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Validation of the 3D Skin Comet assay using full thickness skin models: Transferability and reproducibility

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ABSTRACT

Recently revised OECD Testing Guidelines highlight the importance of considering the first site-of-contact when investigating the genotoxic hazard. Thus far, only *in vivo* approaches are available to address the dermal route of exposure. The 3D Skin Comet and Reconstructed Skin Micronucleus (RSMN) assays intend to close this gap in the *in vitro* genotoxicity toolbox by investigating DNA damage after topical application. This represents the most relevant route of exposure for a variety of compounds found in household products, cosmetics, and industrial chemicals.

The comet assay methodology is able to detect both chromosomal damage and DNA lesions that may give rise to gene mutations, thereby complementing the RSMN which detects only chromosomal damage. Here, the comet assay was adapted to two reconstructed full thickness human skin models: the EpiDerm™- and Phenion® Full-Thickness Skin Models. First, tissue-specific protocols for the isolation of single cells and the general comet assay were transferred to European and US-American laboratories. After establishment of the assay, the protocol was then further optimized with appropriate cytotoxicity measurements and the use of aphidicolin, a DNA repair inhibitor, to improve the assay's sensitivity.

In the first phase of an ongoing validation study eight chemicals were tested in three laboratories each using the Phenion® Full-Thickness Skin Model, informing several validation modules. Ultimately, the 3D Skin Comet assay demonstrated a high predictive capacity and good intra- and inter-laboratory reproducibility with four laboratories reaching a 100% predictivity and the fifth yielding 70%.

The data are intended to demonstrate the use of the 3D Skin Comet assay as a new *in vitro* tool for following up on positive findings from the standard *in vitro* genotoxicity test battery for dermally applied chemicals, ultimately helping to drive the regulatory acceptance of the assay. To expand the database, the validation will continue by testing an additional 22 chemicals.

1. Introduction

In vitro genotoxicity assays are known to reliably identify *in vivo* genotoxicants and rodent carcinogens [1,2]. However, specificity is reduced because non-genotoxic chemicals are often mistakenly identified as genotoxic ('positive'), especially when two or three *in vitro* assays are combined in batteries as requested in different legal sectors (e.g. industrial chemicals) [3–5]. Such positive findings can only be investigated further by time- and cost-intensive follow-up testing. As a consequence, efforts have been undertaken to first improve the

Abbreviations: AK, adenylate kinase; ALI, air-liquid-interface; APC, aphidicolin; BaP, benzo[*a*]pyrene; EURL-ECVAM, European Union Reference Laboratory for alternatives to animal testing; FT, full thickness; HC, historical control range; LMA, low melting agarose; MMS, methyl methane sulfonate; MP, misleading positive; NC, negative control; OECD, Organization for Economic Co-operation and Development; PC, positive control; PM, prediction model; RSMN, Reconstructed Skin Micronucleus; SC, solvent control; TN, true negative; TP, true positive

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predictive capacity of existing *in vitro* genotoxicity assays [6]. Furthermore, several projects developed new *in vitro* methods to follow up on positive findings of the initial test battery [6,7]. These approaches aim to provide alternatives to *in vivo* follow-up testing and are of specific interest due to a growing number of legislations which restrict or ban animal experiments for toxicological safety assessment [3,8].

The limited predictivity of *in vitro* test batteries for *in vivo* genotoxicity is linked to the characteristics of the chosen test systems, as most standard assays are based on monolayer cell cultures. Several are of rodent origin partially lacking normal cell cycle control, e.g. cell lines typically used for micronucleus (MN) or chromosomal aberration (CA) tests like V79, CHO or CHL cells, all of which originate from Chinese hamster. Another general characteristic of these cell cultures is a limited metabolic capacity, which is usually compensated for by adding an external metabolically active supplement, e.g., rat liver S9 mix. However, when compared to the intact liver, the added microsomal mix only provides a limited and imbalanced spectrum of metabolically active enzymes. Additionally, the metabolic capacity of organs other than the liver is poorly represented. The inability to mirror site-specific metabolic capacity and the route of exposure that correctly mimics the *in vivo* situation represents a gap in the standard *in vitro* genotoxicity tests. This has also been emphasized in the recently updated *in vivo* OECD Testing Guidelines [9–11].

Until recently, the genotoxicity of substances with the skin as first site-of-contact, such as ingredients in cosmetics and household products or industrial chemicals, could only be investigated using *in vivo* assays when follow-up testing was triggered by positive results from the standard *in vitro* battery. To close this gap in the current *in vitro* genotoxicity testing toolbox, skin tissues were combined with classical genotoxicity read-out parameters to develop the 3D Skin Comet assay [12], described here in more detail, and the Reconstructed Skin Micronucleus (RSMN) test [13,14]. The Phenion® (Henkel, Germany) and EpiDerm™ Full-Thickness (FT) Skin Models (MatTek, MA, USA) used in the 3D Skin Comet assay, are composed of primary and p53 competent cells of human origin. This not only eliminates the species barrier but also preserves normal cell cycle control in addition to DNA-repair competence, e.g. [15]. The three-dimensional environment supports a phenotype close to native human skin, and has been demonstrated to have similar gene and protein expression patterns [16,17]. As this similarity was also shown for the activity of enzymes involved in xenobiotic metabolism [18–21], the utilization of external metabolizing systems such as rat liver S9 mix is not required. In addition, the topical application of test chemicals on FT skin tissues, which are cultured under air-liquid-interface (ALI) conditions, may help to overcome solubility issues observed with classical submerged cell cultures. Finally, the 3D reconstructed human skin models mimic the route of exposure for dermally applied chemicals, and therefore allow for testing conditions closer to the intended situation of use.

The comet assay is well suited to be applied to skin tissues as it does not rely on proliferating cells and allows for the investigation of DNA damage in any cell culture or tissue that can be subjected to single cell isolation. The method was first introduced by Ostling and Johanson [22] as the single cell gel electrophoresis assay to detect double-strand breaks after cells embedded in a micro-gel were subjected to electrophoresis. The introduction of highly alkaline conditions (pH > 13) during electrophoresis and a prior unwinding step by Singh et al. [23] allowed the detection of a broader range of DNA damage. This includes single-strand breaks which may result from direct interaction of the test chemical with DNA or which are related to incomplete excision repair as well as alkali labile sites [11]. As a result, not only clastogenic DNA damage can be detected but also lesions which may give rise to gene mutation.

Reus et al. [12] were the first to adapt the comet assay methodology to keratinocytes isolated from a human epidermal skin model, EpiDerm™ (MatTek, Ashland, MA). After a transfer of the protocol, five coded chemicals were investigated in three laboratories obtaining a good predictivity of approximately 90%. However, a significant number of experiments were invalid due to high background levels of DNA damage in negative and solvent control groups. As a result, two independent projects collaborated to investigate the suitability of FT skin models as alternative test systems (Table 1). One was funded by the German Federal Ministry for Research and Education, and the second by Cosmetics Europe.

Table 1
Steering Committee members.

Name	Institution
Raphaella Corvi	EURL-ECVAM
Frank Henkler	Federal Institute for Risk Assessment (BfR)
Sebastian Hoffmann	seh consulting + services (statistical analysis)
Jochen Kühnl	Beiersdorf
Cyrille Krul	TNO
Manfred Liebsch	BfR
Stefan Pfuhler	Procter & Gamble
Ralph Pirow	BfR (statistical analysis)
Kerstin Reisinger	Henkel
Astrid Reus	Triskelion
Markus Schulz	BASF

In the following, we describe the identification of suitable FT skin models for the 3D Skin Comet assay methodology and the optimization of protocols by this project. In addition, the results of the first phase of a larger validation exercise are presented. In this phase eight chemicals, including a metabolically activated pro-mutagen and a DNA cross-linker, were tested.

2. Material and methods

2.1. Chemicals

In order to obtain a high level of standardisation and to minimise potential sources of variability, the following reagents were used by all laboratories: Low melting temperature agarose (LMA; SeaPlaque® GTG® Agarose) from Lonza (Basel, Switzerland), agarose (NEEO Ultra-Quality) from Carl Roth (Karlsruhe, Germany), Aphidicolin (APC), DMSO (> 99.7% purity), thermolysin, methyl methane sulfonate (MMS), benzo[*a*]pyrene (BaP) from Sigma-Aldrich (Saint Louis, MO), and SYBR Gold from Life Technologies (Carlsbad, CA). All other reagents were obtained from local suppliers and were not harmonised among laboratories.

2.1.1. Characterization and selection of coded chemicals

Eight chemicals were investigated in the present study. They were selected by external experts (Raphaella Corvi and David Kirkland) from a chemical master list prepared for Cosmetics Europe by a larger group of external experts. In this master list data sets were collated that had previously been investigated in *in vivo* genotoxicity and/or carcinogenicity studies with dermal exposure. This approach led to a short list of appropriate reference chemicals that was only sufficient to support the standardized study described here.

The substances were grouped according to three categories: true negative (TN) and true positive chemicals (TP), with concordant *in vitro* and *in vivo* data, as well as misleading positives (MP) for which positive *in vitro* findings were reported, which were not confirmed in *in vivo* studies. Subsequently, 30 chemicals were selected for the larger validation exercise providing a balanced dataset of 15 genotoxicants (TP) with various modes of action and 15 chemicals with an expected negative outcome (TN and MP), all covering different chemical classes.

