RISK ASSESSMENT OF PRE- (& PRO-HAPTENS)
PROGRESS ON EXPOSURE INFORMATION and NEXT STEPS

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Dr. Matthias Vey
IDEA Management Team
On behalf of the IDEA Hydroperoxides Task Force

Background and Problem Definition

- 4 IDEA Workshops organized so far on pre- and pro-hapten risk assessment
- Aimed to further drive mechanistic understanding of pre- and pro-hapten formation, develop a framework for risk assessment and to bridge findings with clinical data.
- Pro-hapten formation regarded of lower priority as largely covered by existing risk assessment involving in vivo data. However, understanding that with regard to non-animal based approaches, metabolizing systems are often missing at this moment in time (and should be considered?).
- Focus on Hydroperoxides (HP) of widely used terpenes (**Limonene and Linalool**) as important pre-haptens, due to frequent reporting of positive patch test reactions to oxidized terpene fractions, containing these HP's.

Background and Problem Definition

HP are not intentionally added to products, but

- they could be added as impurities from raw materials
- they may form in products if sufficient oxygen is present

Unclear exposure source(s) for induction of HP contact allergy as

- there is very little exact data on HP levels in raw materials and even less for consumer products
- there is no evident pattern of concerned products and missing information on influencing factors like for example product age

Analytical methods for HPs were required

- able to reliably detect them in consumer products
- to allow a thorough market surveillance to better understand the exposure and the role they may play with regard to the clinical observations.

IDEA Activities

- IDEA Hydroperoxides TF (HPTF) established
  - Tasked to develop method(s) for determination and quantification of HPs of Linalool and Limonene in various matrices
  - Resulting exposure information expected to help in interpretation of clinical data.
  - Members of the HPTF: Chanel, DMS, Firmenich, Givaudan, IFF, Universities (of Gothenburg, Lille, Liverpool, Stockholm, Strasbourg), Wala External laboratory (third party): Solvias
- TF looked into various methods
- Methods compared and validated in various comparisons of blind coded samples

IDEA TF: Targets set for analytical method

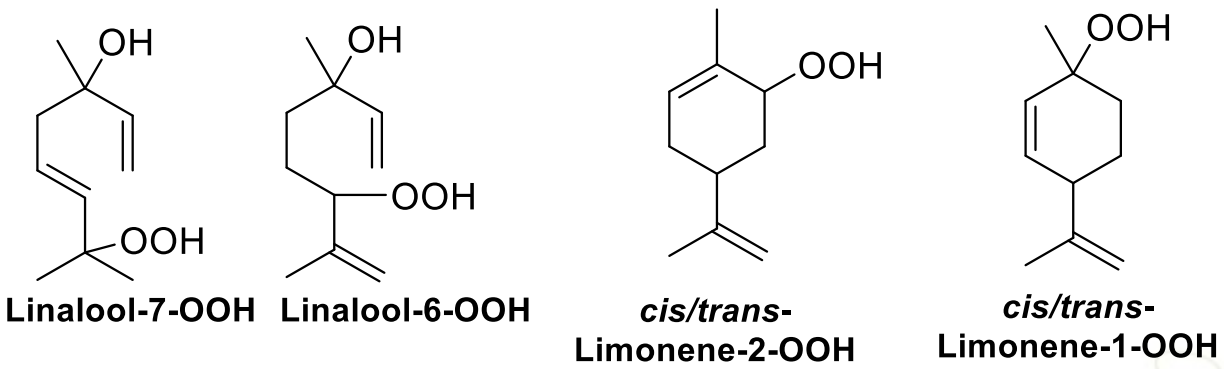
- **Initial analytical target agreed:**
  "Methods should be sensitive, specific, with target limits of quantification (LOQ) below the estimated induction levels and limits of detection (LOD) below the estimated elicitation levels"
  Estimated induction levels:
  - **5000 ppm** taken as a default induction level (based on LLNA EC3 on multiple hydroperoxides)
  - Linalool: Up to now lowest elicitation level in humans: **560 ppm** (based on one published ROAT of limited size)
- **Revised analytical target** – based on improved analytical methods:

<u>50 ppm in final consumer product</u> (defined as 'reporting level')

- This is 100 fold below default induction level
- 10-fold below reported tentative elicitation level
- Note: This lower level is set based on **analytical feasibility**: it does not mean that all levels above 50 ppm are of toxicological concern!

