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The use of Genomic Allergen Rapid Detection (GARD) assays to predict the respiratory and skin sensitising potential of e-liquids

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ABSTRACT

Electronic cigarettes (e-cigarettes) are an increasingly popular alternative to combustible tobacco cigarettes among smokers worldwide. A growing body of research indicates that flavours play a critical role in attracting and retaining smokers into the e-cigarette category, directly contributing to declining smoking rates and tobacco harm reduction. The responsible selection and inclusion levels of flavourings in e-liquids must be guided by toxicological principles. Some flavour ingredients, whether natural extracts or synthetic, are known allergens. In this study, we used the Genomic Allergen Rapid Detection (GARD) testing strategy to predict and compare the respiratory and skin sensitising potential of three experimental and two commercial e-liquids. These novel, myeloid cell-based assays use changes in the transcriptional profiles of genomic biomarkers that are collectively relevant for respiratory and skin sensitisation. Our initial results indicate that the GARD assays were able to differentiate and broadly classify e-liquids based on their sensitisation potential, which are defined mixtures. Further studies need to be conducted to assess whether and how these assays could be used for the screening and toxicological assessment of e-liquids to support product development and commercialisation.

1. Introduction

E-cigarettes have been characterised by Public Health England as being around 95% less harmful than conventional cigarettes (McNeill et al., 2015), with research showing that these devices can assist smokers in replacing conventional cigarettes and reducing their cigarette per day consumption (Brown et al., 2014).

The e-cigarette market is rapidly expanding and, in parallel, the market for e-liquids is growing. Most e-liquids are typically composed of a propylene glycol (PG) and/or vegetable glycerol (VG) base (the aerosol formers) and flavouring substances, with or without nicotine.

A growing body of research shows that flavours play a critical role in attracting and retaining smokers into the vaping category, directly contributing to tobacco harm reduction and declining smoking rates (Farsalinos et al., 2013b; McNeill et al., 2018). A study of 4618 e-cigarette users found that 69% vary their flavours on a daily basis, and that flavours appeared to contribute to perceived pleasure and success in reducing or quitting smoking, while possibly playing a role in reducing relapse into tobacco smoking (Farsalinos et al., 2013b). However, some studies have suggested that flavourings may increase the toxicity of e-liquids (Bahl et al., 2012; Behar et al., 2014; Behar et al., 2017; Farsalinos et al., 2013a; Sherwood and Boitano, 2016). Therefore, the responsible selection and inclusion levels of flavourings in e-liquids must be guided by toxicological principles, including screening and toxicological risk assessment. The screening should cover certain basic criteria. First, it should involve purity requirements, such as pharmaceutical grade for nicotine and humectants and food grade for flavour ingredients. Second, full quantitative disclosure of the individual ingredients should be available, especially for ingredients of natural origin. The compositions of naturals vary depending on their botanical and geographical origins, meteorological conditions, and extraction processes. Third, ingredients that are carcinogenic, mutagenic or toxic to reproduction (CMRs) should be excluded, as mandated by the European Union Tobacco Products Directive (TPD), which is the regulatory framework in the EU that captures e-cigarettes and e-liquids. There is a need for the development of standardised toxicity assessment methods to fill data gaps and add to a weight-of-evidence approach for the risk assessment of ingredients. Methods should be sufficiently fast to support innovation, product development and commercialisation of viable alternatives for adult smokers (Hartung, 2016).

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Some food flavour ingredients, whether natural extracts or synthetic, are known allergens (Scientific Committee on Consumer Safety, 2012). This has led to questions regarding the potential for allergic responses from the use of e-cigarettes and to proposals to restrict the inclusion of allergens in e-liquids (Costigan and Lopez-Belmonte, 2017). Two types of allergy risks are relevant to e-liquid exposure. Respiratory allergy (also referred to as type I immediate IgE-mediated hypersensitivity) is directly relevant as the main intended exposure to e-cigarettes is via inhalation. Skin or contact sensitisation, also known as type IV delayed cell-mediated type hypersensitivity, also needs to be considered in relation to potential accidental and occupational exposures, but also because of evidence that the skin and respiratory sensitisation pathways share similarities.

Currently, there are no specific allergen-related regulatory restrictions for e-liquids under either the TPD in Europe or regulations administered by the Food and Drug Administration in the US. For the purpose of mitigating risks associated with consumer products, e-liquid ingredients should be screened for sensitising effects.

Traditionally, the screening of sensitisers has been performed with animal models. The local lymph node assay is a regulatory accepted method for skin sensitisers (Basketter et al., 2002; OECD, 2010). There is no validated assay for respiratory sensitisers but current approaches include guinea pig testing (Pauluhn et al., 2002), mouse IgE testing (Dearman et al., 1992; Hilton et al., 1996), rat Ig E testing (Arts et al., 1997; Warbrick et al., 2002) and mouse cytokine fingerprinting (Dearman and Kimber, 1999, 2001).