For the first testing phase reported here, eight out of these 30 chemicals were selected and subcategorized as easy, moderate or difficult to identify according to previous experiences in genotoxicity assays and respective modes of action (Table 2). Each laboratory received chemicals from all subcategories. The chemicals were purchased (from

Table 2
List of chemicals tested in the first phase of the 3D Skin Comet assay validation.

Chemical and mode of action	Rationalefor selection and 'ranking of ease of identification' by external experts	Carcinogenicity findings	Genotoxicity profile
TRUE POSITIVES			
Mitomycin C (MMC) (50-07-7) **Mode of action** • Direct acting alkylating agent [56] • Potent DNA cross-linker reacting with guanine residues of 5′-CG-3′ sequences [57] • Induces oxidative damage [58]	• Alkylating activity should be detected but cross-linking activity may confound instead. In line with this, there are conflicting reports of increased and decreased DNA damage in the comet assay. If time is allowed for growth and repair, DNA damage will increase. • Ease of identification: Difficult	• IARC Group 2B (possibly carcinogenic to humans) • Carcinogenic in rats and mice [59] ○ *s.c. − mice, local sarcomas* [60] ○ *p. − rats, peritoneal sarcomas* ○ *v. − rats, lymphosarcomas, abdominal, mammary, lung, bladder, liver, oesophagus, salivary gland, paw*	***In vitro*** • +ve Ames [61,62] • +ve for CA [63,64], MN [65–70], MLA [71–74], HPRT [75] and UDS [76,77] ***In vivo*** • +ve for CA [78,79], MN in bone marrow and blood [48,80–82], and transgenic mutations [83,84] • Induces MN after single and repeated topical dosing to rats [85]
Cadmium chloride (10108 − 64-2) **Mode of action** • Metal carcinogen [86]. Mechanism unknown but possibly involving interaction with DNA, either directly or indirectly [87] Induces metallothionein, indicating oxidative damage	• This inorganic carcinogen induces tumours in multiple organs. It exhibits both clastogenic and aneugenic activity, and the induction of comets is readily detected *in vitro* and *in vivo*. • Ease of identification: Moderate	• IARC Group 1 (probable human carcinogen) [88] • Haematopoietic, lung, prostate and testicular tumours in rats [86,89] • Injection-site sarcomas in mice and rats after s.c. application [88,90,91]	***In vitro*** • +ve Ames [92,93], and −ve Ames [94,95,135] • +ve in MN [65,70,96,97], CA [98,99], and HPRT [100,101]; • +ve in MLA [135] but re-evaluated as "uninterpretable" [102] ***In vivo*** +ve for CA and MN [103–105] and for comets in liver [33,34]
N-ethyl-N-nitrosurea (ENU) (759-73-9) **Mode of action** • Direct acting alkylating agent [115] • Mainly gene mutagen	• Induces comets in multiple organs in rats and mice, including skin after dermal application. • Ease of identification: Easy	• IARC Group 2A (probable Human) carcinogen [106] ○ *Skin – skin and appendages* ○ *p. – skin and appendages* ○ *v. – brain and coverings* • *p.o.–brain and coverings, leukaemia, lung, thorax* Nervous system, small intestine and thyroid tumours in rats (not tested in mice) [114] • Skin tumours in mice after dermal application	***In vitro*** • +ve in Ames [107] • +ve in MN [65] and CA [98], MLA [108], HPRT [109] and UDS [76] ***In vivo*** Induces CA [110], MN [135], comet [48] and transgenic mutations in many tissues in vivo [112,113]
7,12-dimethylbenz(a)anthracene (DMBA) (57-97-6) **Mode of action** • Activated by CYP1B1 [116] • Forms bulky adducts [117]	• DMBA induces increased comet assay values in hairless mice after 24 h (but not after 3 h) and skin tumours in various rodent species after dermal application [38]. Induces increased comet assay values in MCL-5 cells in the presence of repair inhibitors [118]. • Ease of identification: Moderate	• Vascular tumours in female mice (not tested systemically in rats). • Skin tumours in mice, hamsters and gerbils following dermal application [119] ○ *s.c. – site of application* ○ *p. – spleen* ○ *Skin – skin and appendages* ○ *v. – site of application* ○ *p.o.–skin and appendages, transplacental* • Not classified by IARC with regard to human carcinogenicity	***In vitro*** • +ve Ames [107] • Variable CA responses ± S9 [98] • +ve in MLA +S9 [120,159], MN [65–67], HPRT [121,122], and UDS [76,123] ***In vivo*** +ve for MN in bone marrow and blood [80,81,111], CA in bone marrow [124] and gene mutations *in vivo* [112]
MISLEADING POSITIVES			
Propyl gallate (121 − 79-9) **Mode of action** • Possible active oxygen MoA. An antioxidant food additive [125] − generally shown to promote generation of reactive oxygen intermediates at elevated concentrations [126,127], potentially explaining the toxicity and elevated MN response • MN formation was linked to cytotoxicity [128]	• Although this chemicals was positive in rodent and TK6 cells at low concentrations [129] in the absence of S9, it did not induce comets *in vitro* (there are no *in vivo* comet data). • Ease of identification: Moderate	−ve carcinogen in rats and mice [41]	***In vitro*** • −ve Ames [130] but +ve in TA102 [131] • +ve in MLA [132] • +ve for CA [133] • +ve MNT in V79, CHO and CHL [129] • −ve MNT in HuLy, HepG2 and TK6 [129] ***In vivo*** • +ve MN and CA [40]

(*continued on next page*)

Table 2 (*continued*)

Chemical and mode of action	Rationalefor selection and 'ranking of ease of identification' by external experts	Carcinogenicity findings	Genotoxicity profile
Eugenol (97 − 53-0) **Mode of action** MN formation was linked to cytotoxicity [128]	• This chemical is positive in rodent and TK6 cells at low to moderate concentrations but required S9. It also induced comets in repair-proficient but not repair-deficient cells *in vitro* [134] but there are no *in vivo* comet data available. • Ease of identification: Difficult	• − ve carcinogen rats and mice [89,135] • No skin tumours in mice after dermal application (although a short duration of treatment) [136]	***In vitro*** • − ve Ames [107,137,138] • + ve MLA [120] and CA [139,140] due to high concentrations. • + ve MNT in V79, CHO and CHL but − ve MNT in HuLy, HepG2 and TK6 [129] ***In vivo*** • Conflicting MN results: 2 reports of weak + ve [141,142], but -ne in other studies [135,143–146] • − ve for UDS [147] • − ve for transgenic mutations in liver [112]
TRUE NEGATIVES **Di-(2-ethylhexyl)phthalate** (117 − 81-7) **Mode of action** • Non-genotoxic carcinogen [148] • Peroxisome proliferator leading to an excess generation of reactive oxygen species which can contribute to the tumorigenic process.	• This non-genotoxic carcinogen was not only negative *in vitro* tests but also negative for CA, MN, UDS and transgenic mutations *in vivo*. This chemical induced comets in HEK-293 cells, which was attributed to oxidative stress [42]; but did not induce comets in stomach or liver of rats [149]. • Ease of identification: Easy	• Liver carcinogen in rats and mice [135]. • IARC Group 2 B carcinogen	***In vitro*** • − ve Ames [135,150–152] • − ve for CA [135] ***In vivo*** • − ve for CA [135], MN [135], UDS [153] and transgenic mutations in liver [112]
Cyclohexanone (108 − 94-1) **Mode of action: NA**	• The older CA data may be questionable. Using an epidermal 3D skin model, cyclohexanone induced a non-dose-related comet response in 1/3 labs (although it was not considered biologically relevant) [12]. • Ease of identification: Moderate	• − ve carcinogen rats and mice [135] • no *in vivo* genotoxicity data	***In vitro*** • − ve AMES [107] • − ve and + ve in MLA [120,154] • + ve CA [155–157] ***In vivo*** • + *ve CA* [158]

Ames – bacterial reverse mutation assay, CA – chromosome aberration test, HPRT – *in vitro* mammalian cell gene mutation test measuring mutation at hypoxanthine-guanine phosphoribosyl transferase locus, MN – micronucleus test, MLA – mouse lymphoma assay (*in vitro* mammalian cell gene mutation test measuring mutation at thymidine kinase locus), UDS – unscheduled DNA synthesis assay.

Sigma-Aldrich in purities of ≥ 95%), coded and shipped by independent co-workers at the German Federal Institute for Risk Assessment (BfR). Subsequently, they were investigated under blinded conditions, with codes differing among laboratories.

2.2. Skin tissues

Phenion® Full-Thickness Skin Models were purchased from Henkel (Phenion®FT; Düsseldorf, Germany; www.phenion.com) and cultured in small Petri dishes (3.5 cm in diameter) filled with 5 mL pre-warmed air-liquid-interface (ALI) medium. The ALI medium, which was provided by the manufacturer, lacked phenol red and was refreshed one time after an initial overnight equilibration period. EpiDerm™ Full Thickness Skin Model were obtained from MatTek (EpiDermFT™-400; Ashland, MA; www.mattek.com) and cultured in 6-well plates in 2.5 mL/well of provided medium (EFT-400-ASY), which was replaced one time after an initial overnight equilibration period. Both tissues were subjected to experiments after the overnight equilibration at 37 °C and 5% CO_2.