IDEA TF: Selection of Reference Materials

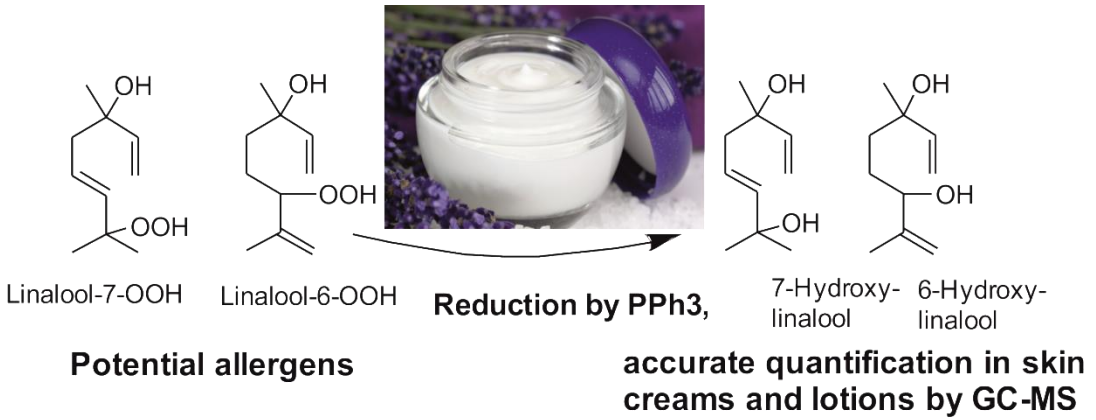
- First study was run with analytical standards containing **mixtures** of hydroperoxides, **not completely purified**
- Key to improve methods: **Highly pure reference standards**
- Substantial investment to have external company prepare and provide 4 highly pure standards
- **These standards served to:**
  - ➢ Prepare exact spiked samples in subsequent ring tests
  - ➢ Calibrate analytical methods



Linalool-7-OOH **Linalool-6-OOH** ***cis/trans*-Limonene-2-OOH** ***cis/trans*-Limonene-1-OOH**

GC-MS-reduction method

- GC-MS-reduction method: HP are reduced to corresponding alcohols
- Alcohols are very stable analytes, which can be analyzed by conventional GC-MS methods



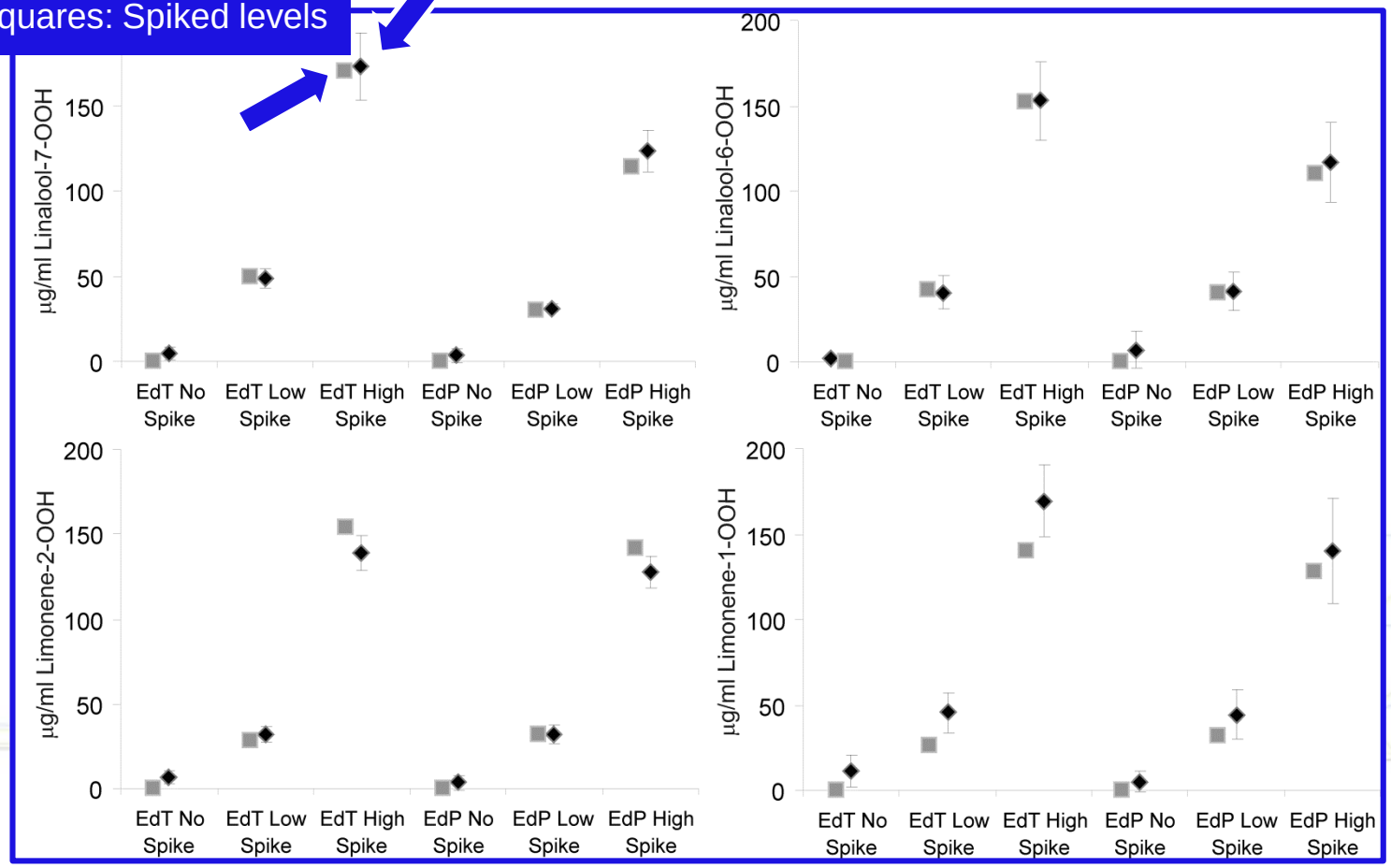
- This method is **very sensitive but** conservative, overestimation possible if alcohol is in product
- Method proven to be highly reproducible by **blind-coded** multi-laboratory trials