In recent years, the focus has been on the development of animal-free, *in vitro*, *in chemico* or *in silico* predictive models (Reisinger et al., 2015). This is in line with the principles of the 3R's and the 21st Century Toxicology framework, in which animal use should be minimized and mechanistic data should be acquired using relevant *in vitro* test systems (Berg et al., 2011; Rovida et al., 2015; Sheldon & Cohes).

Several skin sensitisation *in vitro* assays have been validated by the European Centre for the Validation of Alternative Methods (ECVAM) and by the OECD for regulatory use. These include the direct peptide reactivity assay (DPRA) (Gerberick et al., 2004; OECD, 2015a), KeratinoSens™ (Natsch, 2010; OECD, 2015b) and the human cell line activation test (h-CLAT) (Ashikaga et al., 2006; OECD, 2017; Sakaguchi et al., 2006). *In vitro* data has so far been shown to correlate well with, and to perform equally well or even better than, animal models (Natsch et al., 2009). Despite sustained efforts to develop *in vitro* assays for respiratory sensitisers, no method has yet proven reliable enough to be used for regulatory purposes (Isola et al., 2008; Kimber et al., 2007).

The Genomic Allergen Rapid Detection (GARD) assays are novel, cell-based assays that utilize the innate recognition of xenobiotic substances by dendritic cells. They are based on the human myeloid cell line SenzaCell and changes in the transcriptional profiles of genomic biomarkers that are collectively relevant for the adverse outcome pathways (AOP) of skin and respiratory sensitisation, referred to as GARD Prediction Signatures (GPS) and GARD Respiratory Prediction Signatures (GRPS), respectively (OECD, 2012). A prediction model is used to classify samples according to their sensitising potential.

The GARDskin™ assay predicts the ability of chemicals to induce skin sensitisation based on a set of 200 genomic biomarkers. When challenged with known sensitisers, the sensitivity, specificity and accuracy of the assay was estimated at 94%, 83% and 90%, respectively (Forreryd et al., 2016). While GARDskin provides binary classifications of chemicals as either sensitisers or non-sensitisers, the GARDpotency™ assay predicts three sensitiser potency classes according to the European Classification, Labelling and Packaging (CLP) Regulation, targeting categories 1A (strong), 1B (weak) and no category (non-sensitiser) (Zeller et al., 2017). In the GARDpotency assay, the expression of 52 genomic biomarkers are monitored. When challenged with 18 independent test compounds, the assay provided accurate results for 78% and was shown to be conservative and only underestimated the class label of one chemical (Zeller et al., 2017).

An expansion of the GARD platform was explored in Forreryd et al., (2015), where a gene expression signature, consisting of 389 biomarkers, was used to predict respiratory sensitisers (Forreryd et al., 2015). When challenged with 10 respiratory sensitisers and 22 non-respiratory sensitisers, the sensitivity, specificity and accuracy of the assay were 67%, 89% and 84%, respectively (Forreryd et al., 2015). While the assay is still under development and refinement of the gene signature is ongoing, it is available for experimental work.

The GARDskin assay is currently under validation by the OECD (Test Guideline Program 4.106) and is currently undergoing the final stages in a formal validation procedure under the supervision of the European Reference Laboratory for alternatives to animal testing (EURL ECVAM).

In this study, we used the GARD testing strategy to predict and compare the respiratory and skin sensitisation potential of three experimental and two commercial e-liquids. To our knowledge this is the first published study assessing e-liquids using these novel *in vitro* assays.

2. Material and methods

2.1. Control and test materials

The chemical identities, input concentrations and expected classifications of the controls used in the three assays are listed in Table 1.

2.2. Cell line maintenance and seeding of cells for exposure

The human myeloid leukaemia-derived cell line, SenzaCell, (ATCC Depository PTA-123875) was maintained at 37 °C and 5% CO_2 in Minimum Essential Medium - alpha modification (α-MEM, Thermo Scientific Hyclone, Logan, UT) supplemented with 20% (v/v) fetal calf serum (Life Technologies, Carlsbad, CA) and 40 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF, Bayer HealthCare Pharmaceuticals, Seattle, WA), as described (Johansson et al., 2011). Proliferating progenitor SenzaCell are used for the assay, with no further differentiation steps applied. The cells were split to fresh media every 3–4 days to a concentration of 200,000 cells/ml. The cell stimulations were performed in 12 or 24 well plates at a final cell concentration of 200,000 cells/ml, as described earlier (Johansson et al., 2011).

2.3. Phenotypic analysis

Prior to any chemical exposure, a qualitative phenotypic analysis was performed to ensure that proliferating cells were in an immature stage (Johansson et al., 2013; Johansson et al., 2011).

