTORC1 downstream signaling”, have been linked to inflammation [68] and metabolic responses in immune cell activation [69], respectively. mTOR, a core component of the mTORC1 protein complex, is a serine/threonine kinase considered a master regulator of cellular metabolism. mTORC1 controls the balance between anabolism and catabolism in response to environmental conditions and regulates the production of proteins, lipids and nucleotides while e.g. suppressing catabolic pathways such as autophagy [70]. Sirtuins (SIRT1-7) are histone deacetylase enzymes and in general involved in the regulation of cell stress responses and metabolism [71]. Among them, SIRT2, SIRT6, and SIRT7 are described as key modulators of oxidative stress genes and associated molecular mechanisms [72]. Sirtuins are also able to induce autophagy, e.g. SIRT6-mediated induction of autophagy has been described to be crucial for several processes in normal cells such as the monocyte differentiation [73]. Furthermore, literature strongly supports a role of autophagy for DC function on several levels and it appears both up- and downstream of redox signalling [74] and in inflammatory pathways of allergy and asthma [75].

To this end, when combining our results on regulated proteins and predicted pathways, an association to autophagy processes seems rather clear. Autophagy is a fundamental process for degrading and recycling cellular components and most data available focuses on macroautophagy. This process leads to bulk lysosomal degradation of the cytosol via specialized double-membrane organelles (autophagosomes) [74]. Additionally, mTOR plays a major role in regulating autophagy and is suggested as a pharmacological target for autophagy regulation [76]. p62/SQSTM1, which was found to be one of the proteins regulated with the highest fold changes in this study, binds ubiquitylated protein aggregates and delivers them to the autophagosomes [77].

Generally, upon autophagy activation, p62/SQSTM1 levels decrease, indicating that the cell exposure to POEA and the multi-component formulations could interfere with autophagy flux as they induce p62/SQSTM1 up-regulation as found in our DC cell model. To date, very few reports suggest a role of autophagy in skin sensitization. The sensitizer 1-fluoro-2,4-dinitrobenzene (DNFB) was reported to modulate maturation of THP-1, a myeloid cell line, and to stabilize the transcription factor Nrf2, known to trigger expression of antioxidant proteins, by induction of two autophagy-related genes [78]. Lee et al. reported that treatment with the sensitizer chromium-IV induced apoptosis, autophagy and ROS in keratinocyte HaCaT cells [79]. Furthermore, one study not related to skin sensitization associated glyphosate-induced cell death with autophagy pathways in the rat cell line PC12 [80].

## 5. Conclusions

In summary, we here present an integrative analysis involving advanced technologies to elucidate the molecular mechanisms underlying DC activation in the skin sensitization process triggered by agrochemical materials. Given the frequent use of products consisting of complex mixtures, such as glyphosate formulations, it is crucial that toxicological assessments on both co-formulants and the “real-life” formulations are performed to evaluate their impact on human health. In this regard, the integration of GARD™skin and proteomic data can elucidate the xenobiotic-induced skin sensitization capacity of the agrochemical materials and contribute to the understanding of immunotoxicity-associated molecular mechanisms involved. In this context, the data presented here are in line with autophagy playing a role in the DC response triggered by glyphosate-based formulations and POEA, and likely in skin sensitization more in general. Hence, our data provide a mechanism-based hazard evaluation of xenobiotics that can be used to support human health risk assessment.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2020.103647>.

## Declaration of Competing Interest

The authors declare no competing financial interest.

## Acknowledgements

This work was financially supported by grants from the Swedish Foundation for Strategic Research (IB13-0118), the Swedish Research Council Formas (2017-01030) and the Crafoord Foundation (2017-0650). R. I. de A. received a scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil) (finance code 001). The authors would also like to thank Karin Hansson and Valentina Siino for running the mass spectrometry instrument and providing help during the sample preparation.