2.3. Experimental design

Main experiments comprised at least three dose groups of a test chemical in addition to a negative control (NC) of untreated tissues, a solvent control (SC), and a positive control (PC) with methyl methane sulfonate (MMS; 5 µg/cm^2 in acetone). The NC was omitted if it had been demonstrated in previous experiments that the solvent did not affect background DNA damage. Each control and treatment group was tested in triplicate.

Test chemicals were applied topically in a volume of 16 µL/cm^2 (Phenion®FT: 25 µL, EpiDerm™FT: 16 µL) for 48 h to ensure possible metabolic transformation of test chemicals (Fig. 1). 24 h and 45 h after the first dosing the test chemical was applied again to the same tissue. In particular, the latter time point, was intended to capture damage which may be subjected to immediate DNA repair. Solutions of the test chemicals were prepared fresh daily, shortly before each dosing.

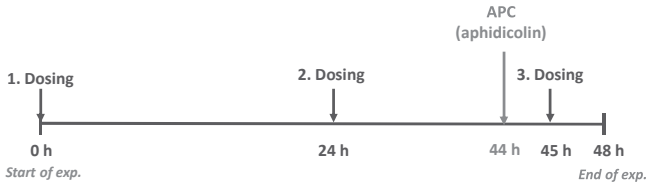

Fig. 1. Treatment schedule of full-thickness skin models. Tissues were exposed with test chemicals for 48 h in total. A maximum of 100 mg/mL in either acetone or 70% ethanol (v/v) was applied three times. In case of negative or equivocal findings, aphidicolin was added 4 h before the end of experiment.

Chemicals that yielded negative or equivocal results in two valid main experiments were tested in an additional confirmatory experiment using aphidicolin (APC). APC (5 µg/mL from a 1000 × stock solution prepared in DMSO) was added to the culture medium 4 h before the end of the exposure period (Fig. 1). In the APC experiment, the pro-mutagen benzo[*a*]pyrene (BaP; 12.5 µg/cm^2 in acetone) was used as positive control instead of MMS to demonstrate the effectiveness of the APC treatment. The positive control, a mid-dose of the test chemical and the respective solvent were applied with and without APC in this experiment.

Immediately after the 48 h exposure period, three slides were prepared from each compartment (epidermis and dermis) of the skin tissue. Two slides were evaluated and the third was maintained as a back-up.

2.4. Study design

The assessment of the genotoxic potential with the 3D Skin Comet assay included (A) a solubility study, (B) a dose range-finding experiment, and (C) at least two valid main experiments:

(A) The maximum applied concentration for soluble chemicals was 10% (w/v), which corresponded to a maximum applied dose of 1600 µg/cm^2. Chemicals were dissolved up to this top dose in acetone with first priority. In case chemicals were not soluble at 10% they were carefully diluted in small increments to determine the maximum soluble concentration unless the visually inspected solubility was below 1% (w/v). In such cases, 70% ethanol (EtOH) (v/v) was used. Precipitation was avoided, as the coverage of skin models with precipitate can interfere with tissue homoeostasis and may lead to incorrect results.

(B) The dose range-finding experiment was designed to define the maximum dose for the main experiments, which could be limited by a) the solubility if it was less than 10%, b) the precipitation of the test chemical present at the end of the experiment, or c) the chemical's cytotoxicity. Cytotoxicity was measured via intracellular adenosine triphosphate (ATP) concentration [24] and the activity of adenylate kinase (AK), which is released from the cells into the culture medium upon cell damage [25].

(C) The main experiments were performed using the design described in Section 2.3. Depending on the outcome of the first main experiment, the dose spacing of the second experiment could be modified, usually by using a tighter spacing. A clear positive finding in the first experiment had to be confirmed in a second experiment. In the event that the test chemical provided two negative or equivocal results (see Section '2.9 Statistical analysis' for a definition of equivocal), an APC experiment was performed.

2.5. Isolation of single cells

After the treatment period of 48 h skin models were washed with 1 mL PBS (w/o Ca^{2+}/Mg^{2+}) and approximately 25% of each tissue was cut off, snap frozen into liquid nitrogen and stored at −80 °C for determining ATP levels. Aliquots of the culture medium were stored at 4 °C for assaying AK activity within the next few days.

Single cells were then isolated using tissue-specific protocols. Phenion®FT Skin Models were first placed on top of 300 µL thermolysin (0.5 mg/mL in buffer containing 10 mM HEPES, pH 7.2–7.5, 33 mM KCl, 50 mM NaCl and 7 mM $CaCl_2$) in a 12-well plate. After incubation at 4 °C for 2 h, the dermis and epidermis were separated using forceps. The tissue layers were transferred separately to 1 mL of cold mincing buffer (20 mM EDTA in HBSS w/o Ca^{2+}/Mg^{2+}, 10% DMSO freshly added, pH 7.0–7.5), and were cut into smaller pieces (20–30 times) using scissors [26]. The tissue suspensions were then incubated for 5 min on ice, resuspended by pipetting, and filtered through 40 µm cell strainers (BD Biosciences, Franklin Lakes, NJ). The mixture of cells and nuclei was harvested by centrifugation (5 min, 250–300*g*), the medium was decanted, and the pellet was resuspended in the residual supernatant.

EpiDerm™FT Skin Models were removed from the culture insert and approximately 25% of each tissue was cut off for ATP measurement. The dermis and epidermis were then separated using forceps before each layer was separately washed in PBS w/o Ca^{2+}/Mg^{2+} (5 min) and PBS containing 0.1% EDTA (5 min). The tissue layers were then transferred to 1 mL of pre-warmed EDTA/trypsin (1 mM/0.25%), cut into smaller pieces (5–10 times) using scissors, and incubated for 15 min (see also [12]). Enzyme activity was stopped by adding 1 mL of cold culture medium supplemented with 10% fetal calf serum. Thorough resuspension by pipetting liberated cells from each layer. Cells were also filtered through 40 µm cell strainers and harvested by centrifugation (5 min, 250–300*g*). After the medium was decanted, cells were resuspended in the remaining supernatant.

Subsequently, keratinocytes and fibroblasts from both FT skin tissues were subjected to the same comet assay procedure.

2.6. Comet assay procedure

The following steps of the alkaline comet assay were carried out according to Singh et al. [23]. Isolated cells were mixed with 0.5% LMA (300–500 µL) and 75 µL were transferred to one of three agarose (1%) coated glass slides. Slides were covered with cover slips and cooled to support solidification of gels. After no more than 5 min, cover slips were removed and cell lysis was carried out by incubating the slides at 2–10 °C overnight in lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris, pH 10, with 1% Triton X-100 and 10% DMSO added shortly before use). For DNA unwinding, slides were then incubated in cold electrophoresis buffer (0.3 M NaOH and 0.001 M Na_2EDTA, pH > 13) for 20 min. Electrophoresis was carried out for 30 min at 39 V and 450 ± 10 mA with fresh buffer using an electrophoresis chamber from Carl Roth (Karlsruhe, Germany, Cat.# N610.1). After electrophoresis, slides were neutralized in 0.4 M Tris-HCl, pH 7.5, for at least 5 min, dehydrated in absolute EtOH, and allowed to dry.

2.7. Slide analysis

The analysis of slides was harmonised among laboratories based on standards recently published [12]. In brief, slides were randomized and coded by a separate person to prevent evaluator bias. Four of the participating laboratories used Comet Assay IV software (Perceptive Instruments, Suffolk, UK), whereas one laboratory used CometImager (MetaSystems, Altlussheim, Germany). DNA was stained for 15 min with a 1:10,000 dilution of SYBR Gold in Tris-EDTA buffer pH 7.2. Tail intensity (% tail DNA) was chosen as parameter to assess genotoxicity. For each tissue compartment, two slides were analyzed, and a third slide was stored as back-up. In detail, 50 cells per slide were analyzed, i.e., 100 cells per single tissue compartment (single epidermis or dermis). For three tissue replicates, this resulted in 300 cells per group of compartments, and lastly, 600 cells total per dose group. Sample size and number of analyzed cells were in line with published recommendations (e.g. [27]). An initial analysis by a statistician involved in the project confirmed the relevance of published criteria for the current project.

2.8. Cytotoxicity assessment

Cell viability was assessed by determining intracellular ATP content in tissue homogenates. Frozen tissue samples (epidermis plus dermis) were homogenized in 1 mL of precooled PBS w/o Ca^{2+}/Mg^{2+} in a TissueLyzer II (Qiagen, Hilden, Germany) set for 5 min and 30 Hz using a 5 mm stainless bead. Homogenates were then heated for 5 min in a heating block at 105 °C, transferred to ice and centrifuged to precipitate any remaining tissue. ATP levels were determined in the supernatants by using the ATPlite Kit (Perkin Elmer, Waltham, MA) according to the manufacturer's instruction. To account for possible variations in the size of the hand-cut skin tissue slices, samples were normalized to the protein content using the Bradford assay (BioRad, Hercules, CA) in four of the participating laboratories. The fifth laboratory used the BCA assay (Thermo Fisher Scientific, Waltham, MA). ATP levels expressed as µg ATP/mg protein were used for further analysis.