Ring study: Method validation in fine fragrances

- Five labs tested **blind-coded** samples
- Eau de Toilette and Eau de parfum spiked with 4 HP at different levels
- Accurate detection with GC-MS reduction by all five labs
- **This method allows accurate quantification in commercial fragrances**

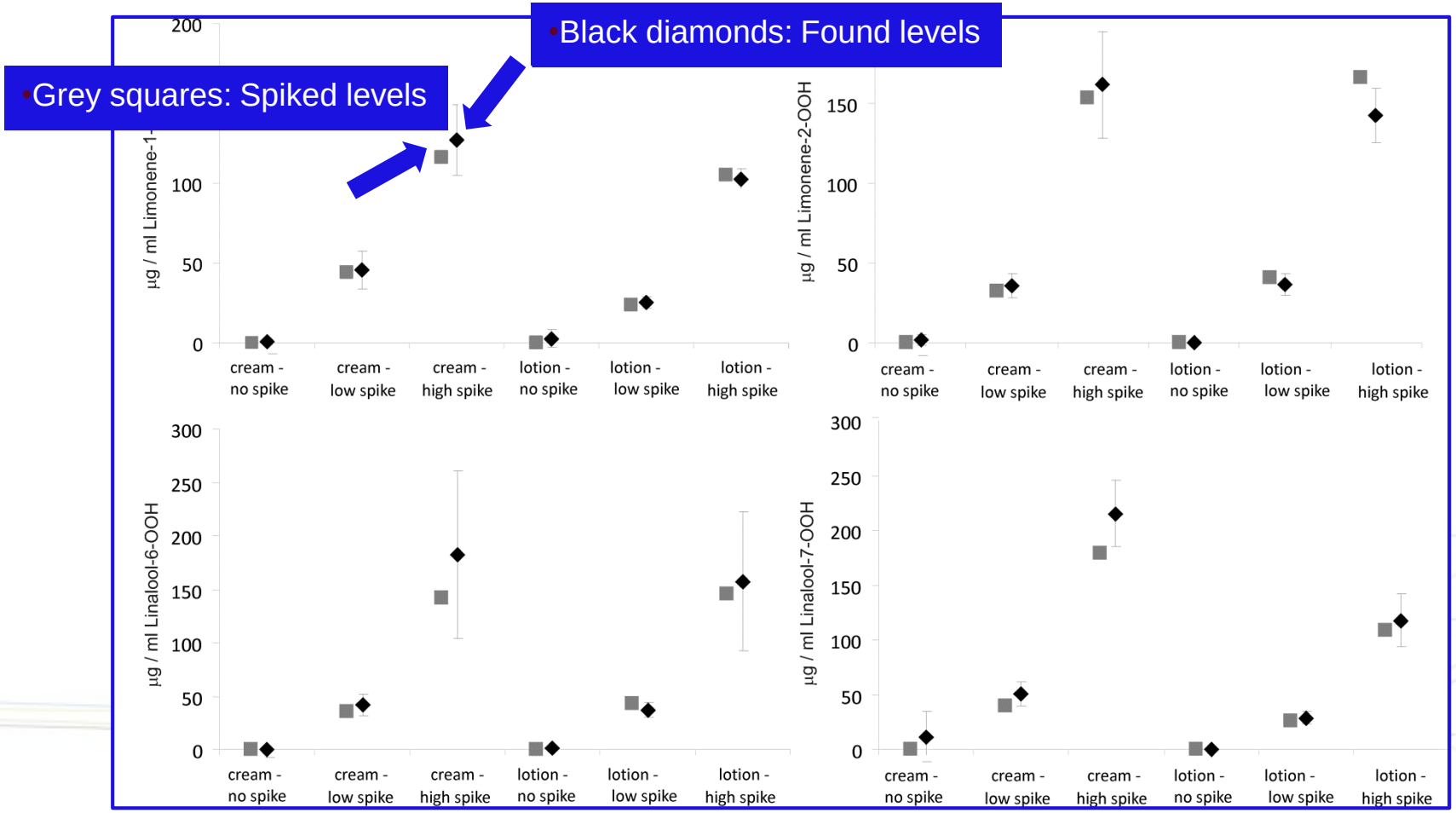
•Black diamonds: Found levels

•Grey squares: Spiked levels



Ring study II: Method validation in creams / lotions

- Five labs tested **blind-coded** samples
- Cream and lotion spiked with 4 HP at different levels
- Accurate detection with GC-MS reduction by all five labs
- **This method allows accurate quantification in complex cosmetic products**



LC-based methods

- LC-method enables to directly detect parent HP
- LC-methods are **more specific** for the hydroperoxides but more prone to matrix interaction
- Three LC-Methods were further validated as confirmatory methods
- Example of results in Eau de Toilette (EdT) and Eau de Parfum (EdP):

	EdT No Spike	EdT Low Spike	EdT High Spike	EdP No Spike	EdP Low Spike	EdP High Spike
LC-Q-TOF MS	nd	90.0	279.0	nd	59.0	200.0
HPLC-CL	nd	79.5	310.7	nd	56.2	203.7
LC-orbitrap-MS	0.2	95.7	398.7	nd	29.1	185.4
spike level added	0.0	92.0	322.0	0.0	70.0	224.0

nd = not detected

Method selection criteria

- GC-MS reduction method provided robust and sensitive and is best for sample screening
- LC-methods, which are more specific for the hydroperoxides, to be used for confirmatory analysis

- Confirmatory analysis recommended for
  1. Samples above reporting level by reduction method, as method may be oversensitive
  2. Confirmatory analysis of suspect samples (e.g. from patients)

Market overview – sample selection

- Samples from consumer homes, which are partly used
- Products should have declared Linalool and Limonene content and batch number /production code / date (to ensure traceability)
- For each aged product we searched for a matched fresh product
  - 31 different products (31 fresh and 31 aged, partly used)
  - Fine fragrances, deodorants, creams, lotions
- Samples from patients, collected by Spanish dermatological network

  - Mainly from patch test positive patients

  - If possible, samples also matched with fresh products

  - 28 samples; 11 samples from patients patch test positive to oxidized Linalool and / or oxidized Limonene
- Specific products with controlled aging

Results aged versus new samples

- 31 products which could be matched with fresh products (62 samples, analyzed for 4 different HPs)
- Only one sample above reporting limit:
  91 ppm of Limonene-1-OH by GC-MS reduction method
- Presence of Limonene-1-OOH verified in this sample by three LC-based methods
- 33% of the analyzed samples contained > 1000 ppm of parent Linalool or Limonene, no indication for oxidative degradation of parent HP
- **No evidence for HP accumulation in aged samples**