## References

- [1] K.Z. Guyton, D. Loomis, Y. Grosse, F. El Ghissassi, L. Benbrahim-Tallaa, N. Guha, C. Scocciati, H. Mattock, K. Straif, Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate, *The Lancet. Oncol.* 16 (2015) 490–491.
- [2] M.J.L. Castro, C. Ojeda, A.F. Cirelli, *Advances in surfactants for agrochemicals*, *Environ. Chem. Lett.* 12 (2014) 85–95.
- [3] H.C. Steinrucken, N. Amrhein, The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase, *Biochem. Biophys. Res. Commun.* 94 (1980) 1207–1212.
- [4] J.P. Myers, M.N. Antoniou, B. Blumberg, L. Carroll, T. Colborn, L.G. Everett, M. Hansen, P.J. Landrigan, B.P. Lanphear, R. Mesnage, L.N. Vandenberg, F.S. Vom Saal, W.V. Welshons, C.M. Benbrook, Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement, *Environ. Health* 15 (2016) 19.
- [5] C.J. Portier, B.K. Armstrong, B.C. Baguley, X. Baur, I. Belyaev, R. Belle, F. Belpoggi, A. Biggeri, M.C. Bosland, P. Bruzzi, L.T. Budnik, M.D. Bugge, K. Burns, G.M. Calaf, D.O. Carpenter, H.M. Carpenter, L. Lopez-Carrillo, R. Clapp, P. Cocco, D. Consonni, P. Comba, E. Craft, M.A. Dalvie, D. Davis, P.A. Demers, A.J. De Roos, J. DeWitt, F. Forastiere, J.H. Freedman, L. Fritsch, C. Gaus, J.M. Gohlke, M. Goldberg,

- E. Greiser, J. Hansen, L. Hardell, M. Hauptmann, W. Huang, J. Huff, M.O. James, C.W. Jameson, A. Kortenkamp, A. Kopp-Schneider, H. Kromhout, M.L. Laramendy, P.J. Landrigan, L.H. Lash, D. Leszczynski, C.F. Lynch, C. Magnani, D. Mandrioli, F.L. Martin, E. Merler, P. Michelozzi, L. Miligi, A.B. Miller, D. Mirabelli, F.E. Mirer, S. Naidoo, M.J. Perry, M.G. Petronio, R. Pirastu, R.J. Portier, K.S. Ramos, L.W. Robertson, T. Rodriguez, M. Roosli, M.K. Ross, D. Roy, I. Rusyn, P. Saldiva, J. Sass, K. Savolainen, P.T. Scheepers, C. Sergi, E.K. Silbergeld, M.T. Smith, B.W. Stewart, P. Sutton, F. Tateo, B. Terracini, H.W. Thielmann, D.B. Thomas, H. Vainio, J.E. Vena, P. Vineis, E. Weiderpass, D.D. Weisenburger, T.J. Woodruff, T. Yorifuji, I.J. Yu, P. Zambon, H. Zeeb, S.F. Zhou, Differences in the carcinogenic evaluation of glyphosate between the International Agency for Research on Cancer (IARC) and the European Food Safety Authority (EFSA), *J. Epidemiol. Community Health* 70 (2016) 741–745.
- [6] C.M. Benbrook, Trends in glyphosate herbicide use in the United States and globally, *Environ. Sci. Eur.* 28 (2016) 3.
- [7] EFSA, Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate, *EFSA J.* 13 (2015) 4302–4409.
- [8] G. Chaufan, I. Coalova, C. Rios de Molina Mdel, Glyphosate commercial formulation causes cytotoxicity, oxidative effects, and apoptosis on human cells: differences with its active ingredient, *Int. J. Toxicol.* 33 (2014) 29–38.
- [9] P.J. Mink, J.S. Mandel, B.K. Scurman, J.I. Lundin, Epidemiologic studies of glyphosate and cancer: a review, *Regul. Toxicol. Pharmacol.* 63 (2012) 440–452.