Adenylate kinase leakage into the culture media was measured using the ToxiLight bioassay kit from Lonza (Basel, Switzerland). Results of the treated tissue groups obtained for both cytotoxicity markers were expressed relative to those of the SC and were used to assess the validity of each experiment.

2.9. Data evaluation

2.9.1. Data processing of genotoxicity data

All laboratories used standardized Microsoft Excel spreadsheets to record the comet assay raw data (% tail DNA) of single experiments, which were submitted to the independent statistician for further analysis. The spreadsheet's data structure mirrored the experimental design which, in the case of a main experiment, consisted of a NC (optional), a SC, at least three dose groups, and a PC (MMS). The experimental design of an APC experiment included six groups comprising a SC with and without APC, a mid-dose group with and without APC, and a PC (BaP) with and without APC. Three tissues were used per control/treatment group, with the two compartments (epidermis and dermis) of a tissue being considered separately, two slides being analyzed per compartment, and 50 comets being scored per slide.

The raw data were aggregated as follows: For each slide, the median was calculated from the 50 comet scores and these median values were then arcsine square-root transformed to achieve normality and variance homogeneity. For each compartment, the transformed medians of the two slides were averaged. This procedure resulted in $n = 3$ values (3 tissues) per control/treatment group for the epidermis and the dermis compartment, respectively, and were used for statistical testing.

2.9.2. Validity criteria

Prior to statistical analysis, the validity of experiments was determined. First, the experiment needed to follow the predefined experimental design (NC, SC, PC and at least three dose groups of the test chemical; each dose group represented by three valid tissues, and two slides per compartment with 50 comets scored per slide). Second, validity criteria for the control groups were applied. NC and SC should display $\leq$ 20% tail DNA; PC (MMS; 5 µg/cm^2) had to show at least a two-fold increase in % tail DNA compared to SC and an absolute increase in % tail DNA by $\geq$15 percentage points above the SC. Third, a dose group was considered valid when the thresholds set for strong cytotoxicity (i.e., 2-fold increase in AK leakage compared to SC and/or 50% reduction in normalized ATP content compared to SC) were not exceeded. In the event that excessive cytotoxicity was observed within a dose group, it was not considered for the evaluation of genotoxicity. If cytotoxicity was seen with both measurements, the more sensitive parameter was used. In rare cases of treatments triggering neither of the above-mentioned cytotoxicity thresholds, a clear decrease in the number of comets observed on slides was used as indication to not consider these high dose groups for genotoxicity assessment.

An experiment with only two valid dose groups could nevertheless be considered valid if (1) the first two doses were positive (in terms of genotoxicity) and the thresholds for strong cytotoxicity were not exceeded; or (2) genotoxic effects were absent in all dose groups and cytotoxicity exceeded the thresholds in the third test group only (because the thresholds were set to prevent misleading positive results). Exceptional cases, in which a single tissue may be missing (e.g. due to issues during handling), were still acceptable if a substance was clearly positive in the remaining two tissues or if the missing tissue belonged to a lower dose for a substance that did not increase % tail DNA at higher dose. Justification needed to be provided in either of these cases.

2.9.3. Statistical analysis and consideration of biological relevance

The experimental design of the main experiment of the 3D Skin Comet assay was a one-way layout with a minimum of three doses that were compared against a SC. A PC (MMS) was included to demonstrate assay sensitivity. The randomized unit was the tissue, whereas three tissues per group were used in a balanced design. Since the tissue consists of two compartments (epidermis and dermis), the statistical analysis was performed separately for each compartment. Two different prediction models, PM1 and PM2, were applied to the data. PM1 used an Analysis of Variance (ANOVA) that, in case of a statistically significant result, was followed by multiple treatment-versus-control comparisons using the one-sided Dunnett test [28] to identify individual dose groups showing a significantly increased response. For the outcome of PM1, the Dunnett result was decisive. PM2 used the one-sided Umbrella-Williams test [29]. This method integrated a test for any increase in individual treatment levels (Dunnett procedure) with a test for an increasing trend against the control (standard Williams procedure) that was additionally protected against downturn effects at high doses. For all tests in PM1 and PM2, the (overall) significance level was set to 0.05.

The experimental design of an APC experiment included a SC with and without APC, a mid-dose with and without APC, and a PC (BaP) with and without APC. A one-sided Student's *t*-test was used to check for a statistically significant increase in the BaP/APC group and in the mid-dose/APC group in relation to the SC/APC group. The latter comparison was relevant to decide on a positive outcome. The significance level was set to 0.05.

For the final conclusion, criteria based on both the statistical significance and the biological relevance were taken into account. These criteria followed the standards of the OECD Test Guideline of the '*In Vivo* Mammalian Alkaline Comet Assay' [11]. Specifically, an experiment was identified as positive if at least one of the two prediction models indicated a significant and dose-related increase in tail intensity for doses that did not exceed the cytotoxicity cut-off. In case only one dose group produced a statistically significant increase in % tail DNA without dose-dependency, the effect had to be reproduced in a second main experiment to trigger a positive call. In both scenarios, at least one dose group needed to be outside the historical control range (laboratory specific: mean of SC of the last experiments plus 2 standard deviations, see also Fig. 2). In any case, a positive call in one cell type/compartment was sufficient to consider a main or an APC experiment as positive.

If none of the criteria were fulfilled, the test chemical was considered negative. In case of two negative main experiments, the following APC experiment was considered positive if the test chemical caused a statistically significant increase in % tail DNA in the presence of APC compared to SC with APC.

In case of equivocal findings where some, but not all, criteria were fulfilled for a positive call, an additional experiment with a modified dose spacing (generally tighter) was recommended.

In addition to applying these decision criteria, each laboratory provided a descriptive judgement for each experiment and for the entire set of experiments.

3. Results and discussion

3.1. Prioritization of skin tissues

In order to identify human skin models that would be most suitable for the 3D Skin Comet assay all commercially available full-thickness skin models were investigated with regard to histology and parameters displaying a possible irritant status after transportation, as proposed by Bätz et al. [30]. Based on these aspects, the Phenion®FT and the EpiDerm™FT Skin Models were selected for further studies addressing (1) the implementation of cell isolation protocols, specific for each skin tissue, and a common comet assay procedure applied to all tissues and (2) the standardisation and optimization of protocols.

Since Reus et al. [12] observed relatively high and variable background levels of DNA damage when using the epidermal model EpiDerm™ (MatTek), we first investigated if the use of FT models could improve the robustness of the 3D Skin Comet assay. Therefore, a direct acting mutagen (MMS) was tested with all three tissues (EpiDerm™, EpiDerm™FT and Phenion®FT) at concentrations leading to a range of slight to marked DNA damage, together with solvent (SC: acetone) and negative controls (Supplemental data Fig. S1). All laboratories obtained a clear dose-dependent increase in % tail DNA with all models. No visual difference was detected between the responses of EpiDerm™FT and

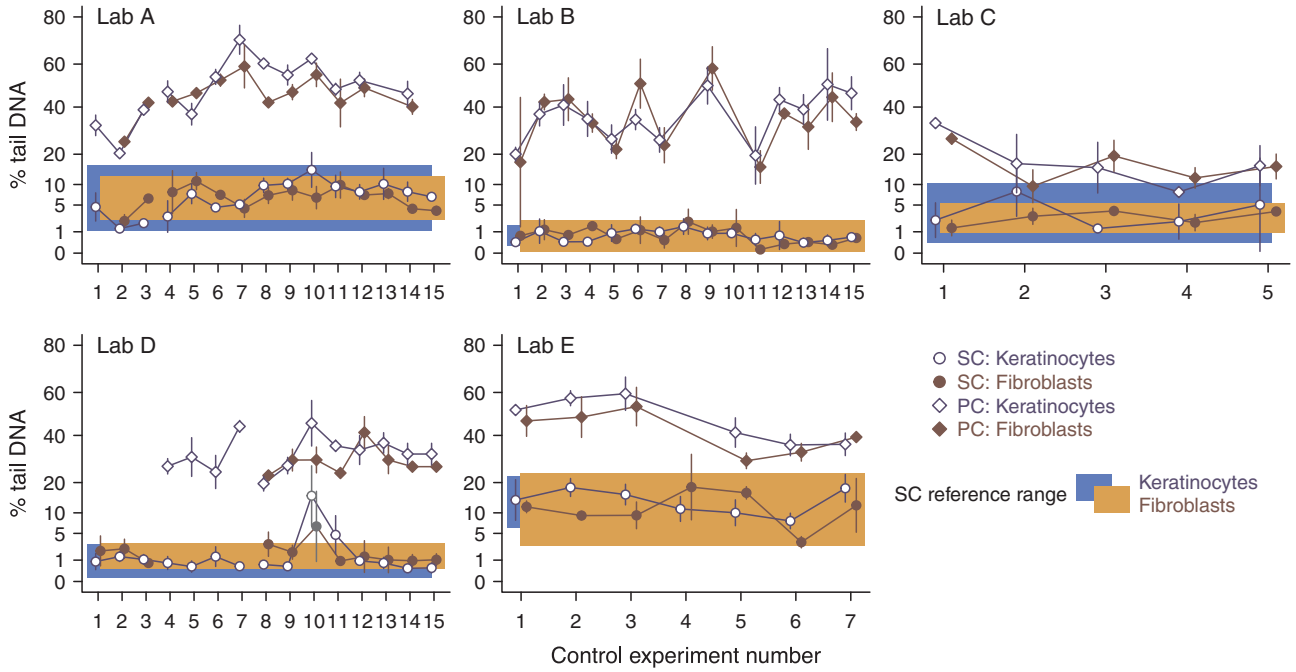

Fig. 2. Historical data. Percentage of tail DNA in the solvent (SC) and positive controls (PC) of individual experiments as obtained with the Phenion®FT during the optimization phase and coded testing are shown. The SC values (circles) and PC values (diamonds) for the keratinocytes (White symbols and blue lines) and fibroblasts (red symbols and lines) are given as mean ± standard deviation. The blue-shaded and orange-shaded areas indicate the reference range for the SC, i.e., historical control, for keratinocytes and fibroblasts, respectively. The y-axis is on the arcsine square-root transformed scale but the axis ticks and tick labels relate to the percentage scale. A single outlier experiment is indicated by gray-colored symbols.