All cell surface staining and washing steps were performed in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA). Cells were incubated with specific mouse monoclonal antibodies (mAbs) for 15 min at 4 °C. The following mAbs were used for flow cytometry: fluorescein isothiocyanate (FITC) conjugated CD1a (DakoCytomation, Glostrup, Denmark), CD34, CD86, and HLA-DR (BD Biosciences, San Diego, CA), phycoerythrin (PE) conjugated CD14 (DakoCytomation), CD54 and CD80 (BD Biosciences). Mouse IgG1, conjugated to FITC or PE were used as isotype controls (BD Biosciences) and propidium iodide (PI) (BD Biosciences) was used to assess cell viability. BD FACSsuit software was used for data acquisition with FACSVerse instrument (BD Bioscience). 10,000 events were acquired, gates were set based on light scatter properties to exclude debris and non-viable cells, and quadrants were set according to the signals from isotype controls. Further data analysis was performed, using FlowJo (TreeStar Inc. Ashland OR, US).

Accepted ranges of listed phenotypic markers are listed in Table 2.

Table 1
Chemical identities, input concentrations and expected classifications for each endpoint of control substances (Johansson et al., 2013; Forreryd et al., 2015; Zeller et al., 2017).

Control substance	GARD input concentration	Expected Respiratory assay classification	Expected GARDskin assay classification	Expected GARDpotency assay classification
p-phenylendiamine	75 µM	Non-sensitiser	Sensitiser	1A
2,4-dinitrochlorobenzene	4 µM	Non-sensitiser	Sensitiser	1A
2-hydroxyethylacrylate	100 µM	Non-sensitiser	Sensitiser	1A
2-aminophenol	100 µM	Non-sensitiser	Sensitiser	1A
2-nitro-1,4-phenylendiamine	300 µM	Non-sensitiser	Sensitiser	1A
Resorcinol	500 µM	Non-sensitiser	Sensitiser	1B
Geraniol	500 µM	Non-sensitiser	Sensitiser	1B
Hexyl cinnamic aldehyde	80 µM	Non-sensitiser	Sensitiser	1B
DMSO	0.1%	Non-sensitiser	Non-sensitiser	No Cat
Chlorobenzene	500 µM	Non-sensitiser	Non-sensitiser	No Cat
1-butanol	500 µM	Non-sensitiser	Non-sensitiser	No Cat
Chloramine T	500 µM	Sensitiser	Non-sensitiser	–
Glutaraldehyde	10 µM	Sensitiser	Non-sensitiser	–
Hexamethylen diisocyanate	120 µM	Sensitiser	Non-sensitiser	–
Isophorone diisocyanate	30 µM	Sensitiser	Non-sensitiser	–
Reactive orange	150 µM	Sensitiser	Non-sensitiser	–
Toluen diisocyanate	100 µM	Sensitiser	Non-sensitiser	–
Trimellitic anhydride	500 µM	Sensitiser	Non-sensitiser	–

The test materials were three experimental base liquids (BL) and two commercial, flavoured e-liquids (CF) obtained from the UK market in March 2017 (CF Blu Cherry 1.6% and CF 1.2%). Their contents were analysed by GC-MS (n = 2) and are reported in Table 2.

Table 2
Phenotypic analysis: accepted ranges of proportion of positive cells for acceptance criteria.

Phenotypic biomarker	Accepted range of positive cells (%)
CD86	10–40
CD54	> 95
HLA-DR	> 60
CD80	< 10
CD34	35–70
CD14	5–50
CD1a	10–60
Propidium Iodide	< 15

2.4. Assessment of cytotoxicity and cell exposure

As some test materials might have a toxic effect on the cells, GARD input concentrations were determined as follows. Pure substance were titrated to concentrations ranging from 1 µM to the maximum soluble concentration in cell media or to 500 µM. Following incubation for 24 h at 37 °C and 5% CO_2, harvested cells were stained with PI (BD Biosciences) and analysed with a flow cytometer. PI-negative cells were defined as viable, and the relative viability of exposed cells compared to non-exposed cells at each concentration in the titration range was determined. The concentration yielding 90% relative viability (Rv90) was considered to demonstrate bioavailability of the compound used for exposure, while not impairing immunological responses. As the test materials were mixtures and do not have one molecular weight, the substance with Rv90 at lowest concentration was directing for all other test substances, i.e. all substances were run at the same concentration. By this procedure, false positive signals from base ingredients are circumvented. Hence, the results correspond to relative sensitisation potencies.

Cells were exposed to the control and test materials at their respective GARD input concentrations for 24 h at 37 °C and 5% CO_2. All assessments of control and test substances were assayed in biological triplicates, performed at different time-points and using different cell cultures. Following incubation, cells from one well were lysed in TRIzol reagent (Life Technologies) and stored at −20 °C until RNA extraction. In parallel, a small sample of exposed cells was taken for PI staining and analysis with flow cytometry, to ensure the expected relative viability of exposed cells has been reached.