- [10] R.I. de Ávila, G.C. Teixeira, D. Veloso, L.C. Moreira, E.M. Lima, M.C. Valadares, *In vitro* assessment of skin sensitization, photosensitization and phototoxicity potential of commercial glyphosate-containing formulations, *Toxicol. in Vitro* 45 (2017) 386–392.
- [11] C.M. Bonefeld, C. Geisler, E. Giménez-Arnau, J.-P. Lepoittevin, W. Uter, J.D. Johansen, Immunological, chemical and clinical aspects of exposure to mixtures of contact allergens, *Contact Dermatitis* 77 (2017) 133–142.
- [12] R.I. de Ávila, D.F.M.C. Veloso, G.C. Teixeira, T.L. Rodrigues, T. Lindberg, M. Lindstedt, S.G. Fonseca, E.M. Lima, M.C. Valadares, Evaluation of *in vitro* testing strategies for hazard assessment of the skin sensitization potential of “real-life” mixtures: the case of henna-based hair-colouring products containing *p*-phenylenediamine, *Contact Dermatitis* 81 (2019) 194–209.
- [13] S.M. Bradberry, A.T. Proudfoot, J.A. Vale, Glyphosate poisoning, *Toxicol. Rev.* 23 (2004) 159–167.
- [14] C.D. Navarro, C.B. Martinez, Effects of the surfactant polyoxyethylene amine (POEA) on genotoxic, biochemical and physiological parameters of the freshwater teleost *Prochilodus lineatus*, *Comp. Biochem. Physiol. Toxicol. Pharmacol.* 165 (2014) 83–90.
- [15] F. Young, D. Ho, D. Glynn, V. Edwards, Endocrine disruption and cytotoxicity of glyphosate and Roundup in human JAr cells *in vitro*, *Integr. Pharm. Toxicol. Genotoxicol.* 1 (2015) 70–76.
- [16] EFSA, Statement of EFSA on the request for the evaluation of the toxicological assessment of the co-formulant POE-tallowamine, *EFSA J.* 13 (2015) 1–13.
- [17] European Union, Commission implementing regulation (EU) 2016/1313 of 1 August 2016 amending Implementation Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance glyphosate, *Off. J. Eur. Union* (2016) L 208/1–L 208/3.
- [18] A. Conrad, C. Schroter-Kermani, H.W. Hoppe, M. Ruther, S. Pieper, M. Kolossa-Gehring, Glyphosate in German adults - time trend (2001 to 2015) of human exposure to a widely used herbicide, *Int. J. Hyg. Environ. Health* 220 (2017) 8–16.
- [19] A. Connolly, M. Leahy, K. Jones, L. Kenny, M.A. Coggins, Glyphosate in Irish adults - a pilot study in 2017, *Environ. Res.* 165 (2018) 235–236.
- [20] D.A. Basketter, P. Evans, R.J. Fielder, G.F. Gerberick, R.J. Dearman, I. Kimber, Local lymph node assay - validation, conduct and use in practice, *Food Chem. Toxicol.* 40 (2002) 593–598.
- [21] NRC, Toxicity testing in the 21st Century: A Vision and a Strategy. Committee on Toxicity Testing and Assessment of Environmental Agents; Board on Environmental Studies and Toxicology, Institute for Laboratory Animal Research; and Division on Earth and Life Studies, National Research Council of the National Academies, The National Academies, 2007.
- [22] J. Strickland, Q. Zang, N. Kleinstreuer, M. Paris, D.M. Lehmann, N. Choksi, J. Matheson, A. Jacobs, A. Lowit, D. Allen, W. Casey, Integrated decision strategies for skin sensitization hazard, *J. Appl. Toxicol.* 36 (2016) 1150–1162.
- [23] EU, Regulation (EC) no 1907/2006 of the European Parliament and of the council of 18 December 2006 concerning the registration, evaluation, authorisation and restriction of chemicals (REACH), establishing a European chemicals agency, amending directive 1999/45/EC and repealing council regulation (EEC) no 793/93 and commission regulation (EC) no 1488/94 as well as council directive 76/769/EEC and commission directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC, *OJ of the European Union*, 396 (2006), pp. 1–1355.