Phenion®FT. However, the level of DNA damage observed with the two full-thickness models for the PC, when compared to the SC, was larger than the damage seen with EpiDerm™. In addition, background levels of DNA damage and variability was higher in EpiDerm™ tissues, resulting in a substantial number of invalid experiments due to SC and NC values exceeding predefined acceptance thresholds (data not shown). These findings supported the observations by Reus et al. [12] and the subsequent protocol optimization phase studies were therefore conducted with EpiDerm™FT and Phenion®FT.

3.2. Protocol optimization – dosing regimen and introduction of aphidicolin

Based on the results by Hu et al. [21] and Wiegand et al. [18], who showed that phase I enzymes in skin tissues and human native skin were clearly induced within 48 h, we topically exposed full thickness tissues with test chemicals for 48 h to facilitate the conversion of promutagens into DNA reactive mutagens. We applied the test substance three times, i.e. at the start of the experiment, at 24 h and at 45 h (Fig. 1). The exposure at 45 h was chosen to allow the detection of DNA damage that may be repaired quickly. Moreover, APC, an inhibitor of DNA polymerases α and δ [31], was added to the ALI medium 4 h before sampling to further investigate chemicals which lacked genotoxic effects in main experiments with the standard protocol. Inhibiting the DNA repair function of the polymerases by APC amplifies probable single strand breaks generated during excision repair which leads to increased comet formation, as proposed by Brinkmann et al. [15]. The suitability of this approach was confirmed by the low background DNA damage in APC-supplemented solvent control groups.

3.3. Protocol optimization – selection of appropriate cytotoxicity measurements

The protocol was complemented with assays measuring general cellular toxicity in parallel to the determination of genotoxicity, as DNA damage can also be triggered as a mechanism secondary to cytotoxicity (e.g. [32]). Therefore, increased DNA damage that occurs only in conjunction with strong cytotoxicity is not considered biologically relevant. Classical *in vitro* genotoxicity assays use proliferation-related parameters to monitor cell division as a central characteristic of fast growing 2D cell cultures. In contrast, FT skin models are more representative of the *in vivo* situation and mirror the quiescent nature of the majority of cells in an adult organism, i.e., in skin only a subset of keratinocytes is dividing whereas parenchymal fibroblasts only proliferate to maintain homeostasis. As a result, parameters reflecting tissue vitality and energy status rather than proliferation were selected for the current project, i.e., the activity of adenylate kinase (AK) released into the culture medium upon cell damage and the intracellular adenosine triphosphate (ATP) concentration. These assays are not only sensitive but also take different modes of cytotoxicity into account. Furthermore, they reveal effects accumulating during the 48 h of exposure including those manifesting only after the last exposure 3 h before the end of the experiment. Importantly, both assays are applicable to the cell isolation protocols used for both FT skin models and cytotoxicity can be analyzed in the same tissue used for genotoxicity assessment.

During the optimization phase, in which both FT skin models were used, it became obvious that the available capacities would only allow testing of one skin model during the validation phase. Therefore, models were prioritized based on the performance of tissues and occurrence of invalid studies in the individual laboratories, of which a majority prioritized the Phenion®FT over the EpiDermFT™. Consequently, it may be possible to conduct the 3D Skin Comet assay with both tissues, however the current project provides detailed data only for the Phenion®FT.

3.4. Results of validation study phase I

The optimized protocol was challenged in the first phase of an ongoing validation study with the investigation of eight chemicals, four with an expected positive and four with an expected negative outcome (Table 3). Each chemical was tested in three of five participating laboratories using the Phenion®FT to provide initial information on (a)

Table 3
Overall calls of studies. Empty boxes indicate that the compound was not tested in the respective laboratory due to the lean design of the study. + Positive call; − Negative call; o − Equivocal; TP − True positive; TN − True negative; MP − Misleading positive; e − expected easy identification with the 3D Skin Comet assay; m − moderate; d − difficult, * − positive calls, which were picked up by the standard protocol (without APC).

No.	Substance	Type	Lab A	Lab B	Lab C	Lab D	Lab E
1	Mitomycin C	TP.d			+	o	+
2	Cadmium chloride	TP.m	+*	+*		−	
3	N-ethyl-N-nitrourea	TP.e		+*	+*		+*
4	7,12-dimethylbenz(a) thracene	TP.m	+	+*	+		
5	Propyl gallate	MP.m	−	−		−	
6	Eugenol	MP.d	−		−		−
7	Di-(2-ethylhexyl)phthalte	TN.e	−		−	−	
8	Cyclohexanone	TN.m		−		−	−

the predictive performance of the assay, after comparing current results with historical *in vivo* genotoxicity and carcinogenicity data, (b) the intra-laboratory reproducibility, and (c) the inter-laboratory reproducibility.

Each of the five laboratories provided a minimum of two valid main experiments and, if both were negative, an APC experiment. The data were statistically analyzed and subjected afterwards to an expert judgement by the performing laboratory, which also took the biological relevance of effects into account. This final conclusion was used for the evaluation of both reproducibility and predictivity.

3.4.1. Database of historical data

As a first step of data analysis, descriptive statistics were performed displaying the percentage of tail DNA for both SC and PC of both cell types in relation to the experiment number (Fig. 2). These data were used to obtain information on mean levels as well as on the variability within and between experiments and to finally derive laboratory-specific reference ranges.

The data of Lab A demonstrated low background DNA damage in the SC and a clear induction of DNA migration with the positive control in both cell types. A similarly pronounced dynamic range between both controls were obtained in Labs B and D, whereas increasingly smaller dynamic ranges were found for Labs E and C. A small dynamic range can lead to overlapping variation ranges of SC and PC as it was the case for the Lab C (both cell types) and Lab E (fibroblasts only). In addition, it can increase the risk of not fulfilling the acceptance criteria for the PC. In the current study the first acceptance criterion for a PC (a 2-fold increase in % tail DNA above SC) was fulfilled in all main experiments. The second criterion (an absolute increase in % tail DNA of 15 percentage points above the SC) was not met in Lab E in one out of 12 main experiments (8%) and in six out of 10 experiments (60%) in Lab C. A possible impact of these findings on the experimental outcome is discussed in the following sections.

3.4.2. Cadmium chloride ($CdCl_2$)

Cadmium chloride was tested up to signs of strong cytotoxicity, i.e., 100 µg/cm^2 (Fig. S2 A), in Lab A and induced a clear dose-dependent increase in % tail DNA in both cell types in both main experiments (Fig. 3A–B, Fig. S2 A–B**)**. Lab B also tested up to signs of strong cytotoxicity (70 µg/cm^2, Fig. S2 C) and observed a clear increase in % tail DNA in both main experiments, which concentrated on the fibroblasts in the second experiment (Fig. S2 C–D). Lab D detected a slight dose-dependent increase in % tail DNA in both cell types in the first main experiment, which was statistically significant for the keratinocytes (Fig. S2 E). However, the values were within the range of the laboratory specific historical control (HC). In the second main experiment, in which the dose range was adapted according to cytotoxicity observed in the first experiment at doses above 100 µg/cm^2 $CdCl_2$, a statistically significant increase in % tail DNA could only be observed with one dose group slightly outside the HC while no dose-dependency was observed (Fig. S2 F). As in the subsequent APC experiment values for both cell types were within HC (Fig. S2 G), Lab D classified $CdCl_2$ as negative (Table 3).

The results in Lab A and B were in line with findings showing that exposure of cadmium salts is linked to an increase in mutation and micronucleus frequencies both *in vitro* and *in vivo* as well as to tumor formation (Table 2). However, $CdCl_2$ was classified as 'moderate to test' as conflicting results have been reported with the Reverse Bacterial Mutation test (Table 2). Notably, inconsistent results were observed with the *in vivo* comet assay as the outcomes of a validation study with rats exposed for 3 days of daily treatment were considered positive for stomach and equivocal for liver [33]. After a re-evaluation of the data, the findings for both organs were determined to be equivocal [34]. Furthermore, negative results have been reported for mice treated with a single i.p. injection in another *in vivo* comet assay study in which samples were taken 3, 8, and 24 h after dosing [35]. In all of the eight organs investigated (stomach, colon, liver, kidney, urinary bladder, lung, brain, bone marrow), no increase in DNA migration could be detected.