2.5. RNA isolation, Nanostring, cDNA preparation and hybridization to microarrays

RNA isolation from lysed cells was performed using spin columns as per supplier's instructions (Direct-Zol RNA MiniPrep, Zymo Research, Irvine, CA). Total RNA was quantified and quality controlled using BioAnalyzer equipment (Agilent, Santa Clara, CA) and NanoDrop (ThermoFisher Scientific).

For the GARDskin assay, the Nanostring technology was used according to the manufactrer's instructions (Nanostring, Seattle, WA). In brief, 100 ng RNA was hybridized to the GPS code set and prepared on a chip using the nCounter prepstation. The individual transcripts in the GPS were quantified using a Nanostring Digital Analyzer.

A minimum of 300 ng total RNA was required to perform preparation of cDNA. The preparation of labeled sense DNA was performed according to Affymetrix GeneChip™

Whole transcript (WT) sense target labeling assay (100 ng Total RNA labeling protocol), using the recommended kits and controls (Affymetrix, Santa Clara, CA). Hybridization, washing and scanning of the Human Gene 1.0 ST arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA).

2.6. Data analyses

All further downstream analyses were performed in the statistical environment R, an open source software environment for statistical computing and graphics (R Foundation for Statistical Computing).

For assessment of skin and respiratory sensitisation, a Support Vector Machine (SVM) was modelled on a training data set corresponding to samples used for assay development (Forreryd et al., 2015). Batch variations between the training data set and the test data set were eliminated using the BARA-method (SenzaGen unpublished data), using unstimulated cells as a reference control. Each sample in the test set were assigned a decision value (DV), based on its transcriptional levels of the GPS or GRPS biomarker signature. A positive average DV (n = 3) means a sample is a sensitiser in the assay, and a negative average DV (n = 3) means a sample is a non-sensitiser.

For assessment of skin sensitising potency, a Random Forest (RF) was modelled on a training data set corresponding to samples used for assay development (Zeller et al., 2017). Each sample in the test set were assigned a probability value (PV) for each of the CLP potency categories

(1A, 1B, no cat), based on its transcriptional levels of the GPS biomarker signature. Classifications of each test substance into CLP potency were performed by a majority vote (n = 3).

2.7. Data visualization

To create a comprehensible visualization of the generated PVs in the potency classification, the numbers were recalculated to fit a regular x-y- plot according to the following equations:

$$if\ prediction = 1A\text{: } 1 + +p(1A) - \frac{p(1B) + p(No\ Cat)}{2}(Ranges\ between\ 1\&2) \quad (a)$$

$$if\ prediction = 1B\text{: } 0.5 + p(1A) - p(No\ Cat)(Ranges\ between\ 0\&1) \quad (b)$$

$$if\ prediction = 1B\text{: } 0.5 + p(1A) - p(No\ Cat)\ (Ranges\ between\ 0\ \&\ 1) \quad (c)$$

3. Results

Prior to chemical challenge, cells were quality controlled by measuring the cellular expression of common myeloid and dendritic cell markers using flow cytometry, as described in Materials and Methods. Results correlated with previously published phenotypic profiles, ensuring that cells were successfully maintained in an immature state (Johansson et al., 2011), Supplementary Table 1. During one of the cell stimulations the CD86 signal was too high. This is an unrefined marker for activation and to ensure further activation by the test substances the stimulated cells were checked to have a higher CD86 level. Further, it has been noted that a high CD86 level before stimulation do not influence the final prediction (unpublished data).

GARD input concentrations for each test substance were established by cytotoxicity screening as described in Material and Methods. Results from this screening and resulting GARD input concentrations are presented in Table 3.

As expected, the cytotoxicity of the base liquid increased with the addition of nicotine, as indicated by decreases in Rv90. At equivalent nicotine concentrations, commercial e-liquids containing other substances, including flavourings, were more cytotoxic than base liquids made exclusively of humectants and nicotine (see Table 4 for test material compositions), as determined by Rv90.

To ensure an appropriate comparison of the mixtures and avoid false positives from base ingredients, all test substances were assessed at the same concentration as the most cytotoxic test substance (Rv90 at lowest concentration), i.e., at 0.55% (v/v).

Binary predictions of respiratory sensitising potential

All replicates of test substances and controls were assigned DVs using the respiratory sensitisation prediction model. A graphical representation based on DVs from controls and test substances is presented in Fig. 1.

Out of the 7 assayed respiratory sensitisers, 5 were accurately classified as such. No false positives were generated (Fig. 1A). Chloramine T and Trimellitic anhydride were incorrectly classified as non-respiratory sensitisers. Thus, the sensitivity and specificity were estimated to 71% (5/7) and 100% (12/12), respectively, with an overall predictive accuracy of 89% (17/19).