- [24] EUR-Lex, Commission Regulation (EU) 2017/706 of 19 April 2017 amending Annex VII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards skin sensitisation and repealing Commission Regulation (EU) 2016/1688 (Text with EEA relevance), (2017).
- [25] EU, Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products, *OJ of the European Union*, (2009), p. 342.
- [26] R.I. de Ávila, M. Lindstedt, M.C. Valadares, The 21st Century movement within the area of skin sensitization assessment: from the animal context towards current human-relevant *in vitro* solutions, *Regul. Toxicol. Pharmacol.* 108, 2019, p. 104445.
- [27] OECD, The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins, Part 1: Scientific Evidence, Environment, Health and Safety Publications, Series on Testing and Assessment No. 168. ENV/JM/MONO, 1 (2014), pp. 1–59.
- [28] T. Simões, S.C. Novais, T. Natal-da-Luz, B. Devreese, T. de Boer, D. Roelofs, J.P. Sousa, N.M. van Straalen, M.F.L. Lemos, An integrative omics approach to unravel toxicity mechanisms of environmental chemicals: effects of a formulated herbicide, *Sci. Rep.* 8 (2018) 11376.
- [29] A. Lanzoni, A.F. Castoldi, G.E. Kass, A. Terron, G. De Seze, A. Bal-Price, F.Y. Bois, K.B. Delclos, D.R. Doerge, E. Fritsche, T. Halldorsson, M. Kolossa-Gehring, S. Hougaard Bennekou, F. Koning, A. Lampen, M. Leist, E. Mantus, C. Rousselle, M. Siegrist, P. Steinberg, A. Tritscher, B. Van de Water, P. Vineis, N. Walker, H. Wallace, M. Whelan, M. Younes, Advancing human health risk assessment, *EFSA J.* 17 (S1) (2019) e170712.
- [30] H. Johansson, M. Lindstedt, A.S. Albrekt, C.A. Borrebaeck, A genomic biomarker signature can predict skin sensitizers using a cell-based *in vitro* alternative to animal tests, *BMC Genomics* 12 (2011) 399.
- [31] A. Forreryd, K.S. Zeller, T. Lindberg, H. Johansson, M. Lindstedt, From genome-wide arrays to tailor-made biomarker readout - Progress towards routine analysis of skin sensitizing chemicals with GARD, *Toxicol. in Vitro* 37 (2016) 178–188.
- [32] H. Johansson, A.S. Albrekt, C.A. Borrebaeck, M. Lindstedt, The GARD assay for assessment of chemical skin sensitizers, *Toxicol. in Vitro* 27 (2013) 1163–1169.
- [33] K.S. Zeller, A. Forreryd, T. Lindberg, R. Gradin, A. Chawade, M. Lindstedt, The GARD platform for potency assessment of skin sensitizing chemicals, *Altex* 34 (2017) 539–559.
- [34] A. Forreryd, U. Norinder, T. Lindberg, M. Lindstedt, Predicting skin sensitizers with confidence — using conformal prediction to determine applicability domain of GARD, *Toxicol. in Vitro* 48 (2018) 179–187.
- [35] H. Johansson, R. Gradin, A. Forreryd, M. Agemark, K. Zeller, A. Johansson, O. Larne, E. van Vliet, C. Borrebaeck, M. Lindstedt, Evaluation of the GARD assay in a blind Cosmetics Europe study, *Altex* 34 (2017) 515–523.
- [36] M. Stevenson, L. Czekala, L. Simms, N. Tschierske, O. Larne, T. Wale, The use of Genomic Allergen Rapid Detection (GARD) assays to predict the respiratory and skin sensitizing potential of e-liquids, *Regul. Toxicol. Pharmacol.* 103 (2019) 158–165.
- [37] A. Chawade, E. Alexandersson, T. Bengtsson, E. Andreasson, F. Levander, Targeted proteomics approach for precision plant breeding, *J. Proteome Res.* 15 (2016) 638–646.