The genotoxic mechanism of cadmium salts remains to be fully described, but indirect mechanisms have been proposed as cadmium does not cause DNA damage in cell extracts or when using isolated DNA [36]. In fact, among other mechanisms, the generation of reactive oxidative species and the inhibition of DNA repair are suggested [36]. There is evidence that the dosing and sampling schedule as defined for the 3D Skin Comet assay was suitable in the majority of labs to reveal cadmium-linked effects despite the discussed varying kinetics, an aspect that might be considered for the *in vivo* comet studies.

3.4.3. N-ethyl-N-nitrosourea (ENU)

ENU induced a clear and dose-dependent increase in % tail DNA in both main experiments and both cell types in each of the three laboratories which investigated the chemical (Fig. S3 A–G). The maximum dose was either determined by a disappearance of analyzable comets from slides at high doses in Lab B, or by cytotoxicity exceeding the agreed thresholds in Lab E. After Lab C did test up to strong cytotoxicity in the dose-range finder, for the main experiments three dose groups without cytotoxicity but a clear increase in DNA migration were chosen. In parallel, the increase in % tail DNA with the PC was less clear and not sufficient to meet all acceptance criteria in an experiment in Lab C (Fig. S3 D). Due to the clear response with ENU which demonstrated the responsiveness of the tissues, the experiment was nevertheless considered valid. A similar experiment in Lab E (Fig. S3 E) was not considered valid due to strong cytotoxicity in all three dose groups. These are two examples in which the reduced dynamic range in Labs C and E had no impact on the predictive capacity.

The ENU results were consistent with publications characterizing the chemical as a potent direct-acting alkylating agent, which induces transversions and transitions [37]. This is reflected by a variety of positive *in vitro* findings and the frequent use of ENU as positive control in *in vivo* comet assay studies as ENU induces DNA migration in cells from multiple organs after oral or dermal exposure [34] (Table 2). Therefore, ENU was considered 'easy to test' for the current study.

3.4.4. 7,12-dimethylbenz[a]anthracene (DMBA)

In Lab A, the top dose for DMBA was limited by precipitation on the surface of the skin models. DMBA failed to increase % tail DNA in two main experiments but clearly induced DNA migration in APC follow-up experiments (Fig. 3C–E, Fig. S4 A–E). Likewise, in Lab C a statistically significant increase in % tail DNA was not observed after DMBA treatment in main experiments but when administered together with APC (Fig. S4 I–K). In this second study, precipitation was not observed and therefore higher concentrations up to maximum solubility were tested. The positive finding in the APC experiment was of particular importance as the PC in the first two main experiments showed a

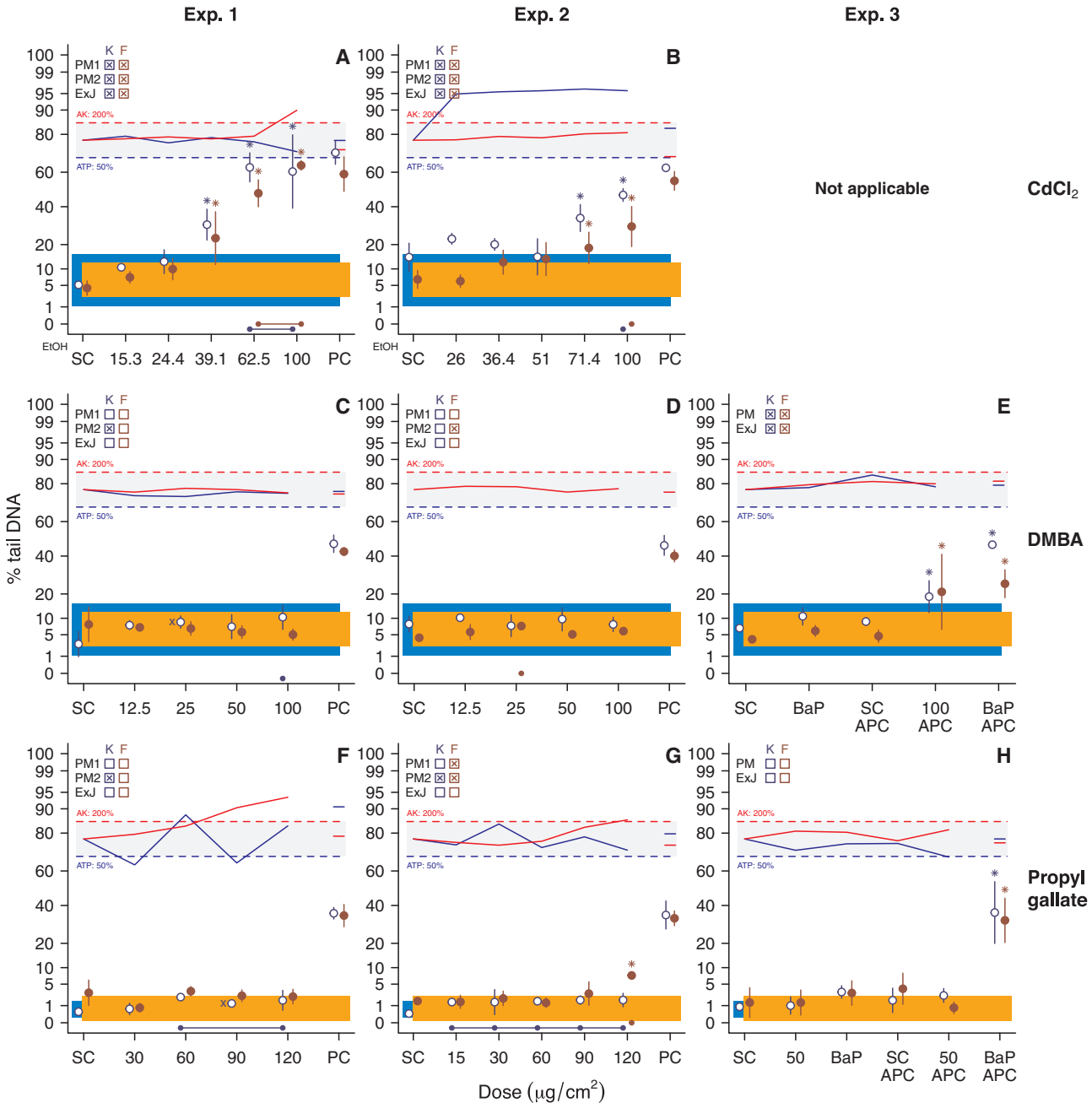

Fig. 3. Representative studies (each from one of three laboratories having tested the same compound) using the Phenion®FT. The percentage of % tail DNA for keratinocytes (white circles) and fibroblasts (red circles) is given as mean ± standard deviation. ATP content and AK leakage (both in % of the SC) are indicated by blue and red lines on a log-transformed scale; thresholds for strong cytotoxicity (50% ATP, 200% AK) are marked. SC − solvent control; PC − positive control (MMS, 5 µg/cm²); * − statistical significance of an increase in % tail DNA in a cell type within a treatment group with prediction model 1 (PM1) for main experiments or with the *t*-test for APC experiments. The dark blue and red filled (individual or linked) circles below the references range indicate single or pooled dose groups with the smallest significant *p*-value as obtained with PM2. The blue-shaded and orange-shaded areas indicate the reference range for the SC, i.e., historical control, for keratinocytes and fibroblasts, respectively. Invalid treatment groups (i.e., groups with less than three valid tissues) are indicated by an "x" letter. The label "EtOH" indicates that 70% ethanol was used instead of the standard solvent acetone.

sufficiently increased DNA migration (2-fold compared to the SC), but not an absolute increase in % tail DNA by ≥ 15 percentage points above SC. In contrast, Lab B observed a statistically significant increase in DNA migration in three valid main experiments, for which the maximum dose was defined by the lowest precipitating dose (Figs. S4F–H). Since in two experiments half or all of the dose groups were also outside the HC (Figs. S4 F and H), these experiments and therefore the study was considered positive and APC experiments were not performed. ATP values were of low priority for defining cytotoxicity in this study due to lacking dose-dependency of the effects observed.

Taken together, the three laboratories correctly predicted DMBA (Table 2), which has been classified as 'difficult to test' because it requires activation by cytochrome P450 1B1 (CYP 1B1) to interact with DNA, i.e. it is a pro-mutagen.

Furthermore, the study is suited to reflect the xenobiotic metabolism in skin. Basal phase I enzyme expression is low in both native human skin and reconstructed human skin tissues, but it can be upregulated within 24–72 h [18,21]. In accordance, two pro-mutagens, BaP and DMBA, induced DNA migration in the skin of hairless mice only after a treatment period of 24 h but not after 3 h [38]. In the present study, CYP1B1 expression, which may have been induced during the 48-h treatment period, seemed to be sufficient in Lab B to activate

DMBA and to obtain a slight, but significant, rise in comet tail formation in two out of three experiments without the need to add APC to amplify comet formation.