None of the base liquids nor commercial e-liquids were classified as respiratory sensitisers in the assay (Fig. 1B). Considering the relative level introduced by the study design the values indicate the same result.

Table 3
Determination of GARD input concentrations for test substances.

Test substance	Max. screen[a]	Rv90[b]	GARD input concentration[c]
BL 0%	5	3.5	0.55
BL 1.6%	5	2.5	0.55
BL 4.5%	5	1.125	0.55
CF Blu Cherry 1.6%	5	0.55	0.55
CF 1.2%	5	1.125	0.55

[a] The highest concentration used in screening titration range. Concentration is given in % (v/v).
[b] Concentration yielding 90% relative viability. Concentration is given in % (v/v).
[c] Based on Max. screen and Rv90. Concentration is given in % (v/v).

Table 4
Composition of test materials. PG: Propylene glycol; VG: Vegetable glycerol; BL: base liquid; CF: commercially available flavoured e-liquid.

Test material	Content [w/w %]			
	PG	VG	Nicotine	Other substances (e.g. water, flavourings)
BL 0%[a]	50	50	–	–
BL 1.6%[a]	49.2	49.2	1.6	–
BL 4.5%[a]	47.75	47.75	4.5	–
CF Blu Cherry 1.6%[a]	40.4	53.4	1.6	4.6
CF 1.2%[a]	66.5	18.8	1.1	13.6

[a] % refer to % nicotine content.

GARDskin binary predictions of skin sensitising potential

All replicates of test substances and controls were assigned DVs using the GARDskin prediction model. A graphical representation based on DVs from controls and test substances is presented in Fig. 2.

The negative and positive controls of relevance for binary categorization were accurately classified, as compared to the expected outcome.

Non-flavoured BLs, whether or not they contained nicotine, were classified as non-sensitisers. The DVs for two out of the three replicates of each commercial, flavoured e-liquid indicated a sensitisation potential, thereby classifying the CFs as potential skin sensitisers in the binary prediction. Considering the relative level introduced by the study design the values indicate the same result.

GARDpotency categorical ranking of skin sensitising potency

All replicates of test substances and controls were assigned PVs corresponding to the CLP potency categories using the GARDpotency prediction model. A graphical representation based on PVs and equation *a*, *b* and *c* from benchmark controls and test substances is presented in Fig. 3.

All 12 controls of relevance for potency categorization were accurately classified as compared to the expected outcome (Fig. 3A).

Non-flavoured BLs, whether or not they contained nicotine, were classified as non-sensitisers (no category). The PVs for two out of the three replicates of each commercial, flavoured e-liquid indicated a sensitisation potential, thereby classifying them as potential skin sensitisers Category 1 B (Fig. 3B). Considering the relative level introduced by the study design the values indicate the same result.

Table 5 summarizes the test substance classifications obtained with the GARD assay for respiratory sensitisers, GARDskin and GARDpotency assays.

4. Discussion

In this study, we used the GARD testing strategy, including the GARD assay for respiratory sensitisers, GARDskin and GARDpotency assays, to predict and compare the sensitisation potential of three