- [38] M.C. Chambers, B. Maclean, R. Burke, D. Amodei, D.L. Ruderman, S. Neumann, N. Gatto, B. Fischer, B. Pratt, J. Egertson, K. Hoff, D. Kessner, N. Tasman, N. Shulman, B. Frewen, T.A. Baker, M.Y. Brusniak, C. Paulse, D. Creasy, L. Flashner, K. Kani, C. Moulding, S.L. Seymour, L.M. Nuwaysir, B. Lefebvre, F. Kuhlmann, J. Roark, P. Rainer, S. Detlev, T. Hemenway, A. Huhmer, J. Langridge, B. Connolly, T. Chadick, K. Holly, J. Eckels, E.W. Deutsch, R.L. Moritz, J.E. Katz, D.B. Agus, M. MacCoss, D.L. Tabb, P. Mallick, A cross-platform toolkit for mass spectrometry and proteomics, *Nat. Biotechnol.* 30 (2012) 918–920.
- [39] B. Zhang, M. Pirmoradian, A. Chernobrovkin, R.A. Zubarev, DeMix workflow for efficient identification of cofragmented peptides in high resolution data-dependent tandem mass spectrometry, *Mol. Cell. Proteomics* 13 (2014) 3211–3223.
- [40] J. Häkkinen, G. Vincic, O. Månsson, K. Wårell, F. Levander, The proteios software environment: an extensible multiuser platform for management and analysis of proteomics data, *J. Proteome Res.* 8 (2009) 3037–3043.
- [41] S. Kim, P.A. Pevzner, MS-GF+ makes progress towards a universal database search tool for proteomics, *Nat. Commun.* 5 (2014) 5277.
- [42] E.W. Deutsch, Z. Sun, D.S. Campbell, P.-A. Binz, T. Farrah, D. Shteynberg, L. Mendoza, G.S. Omenn, R.L. Moritz, Tiered human integrated sequence search databases for shotgun proteomics, *J. Proteome Res.* 15 (2016) 4091–4100.
- [43] J. Teleman, A. Chawade, M. Sandin, F. Levander, J. Malmstrom, Dinosaur: a refined open-source peptide MS feature detector, *J. Proteome Res.* 15 (2016) 2143–2151.
- [44] M. Sandin, A. Ali, K. Hansson, O. Mansson, E. Andreasson, S. Resjo, F. Levander, An adaptive alignment algorithm for quality-controlled label-free LC-MS, *Mol. Cell. Proteomics* 12 (2013) 1407–1420.
- [45] J. Willforss, A. Chawade, F. Levander, NormalizerDE: online tool for improved normalization of omics expression data and high-sensitivity differential expression analysis, *J. Proteome Res.* 18 (2018) 732–740.
- [46] Y. Karpievitch, J. Stanley, T. Taverner, J. Huang, J.N. Adkins, C. Ansong, F. Heffron, T.O. Metz, W.-J. Qian, H. Yoon, R.D. Smith, A.R. Dabney, A statistical framework for protein quantitation in bottom-up MS-based proteomics, *Bioinformatics* 25 (2009) 2028–2034.
- [47] J.A. Vizcaíno, A. Csordas, N. del-Toro, J.A. Dianas, J. Griss, I. Lavidas, G. Mayer, Y. Perez-Riverol, F. Reisinger, T. Ternent, Q.-W. Xu, R. Wang, H. Hermjakob, 2016 update of the PRIDE database and its related tools, *Nucleic Acids Res.* 44 (D1) (2015) D447–D456.
- [48] W.E. Johnson, C. Li, A. Rabinovic, Adjusting batch effects in microarray expression data using empirical Bayes methods, *Biostatistics (Oxford, England)* 8 (2007) 118–127.
- [49] J.T. Leek, W.E. Johnson, H.S. Parker, A.E. Jaffe, J.D. Storey, The sva package for removing batch effects and other unwanted variation in high-throughput experiments, *Bioinformatics* 28 (2012) 882–883.