3.4.5. Propyl gallate

Propyl gallate was tested up to signs of cytotoxicity in Lab B (Fig. 3F–H, Fig. S5 D–F). Several dose groups showed statistical significance supported by low background DNA damage in the SC and low standard deviations observed with dose and control groups. The majority of those dose groups were within HC with two exemptions. The highest dose in the second main experiment induced strong cytotoxicity and was therefore not considered for genotoxicity assessment. In consequence, the finding in experiment 1 (60 µg/cm^2 dose group outside HC and statistically significant) could not be reproduced neither in the second nor in the APC experiment. Therefore the study was considered negative. It should be noted that experiment 1 was considered valid since the ATP effects missed dose-dependency in this study in contrast to the AK response curve which was therefore considered more relevant. Lab A obtained comparable findings. While propyl gallate was tested up to signs of strong cytotoxicity, a slight and statistically significant increase in DNA migration in the second main experiment was only observed at the lowest dose in one cell type. Since the increased value was within HC and could not be reproduced, the compound was also considered negative (Fig. S5 A–C). Lab D also did not observe effects on DNA migration in the main experiments in dose groups which showed strong cytotoxicity in parallel (Fig. S5 G–I). As the dose in the APC experiment marked with statistical significance was clearly within HC the third study was also considered negative. The appearance of strong cytotoxicity in parallel was considered as a maximization of exposure confirming the relevance of the genotoxic assessment.

The results were consistent with the genotoxic assessment of propyl gallate by the European Food Safety Authority [39]. Its ability to generate reactive oxygen intermediates might be the reason for several positive *in vitro* findings (Table 2). However, when applying current standards such as the usage of human p53 competent cells, negative *in vitro* findings were reported recently (Table 2). In contrast, a summary of *in vivo* MN and CA studies listed positive findings [40]. As neither the purity of the chemical batches nor the HC from varying laboratories was reported the relevance of the findings cannot fully be determined. Due to this heterogeneous data set propyl gallate was classified as 'difficult to test'. However, the classification of propyl gallate as misleading positive is supported by a negative carcinogenicity study [41] and the current EFSA statement.

3.4.6. Eugenol

Lab A and C tested eugenol in doses up to signs of strong cytotoxicity without observing genotoxic effects, neither in the two main nor in the final APC experiments (Fig. S6 A–C, D–F). The fact that ATP values in the test chemical/APC dose group in Lab C were even shortly below the set threshold indicating strong cytotoxicity strengthened the relevance of the negative call by maximization of the applied dose. Lab E observed a statistically significant increase in % tail DNA in the first main experiment in the lowest dose group in both cell types, one of which was outside the HC (Fig. S6 G). However, this finding could not be reproduced in any of the subsequent three experiments, including an APC experiment (Fig. S6 H–J). Again, ATP values below the threshold in the test chemical/APC dose group were considered as exposure maximization and an emphasis of the negative call in Lab E. Further, the missing clear increase of the PC in the second main experiment had no impact on the study outcome as a third and valid experiment was provided.

Despite that eugenol was classified as 'difficult to test' due to the variety of inconsistent data, all three laboratories came to the same and correct result, which was in line with published data on *in vivo* mutagenicity and carcinogenicity (Table 2).

3.4.7. Di-(2-ethylhexyl)phthalate (DEHP)

Lab A investigated DEHP up to the maximum dose of 1600 µg/cm^2 in the first main experiment observing strong cytotoxicity with the highest dose (data not shown). Experiments 2 and 3 with a modified dose range reproduced the lack of genotoxic effects in parallel to first indications of cytotoxicity (Fig. S7 A-C). In the APC experiment the mid-dose produced a statistically significant increase in % tail DNA in the keratinocytes, but was within the HC (Fig. S7 D). Therefore, the increase was considered not of biological relevance and DEHP was classified as negative. Similar results were observed in Lab C, i.e., no increase in % tail DNA in both main experiments in which the maximum dose of 1600 µg/cm^2 was applied (Fig. S7 E–F). Since the parallel application of APC did ńot induce DNA migration, the study was considered negative (Fig. S7 G). This negative outcome of the confirmatory APC experiment was of particular importance as the PC in the first two main experiments met one validity criterion (2-fold increase compared to the SC), but not the second one of an absolute difference in % tail DNA by ≥15 percentage points between SC and PC. Also, Lab D tested up to the maximum dose without detecting signs of genotoxicity in the two main experiments (Fig. S7 H–I). As the dose marked with statistical significance in the APC experiment was inside HC, the third study was also considered negative (Fig. S7 J).

DEHP, a peroxisome proliferator, is classified as non-genotoxic carcinogen generating reactive oxygen species (Table 2), which may account for the positive findings in an *in vitro* comet study using HEK-293 cells [42]. Beyond this exception, only negative *in vitro* and *in vivo* genotoxicity findings have been published for DEHP leading to an 'easy to test' classification (Table 2).

3.4.8. Cyclohexanone

Lab B detected statistically significant increases in % tail DNA among the two mid-doses of the second main experiment, which were slightly outside the HC but without statistical significance when compared to SC (Fig. S8 A-D). Since these findings could not be reproduced in subsequent main or APC experiments, cyclohexanone, which was tested up to maximum dose limited by strong cytotoxicity, was considered negative overall. Lab D tested up to 800 µg/cm^2, which induced signs of strong cytotoxicity, and did not observe evidence for any genotoxic effect in the three experiments performed (Fig. S8 E-G). After Lab E observed strong cytotoxicity with the maximum dose of 1600 µg/cm^2 in the dose range finder, a modified spacing was applied in the three main experiments without observing cytotoxicity or genotoxicity (Fig. S8 H–J), which was confirmed in the final APC experiment (Fig. S8 K). In this study, three main experiments were performed because of a prior invalid experiment due to an invalid PC.

The results were in line with negative carcinogenicity data (Table 2). However, due to a heterogeneous *in vitro* data set (Table 2), cyclohexanone was categorized as 'moderate to test'.

3.4.9. Mitomycin C (MMC)

Lab C observed a slight and statistically significant increase in DNA migration across valid dose groups in the first and second experiment (Fig. 4A, Fig. S9 A), however only in the APC experiment DNA migration increased to a level above the HC (Fig. S9 B–C). The positive finding of the APC experiment was of particular importance as the PC in the first two main experiments met the first acceptance criteria (2-fold increase compared to the SC), but not the second one of an absolute increase in ≥15 % tail DNA above SC. In Lab D, both keratinocytes and fibroblasts showed a statistically significant increase in % tail DNA in several dose groups outside the HC in the two main experiments while the chemical was tested up to maximum solubility in 70% ethanol (Fig. S9 D-F, low ATP values in the second experiment were considered of less relevance due to missing dose dependency and missing alignment to the two other experiments). Since this effect progressively disappeared with higher doses, and approached SC levels, MMC was classified as equivocal in the final expert judgement of Lab D (Table 3).

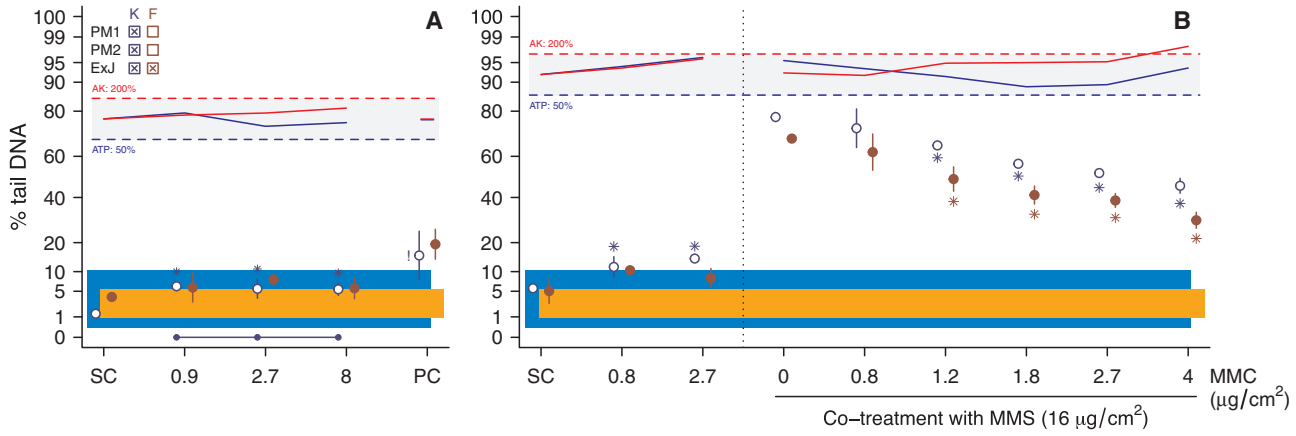

Fig. 4. Representative comet assay experiments using Phenion® FT displaying the assessment of MMC in the indicated concentrations differentiated in keratinocytes (white circles) and fibroblast (red). The blue-shaded and orange-shaded areas indicate the reference range for the SC, i.e., historical control, for keratinocytes and fibroblasts, respectively. ATP content and AK leakage (both in % of the SC) are indicated by blue and red lines. (A) Representative experiment from a study conducted with the coded compound. (B) Effects of MMC alone and of the co-treatment with MMC on the increased % tail DNA induced by MMS. SC − solvent control, PC − positive control (MMS, 5 µg/cm^2), * − statistical significance of (i) an increase in an MMC-only treatment group in relation to the SC and of (ii) a decrease in a MMC/MMS co-treatment group in relation to the MMS-only control group. Statistical testing was done using the Dunnett procedure. For further graphical details, see Figure legend 3.