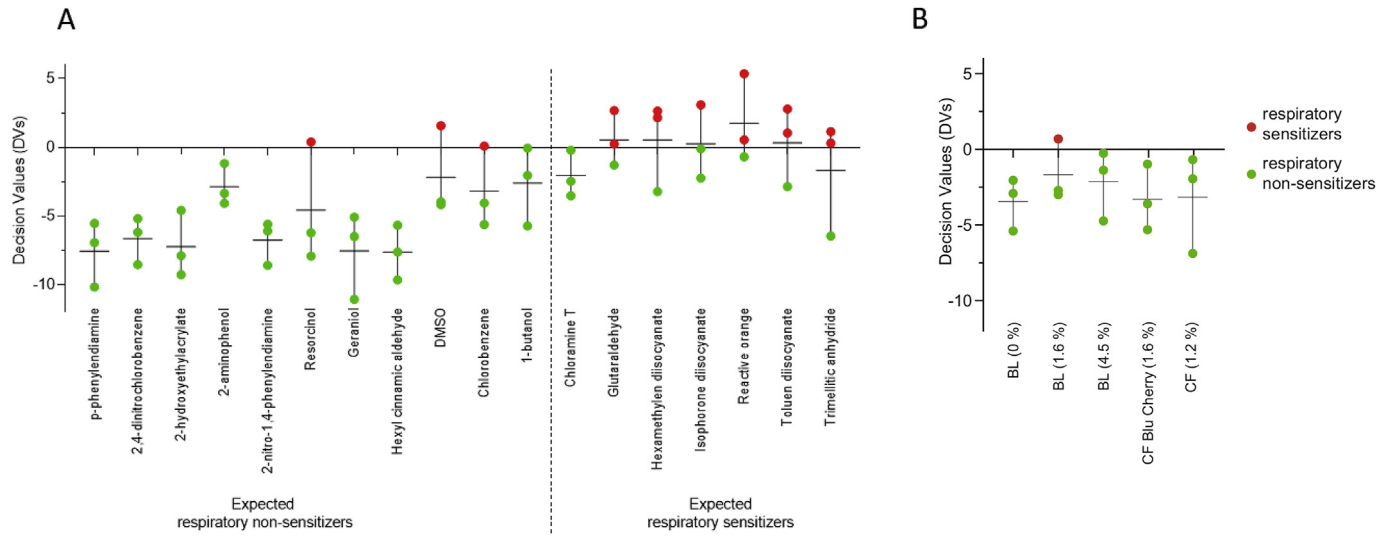

Fig. 1. Decision values for biological triplicate samples of control (A) and test (B) substances in the GARD assay for respiratory sensitisers. The chemical identities, input concentrations and expected classifications of the controls are listed in Table 1. A positive average DV means a sample is a sensitiser in the assay, and a negative average DV means a sample is a non-sensitiser.

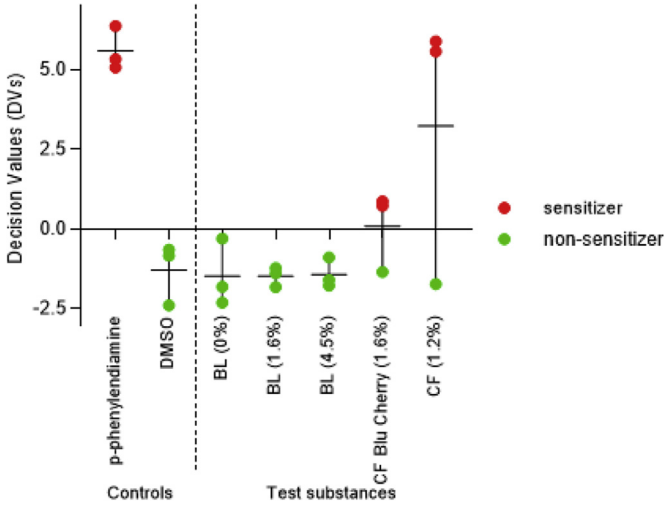

Fig. 2. GARDskin decision values for biological triplicate samples of control and test substances. DMSO 0.1% is the negative control and p-phenylendiamine (PPD) 75 µM the positive control. A positive average DV means a sample is a sensitiser in the assay, and a negative average DV means a sample is a non-sensitiser.

experimental and two commercial e-liquids. To our knowledge this is the first application of these *in vitro* assays to e-liquid research. The GARD assays were originally developed to assess the sensitisation potential of pure chemicals. Our initial results indicate that these *in vitro* assays can effectively be used to differentiate and broadly classify e-liquids, which are defined mixtures.

At the outset, the three experimental and two commercial e-liquids were screened for their cytotoxicity in the PI cell viability test in order to derive input concentrations. Both the presence of nicotine and the presence of other substances, including flavourings, increased the cytotoxicity of the e-liquids in this assay. Nicotine is a known toxicant and has been shown to be cytotoxic in several *in vitro* assays (Gonzalez-Suarez et al., 2017). A variety of published work has been conducted on e-liquids with cytotoxicity as an endpoint, with variable results (Bahl et al., 2012; Behar et al., 2014; Behar et al., 2017; Farsalinos et al., 2013b; Sherwood and Boitano, 2016). Some studies found that e-liquids and their aerosols are not cytotoxic or have relatively low cytotoxicity. Other studies found some cytotoxic effects of e-liquids with significant variability depending on the cell system used and reported cytotoxic effects. These effects were possibly related to the presence of flavourings in some studies. However, *in vitro* studies comparing the cytotoxicity of e-liquid aerosols, with and without flavourings, with the one of combustible cigarette smoke, have consistently shown e-liquids to be less cytotoxic than cigarette smoke (Cervellati et al., 2014; Scheffler et al., 2015). For a fair comparison of the mixtures, all test substances were assessed at the same concentration as the most cytotoxic test substance. At this use level, the toxicity of the test materials did not hamper the use of the GARD assays.

Base liquids, whether they contained nicotine or not, were classified as non-sensitisers in all three GARD assays. This finding is in agreement with the known properties of the BL components. None of the BL ingredients, PG, VG and nicotine, have been reported as respiratory sensitisers. Nicotine, while toxic in high doses in humans, does not seem to cause skin sensitisation (Bonamonte et al., 2016). The scientific evidence on glycerol shows that it is not a skin sensitiser (OECD, 2002). The evidence on propylene glycol indicates it is a very weak to weak contact sensitiser, if at all (Jacob et al., 2017; Lessmann et al., 2005). In our study, at the concentrations tested, PG did not display sensitising properties as assessed by the GARD assays.