- [50] R Core Team, R: A Language and Environment for Statistical Computing, (2016).
- [51] KPA, Key Pathway Advisor by Clarivate Analytics (Formerly the IP & Science business of Thomson Reuters), <http://ipsience.thomsonreuters.com/product/metacore/>, (2016).
- [52] S. Meaney, Epigenetic regulation of cholesterol homeostasis, *Front. Genet.* 5 (2014) 311.
- [53] D.D. Anderson, C.M. Quintero, P.J. Stover, Identification of a de novo thymidylate biosynthesis pathway in mammalian mitochondria, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 15163–15168.
- [54] M.L. Seibenhener, J.R. Babu, T. Geetha, H.C. Wong, N.R. Krishna, M.W. Wooten,

- Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation, *Mol. Cell. Biol.* 24 (2004) 8055–8068.
- [55] A.B. Daniel, J. Strickland, D. Allen, S. Casati, V. Zuang, J. Barroso, M. Whelan, M.J. Régimbald-Krnel, H. Kojima, A. Nishikawa, H.-K. Park, J.K. Lee, T.S. Kim, I. Delgado, L. Rios, Y. Yang, G. Wang, N. Kleinstreuer, International regulatory requirements for skin sensitization testing, *Regul. Toxicol. Pharmacol.* 95 (2018) 52–65.
- [56] EC, Communication from the commission to the council - the combination effects of chemicals, *Chem. Mix. Com.* (2012) 252.
- [57] D. Reker, S.M. Blum, C. Steiger, K.E. Anger, J.M. Sommer, J. Fanikos, G. Traverso, "Inactive" ingredients in oral medications, *Sci. Transl. Med.* 11 (2019) eaau6753.
- [58] J. Strickland, A.B. Daniel, D. Allen, C. Aguila, S. Ahir, S. Bancos, E. Craig, D. Germolec, C. Ghosh, N.L. Hudson, A. Jacobs, D.M. Lehmann, J. Matheson, E.N. Reinke, N. Sadrieh, S. Vukmanovic, N. Kleinstreuer, Skin sensitization testing needs and data uses by US regulatory and research agencies, *Arch. Toxicol.* 93 (2019) 273–291.
- [59] A. Knight, Animal experiments scrutinised: systematic reviews demonstrate poor human clinical and toxicological utility, *Altex* 24 (4) (2007) 320–325.
- [60] W. Luu, G. Hart-Smith, L.J. Sharpe, A.J. Brown, The terminal enzymes of cholesterol synthesis, DHCR24 and DHCR7, interact physically and functionally, *J. Lipid Res.* 56 (2015) 888–897.
- [61] M.B. Fessler, Regulation of adaptive immunity in health and disease by cholesterol metabolism, *Curr Allergy Asthma Rep* 15 (2015) 48.
- [62] S.S. Im, L. Yousef, C. Blaschitz, J.Z. Liu, R.A. Edwards, S.G. Young, M. Raffatellu, T.F. Osborne, Linking lipid metabolism to the innate immune response in macrophages through sterol regulatory element binding protein-1a, *Cell Metab.* 13 (2011) 540–549.
- [63] E. Araldi, M. Fernandez-Fuertes, A. Canfran-Duque, W. Tang, G.W. Cline, J. Madrigal-Matute, J.S. Pober, M.A. Lasuncion, D. Wu, C. Fernandez-Hernando, Y. Suarez, Lanosterol modulates TLR4-mediated innate immune responses in macrophages, *Cell Rep.* 19 (2017) 2743–2755.
- [64] J.S. Roh, D.H. Sohn, Damage-associated molecular patterns in inflammatory diseases, *Immune Netw.* 18 (2018) e27.
- [65] A.G. Kutikhin, A.E. Yuzhalin, E.A. Tsitko, E.B. Brusina, Pattern recognition receptors and DNA repair: starting to put a jigsaw puzzle together, *Front. Immunol.* 5 (2014) 343.