Further support for the conclusion came from the APC experiment where a statistically significant increase was seen but this was not considered as an unequivocal positive response by Lab D since values were only slightly outside the HC (Fig. S9 F). Finally, Lab E observed increased DNA migration in both main experiments 1 and 3, which was statistically significant and outside the HC in experiment 3, a finding that was confirmed in the APC experiment (Fig. S9 G-J). Experiment 3 was added because of a prior invalid experiment due to an invalid SC. The variable cytotoxicity observed with ATP was considered of minor relevance due to lacking dose dependency and because no effect was observed with AK except for the highest dose tested.

MMC possesses alkylating properties at low concentrations whereas after accumulating at higher doses, a DNA crosslinking effect becomes prominent (Table 2), which may complicate its detection. Chemicals with crosslinking activities are not easy to detect with the standard comet assay protocol but are more easily identified by the *in vitro* MN test when investigated in a standard genotoxicity test battery. Therefore, MMC was classified as 'difficult to test' in the current comet assay validation. The alkylating properties of MMC could indeed be detected with the standard 3D Skin Comet assay protocol by the moderate increase in % tail DNA at low concentrations in all three laboratories. The crosslinking activities were demonstrated by the down-turn effect at higher doses in Labs D (Fig. S9 D-E), being indicative of reduced migration of larger DNA fragments. However, as the background DNA damage in solvent controls was low and the MMC-related increase in % tail DNA small, it was unclear whether the observed down-turn effect was of biological relevance. Therefore, a specific protocol [43] was used to reveal the chemicaĺs crosslinking mode of action after decoding: MMS was applied to the skin tissues to induce a high background level of DNA damage, which decreased in a dose-dependent manner when increasing MMC concentrations were added (Fig. 4B).

As MMC induces different types of DNA damage, which are linked to various repair mechanisms and kinetics, e.g., [44,45], appropriate timing for dosing and sampling is needed to detect all types of damage. The data suggest that the protocol for the 3D Skin Comet assay indeed provides an appropriate time schedule for exposure and sampling. However, this may not apply to all reported *in vivo* comet assay studies. Besides two positive mice studies [46,47], a negative finding with hairless mice [38] was reported in which MMC was applied topically. Mixed results were observed in an oral comet assay/micronucleus combination study [48]. Statistically significant increases in DNA migration after treatment with MMC were only observed in the stomach while liver and blood were negative. MMC, however, gave clear positive results in the micronucleus test [48].

3.5. Predictivity of the 3D skin comet assay after phase I of the validation study

The assessment of predictivity values was based on the final calls provided in Table 3, which considered both the statistical significance and the biological relevance of the findings.

(1) The analysis of the intra-laboratory reproducibility resulted in a 100% concordance of main experiments in three laboratories: Lab A (which provided 12 main experiments for 5 chemicals), Lab C (10 main experiments for 5 chemicals), and Lab E (12 main experiments for 4 chemicals). Lab B performed 12 main experiments for 5 chemicals and obtained concordant results for four out of five chemicals (80% concordance). The fifth chemical, the pro-mutagen DMBA, produced two positive and one negative call. Lab D performed 10 main experiments for 5 chemicals, three of which gave concordant results, one was considered equivocal (70% concordance). The fifth, $CdCl_2$ induced a slight and statistically significant positive response, which was not reproduced in the second main experiment.

APC experiments were not included in the assessment of the intra-laboratory reproducibility as they are able to pick up pro-mutagens and are thus expected to provide a different pattern of results compared to main experiments. The results of this first phase showed that the APC approach not only successfully supported the correct prediction of a pro-mutagen, but also helped to identify the crosslinking agent MMC. Importantly, all chemicals with an expected negative outcome remained negative when tested with APC, i.e., no false positive predictions were observed.

(2) The analysis of the inter-laboratory reproducibility showed that for six substances, predictions were concordant between the respective three laboratories. For two substances, $CdCl_2$ and MMC, predictions differed between laboratories.

(3) The overall accuracy was 100% in four laboratories and 70% in Lab D which incorrectly predicted one (of five) chemicals and considered one as equivocal (Table 3). The reduced dynamic range observed for Labs C and E had no impact on the predictivity since all chemicals were identified correctly as discussed above.

In summary, the predictivity observed in phase I of the validation study was promising. The relevance will be further investigated by testing an additional set of 22 chemicals in the next phase of the validation exercise.

3.6. Impact of the phase I results on phase II procedures

In most experiments that were considered positive, both cell types exhibited an increase in % tail DNA. In the rare cases in which only one cell type showed genotoxic effects, results were confirmed in a subsequent experiment in which both cell types were positive. We can therefore conclude that testing in one cell type may be sufficient. This would reduce the overall workload of the assay, but would need to be confirmed with more data, which will likely be produced in the following phase of the validation study.

Likewise, the project team agreed to also include both cytotoxicity markers, ATP and AK, in the next phase. AK has been shown to be more reliable, but not all cytotoxic chemicals induce a disintegration of cellular membranes. Therefore, both markers will be used in the dose-range-finding experiments, and only in case AK does not show a dose-dependent response, the main experiment should also proceed with the detection of cellular ATP.

In addition to the above-described evaluation that is based on the final calls, which included the assessment of the biological relevance of effects observed, the performance of the prediction models (PM) themselves was analyzed. Whereas PM1 is based on a pairwise treatment-to-control comparison with the Dunnett test, PM2 used the Umbrella-Williams test, which integrates the Dunnett test comparisons with a trend test that is additionally protected against downturn effects at high doses allowing statements about global and partial trends. Therefore, the same dose groups were not always flagged by both PMs (Figs. S2–S9). In brief, both PMs did flag the experiments of the four positive chemicals except the main experiments of Labs A with DMBA and one $CdCl_2$ experiment in Lab D which were only flagged by PM2. In addition, several dose groups in experiments of chemicals with an expected negative outcome were flagged by both of the PMs: three experiments by PM1 and five by PM2, neither of which were considered biologically relevant. A final preference for one of the statistical methods can only be made once a sufficiently high number of cases is available, i.e. after the evaluation of the entire validation data set, and therefore both PMs will be applied in the next phase.

Two modifications of the protocol that are supported by the data from phase I were accepted by the Steering Team for the upcoming phase II: 1) omission of the NC since all laboratories found that the SC reflected well the background DNA damage of the FT skin models when using the standard solvents (acetone and 70% EtOH) and 2) an optimized design of the APC experiment. Specifically, a negative finding in the first main experiment is directly followed by an APC experiment. Since APC is intended to amplify DNA migration, provided that DNA damage is present, the absence of DNA migration will be confirmed in an experiment with three dose groups of the test chemical plus APC. This dosing regimen is supported by the fact that the intra-laboratory reproducibility of negative chemicals in phase I was 100%.

3.7. Relevance of the 3D Skin Comet assay for toxicological safety assessment

The 3D Skin Comet assay is one of two recently developed *in vitro* genotoxicity test methods for which standard genotoxic read-out parameters were combined with reconstructed human skin models to address the dermal route of exposure on the *in vitro* level for the first time. The other is the Reconstructed Skin Micronucleus (RSMN) assay [13,49], in which micronuclei are analyzed in EpiDerm™ tissues. Applied in a test battery, they are able to address the different types of DNA damage required to be assessed for regulatory proposes. These characteristics make them ideal tools to follow up on initial positive findings of standard *in vitro* test batteries, which suffer from low predictivity [1].

The intended use of the 3D Skin Comet assay has already been demonstrated in the safety assessment of cosmetic ingredients. For example, Basic Brown 17, a hair dye ingredient, was reported positive in the Bacterial Reverse Mutation Test [50]. This initial finding was followed up by utilizing the 3D Skin Comet assay in addition to *in vitro* mammalian cell gene mutation tests (according to OECD TGs 476, 490) [51,52]. The negative findings in all three assays were accepted in a weight-of-evidence approach by the Scientific Committee on Consumer Safety (SCCS), an independent expert panel of the European Commission [53].

In addition, the SCCS provides guidance on the testing of cosmetic ingredients and revised its guidance for genotoxicity testing to reflect progress made with the characterization and validation of the reconstructed skin model-based assays [5]. The SCCS calls these assays a 'good alternative to bridge the gap between *in vitro* and *in vivo* testing in terms of final hazard assessment' [5] and recommends using both the 3D Skin Comet assay and the RSMN as follow-up on suspected misleading positive results from the *in vitro* standard testing battery.

Meanwhile, the toxicological safety assessments of two additional hair dye ingredients have successfully been supported by negative findings of the 3D Skin Comet assay using the Phenion®FT [54,55].

In summary, the Phenion® Full-Thickness Skin Model has been shown to be suitable for the use in the 3D Skin Comet assay. The assay will be further investigated using an additional set of 22 coded chemicals. Based on observed high intra- and inter-laboratory reproducibility, each chemical will be investigated in one laboratory only. A successful validation is considered key for a wider regulatory acceptance, especially for product categories where dermal exposure is the most relevant route of exposure to be considered for risk assessment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mrgentox.2018.01.003.

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