The two commercial e-liquids were not classified as respiratory sensitisers in the GARD respiratory assay. As the main intended route of exposure to e-liquids is via inhalation of their aerosols, respiratory allergenicity is an important toxicological endpoint. Chemical respiratory allergy is much less common than contact sensitisation, however, the potential adverse effects are much more severe. Owing to the uncertainties involved in potency determination and the derivation of a tolerable level for respiratory sensitisation, it has been recommended that the use of known respiratory sensitisers should be avoided completely in e-liquids (Costigan and Lopez-Belmonte, 2017; Costigan and Meredith, 2015). Indeed all novel e-liquid ingredients should be screened for respiratory sensitising activities based on published literature and *in silico* techniques and exclude those for use in e-liquids. A prediction of respiratory sensitisation remains a significant challenge due to a limited understanding of the underlying biological mechanisms. It is therefore critical that assays like the GARD assay for respiratory sensitisers are further developed and validated to support consumer product development, including the development of e-liquids.

In the GARDskin and GARDpotency assays, the two commercial e-liquids were classified as potential skin sensitisers and Cat 1 B weak

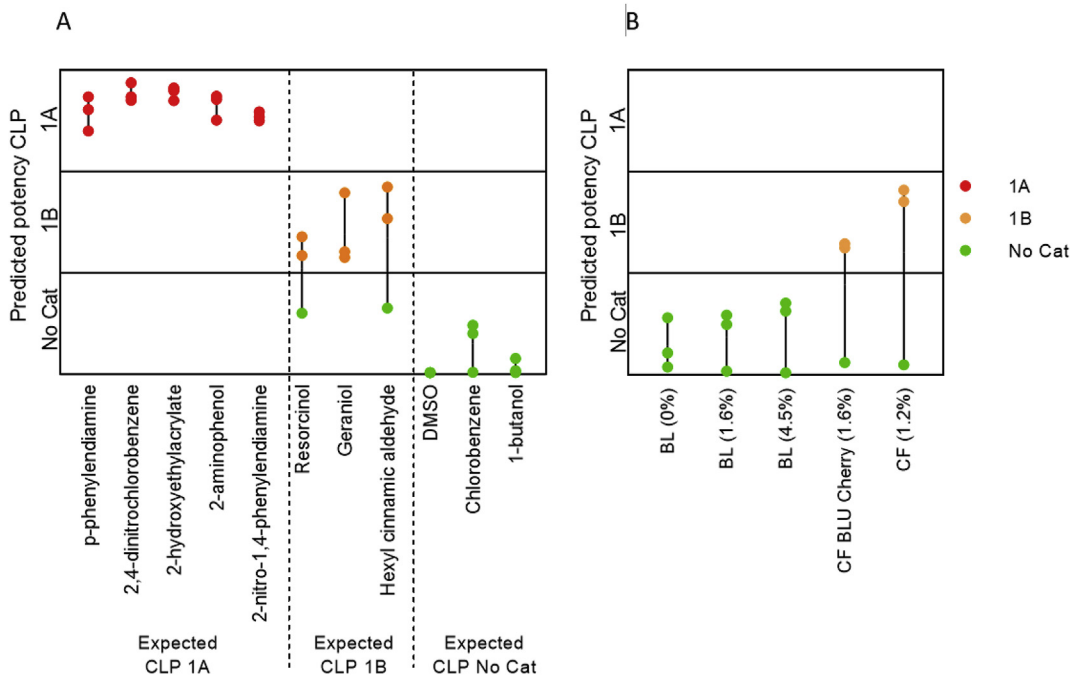

Fig. 3. GARDpotency predictions for biological triplicate samples of control (A) and test (B) substances. The chemical identities, input concentrations and expected classifications of the controls are listed in Table 1. The figure presented is generated by incorporation of the PVs incorporated in to equation *a, b* and *c*. The potency CLP prediction is based on a majority vote of the triplicates classifying the samples into CLP sensitiser potency classes 1A (strong), 1B (weak) and No Cat (non-sensitiser).

Table 5
Test substance classification with the GARD assay for respiratory sensitisers, GARDskin and GARDpotency assays.

	Respiratory assay	GARDskin	GARDpotency
BL 0%	Non-Sensitiser	Non-Sensitiser	No Cat
BL 1.6%	Non-Sensitiser	Non-Sensitiser	No Cat
BL 4.5%	Non-Sensitiser	Non-Sensitiser	No Cat
CF Blu Cherry 1.6%	Non-Sensitiser	Sensitiser	1 B
CF 1.2%	Non-Sensitiser	Sensitiser	1 B

sensitisers, respectively, as the DVs for two out of the three replicates of each CF indicated a sensitisation potential. These findings warrant further analysis of the individual e-liquid components to assess the risks associated with single ingredients and whether the level of any sensitiser is sufficiently low that it is not expected to elicit reactions, even in pre-sensitised individuals.