- [66] Z. Li, X. Xu, X. Leng, M. He, J. Wang, S. Cheng, H. Wu, Roles of reactive oxygen species in cell signaling pathways and immune responses to viral infections, *Arch. Virol.* 162 (2017) 603–610.
- [67] I.G. Luzina, A.D. Keegan, N.M. Heller, G.A.W. Rook, T. Shea-Donohue, S.P. Atamas, Regulation of inflammation by interleukin-4: a review of alternatives, *J. Leukoc. Biol.* 92 (2012) 753–764.
- [68] M. Lappas, Anti-inflammatory properties of Sirtuin 6 in human umbilical vein endothelial cells, *Mediat. Inflamm.* 2012 (2012) 11.
- [69] M. Linke, S.D. Fritsch, N. Sukhbaatar, M. Hengstschläger, T. Weichhart, mTORC1 and mTORC2 as regulators of cell metabolism in immunity, *FEBS Lett.* 591 (2017) 3089–3103.
- [70] R.A. Saxton, D.M. Sabatini, mTOR signaling in growth, metabolism, and disease, *Cell* 168 (2017) 960–976.
- [71] Z. Mei, X. Zhang, J. Yi, J. Huang, J. He, Y. Tao, Sirtuins in metabolism, DNA repair and cancer, *J. Exper. Clin. Cancer Res.* 35 (2016) 182.
- [72] C.K. Singh, G. Chhabra, M.A. Ndiaye, L.M. Garcia-Peterson, J.N. Mack, N. Ahmad, The role of sirtuins in antioxidant and redox signaling, *Antioxid. Redox Signal.* 28 (2018) 643–661.
- [73] A. Singh, E. Sen, Reciprocal role of SIRT6 and Hexokinase 2 in the regulation of autophagy driven monocyte differentiation, *Exp. Cell Res.* 360 (2017) 365–374.
- [74] G. Ghislat, T. Lawrence, Autophagy in dendritic cells, *Cell. Mol. Immunol.* 15 (2018) 944–952.
- [75] H. Renz, Autophagy: Nobel prize 2016 and allergy and asthma research, *J. Allergy Clin. Immunol.* 140 (2017) 1548–1549.
- [76] Y.C. Kim, K.L. Guan, mTOR: a pharmacologic target for autophagy regulation, *J. Clin. Invest.* 125 (2015) 25–32.
- [77] M. Komatsu, S. Waguri, M. Koike, Y.-s. Sou, T. Ueno, T. Hara, N. Mizushima, J.-i. Iwata, J. Ezaki, S. Murata, J. Hamazaki, Y. Nishito, S.-i. Iemura, T. Natsume, T. Yanagawa, J. Uwayama, E. Warabi, H. Yoshida, T. Ishii, A. Kobayashi, M. Yamamoto, Z. Yue, Y. Uchiyama, E. Kominami, K. Tanaka, Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice, *Cell* 131 (2007) 1149–1163.
- [78] A. Luis, J.D. Martins, A. Silva, I. Ferreira, M.T. Cruz, B.M. Neves, Oxidative stress-dependent activation of the eIF2alpha-ATF4 unfolded protein response branch by skin sensitizer 1-fluoro-2,4-dinitrobenzene modulates dendritic-like cell maturation and inflammatory status in a biphasic manner, *Free Radic. Biol. Med.* 77 (2014) 217–229.
- [79] Y.H. Lee, S.B. Su, C.C. Huang, H.M. Sheu, J.C. Tsai, C.H. Lin, Y.J. Wang, B.J. Wang, N-acetylcysteine attenuates hexavalent chromium-induced hypersensitivity through inhibition of cell death, ROS-related signaling and cytokine expression, *PLoS One* 9 (2014) e108317.
- [80] Y.X. Gui, X.N. Fan, H.M. Wang, G. Wang, S.D. Chen, Glyphosate induced cell death through apoptotic and autophagic mechanisms, *Neurotoxicol. Teratol.* 34 (2012) 344–349.