Skin sensitisation is firstly relevant to accidental and occupational exposures to e-liquids. In the EU, product standards set out in the EU Tobacco Products Directive aim at minimising the risk of accidental dermal exposure to e-liquids and, hence, the risk of exposure to potential skin irritants and sensitisers. The Directive sets forth that e-cigarettes and refill containers should be child- and tamper-proof, be protected against breakage and leakage and have a mechanism that ensures refilling without leakage (European Parliament and Council, 2014).

It is an interesting finding that the commercial e-liquids are predicted to be skin sensitizers, but not respiratory sensitizers. This may reflect that even though the signalling pathways share similarities, there are evidence that they are not precisely the same. For instance, it is known that although respiratory sensitizers can be absorbed through skin, they specifically induce a Th2-type response opposite to skin sensitizers that induce a Th1-type response (Kimber et al. 2011). Relevant approaches to manage any identified skin sensitisation risk need to be devised. The usual approach to protecting consumers is to define levels above which consumers should be informed of the presence of known sensitisers, so they may decide whether to use the product if they know of, or suspect, a sensitivity. This approach is analogous to that taken in the EU Cosmetics Directive 76/768/EEC and in the REACH CLP legislation. For e-liquids, it has been proposed to limit the use of allergens depending on their strength, with a tolerable no effect level of 1000 ppm in e-liquids, below which the chance of induction of contact sensitisation and eliciting effects in pre-sensitised people is considered tolerable. It has also been proposed that known contact sensitisers should be mentioned in the product information if present at levels above 0.1% in the e-liquid (Costigan and Lopez-Belmonte, 2017; Costigan and Meredith, 2015).

Chemically induced allergic reactions are complex biological processes that cannot be evaluated accurately using single events or biomarkers. These allergic reactions develop in two basic phases: sensitisation and elicitation of a reaction (Kimber et al., 2002). Induction of Sensitisation primes an individual's immune system to a specific substance by inducing immunological memory to an allergen. After induction, further exposure can elicit the classic inflammatory reaction associated with contact dermatitis. Both induction of sensitisation and elicitation are threshold mechanisms. The threshold for elicitation, however, is typically lower than that for induction. Validated *in vitro* sensitisation tests are based on single or few biomarkers and cover specific key events within the skin sensitisation AOP. None of the currently validated non-animal methods can account for the whole series of events and, from a regulatory perspective, tests should always be considered in combinations and/or with other information, and should be integrated in a weight-of-evidence approach (European Chemicals Agency, 2018). The high informational content of the GARD assays aims at better capturing the complexity of the AOP (Forreryd et al., 2016; Malmborg and Borrebaeck, 2017). However, they are still focused on a single key event, key event 3 of the skin sensitisation AOP, dendritic cell activation (Zeller et al., 2017). Hence, additional assays should be used to complete the weight-of-evidence assessment of the skin sensitisation potency of the e-liquids.

This preliminary study had several limitations. We tested only two commercial e-liquids. E-liquids are a highly heterogeneous product

category and the feasibility of using the GARD assays with a whole range of diverse e-liquids should be further assessed.

Future work should focus on the elucidation of the potential role of specific flavours and other components in the commercial samples which appear to be contributing to the sensitising potential of the e-liquids. E-liquids are highly complex mixtures, in particular if they include natural flavourings.

In order to formulate an efficient *in vitro* sensitisation testing strategy for e-liquids and their flavourings, the relevance of testing e-liquid formulations or their in-going ingredients also needs to be assessed. Sensitisation tends to be compound-specific. However, mixture effects and cross-reactivity between similar molecules cannot be excluded. Quantitative evidence for levels of cross-reactivity exists for only a limited number of compounds and is not currently available for the bulk of flavours used in e-liquids.

From a research and product development perspective, the GARD assays are interesting because they are being developed with a view to meet industrial and commercial demands for reliability, cost effectiveness and sample capacity. The assays are simple to perform, with a majority of the laboratory steps being conducted according to standardised protocols provided by platform suppliers, thus constituting an attractive replacement for animal tests.

5. Conclusion

The market for e-liquids is rapidly expanding and there is a pressing need for the development of standardised toxicity assessment methods to support product development and commercialisation of viable alternatives to tobacco products for adult smokers. Our initial results indicate that the GARD testing strategy, including the GARD assay for respiratory sensitisers, GARDskin and GARDpotency assays, were able to differentiate and broadly classify e-liquids based on their sensitising potentials. Further studies need to be conducted to assess whether these *in vitro* assays could be part of a broader assessment framework for the screening and toxicity assessment of e-liquids.

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Appendix A. Supplementary data

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Transparency document

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