Results products from patients

- 28 products obtained from patients over Spanish dermatological network, suspected for being causative of skin reactions
- 11 of these samples from patients which were patch test positive to oxidized Linalool or Limonene
- None of these samples contained above 50 ppm by GC-MS method, confirmed by the three LC-MS methods
- Induction in these patients cannot be explained by HP level in the sampled, suspected products (based on todays knowledge)

Example of a patient product

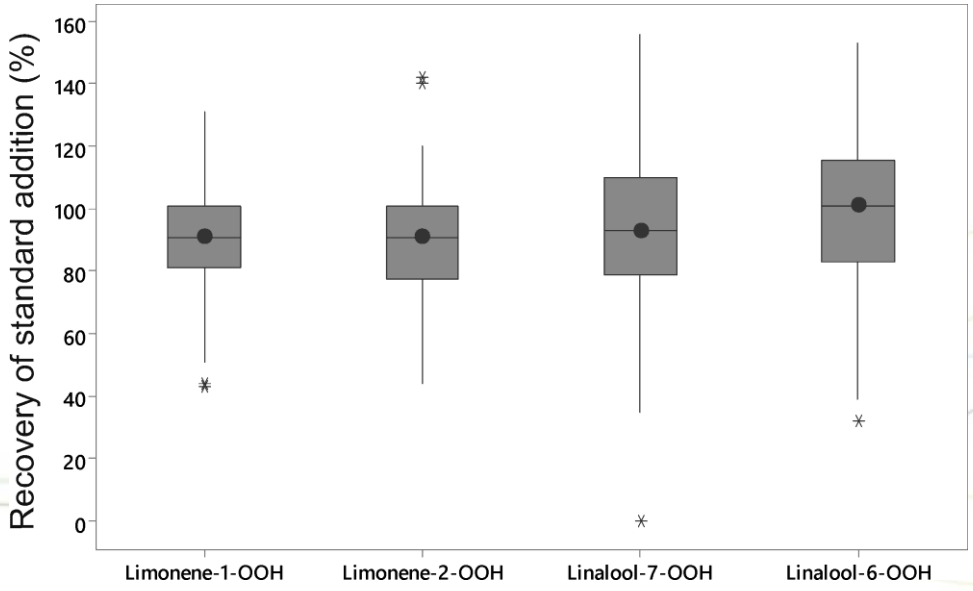
Sample and history of donating patient	Analytical methods	Limonene-1-OOH	Limonene-2-OOH	Linalool-7-OOH	Linalool-6-OOH
O12, Body cream, Positive some fragrances, Positive Limonene ox	GC-MS red. (µg/ml)	<22	<22	<22	<22
	GC-MS red. (% recovery)	69%	70%	59%	84%
	LC-Orbitrap-MS (µg/ml)	NF	nr	NF	NF
	LC-Q-ToF-MS (µg/ml)	<5	<5	<5	11
	LC-CL (µg/ml)	NF	NF	NF	NF

NF: Not found

⇐ Reduction method
⇐ Spike recovery
⇐ LC-MS method 1
⇐ LC-MS method 2
⇐ Chemilum. method

Repeat analysis: standard addition

- In this work we report many negative results: The vast majority of samples do not contain hydroperoxides
- It is very important to validate these results to increase confidence that we can analyze the HP in these very different products.
- Thus each sample was analyzed in duplicate – once spiked with all four synthetic hydroperoxides at the reporting level (50 ppm)
- Spike could always be positively detected (one exception in 416 single determinations)
- Spike recovery in general > 70%, and close to 100% on average



Recovery of standard addition (50μg/g) of four HP added to 104 products analysed by the GC-MS-reduction method.

Interpretation – Sensitivity and detected levels vs. toxicological / clinical data

Dose per area calculations for limonene-1-OOH

	Dose of hydroperoxide in test preparation	Dose per area
LLNA Dose inducing sensitisation (EC3)	3300 µg/g (0.33%)	82.5 µg/cm^2
Patch test limonene-HPs *, **routine diagnostic level**	3300 µg/g (0.33%)	132 µg/cm^2
Patch test limonene-1-OOH **, diagnostic level	5000 µg/g (0.5%)	200 µg/cm^2
Defined reporting limit	50 µg/g	0.1 – 0.5*** µg/cm^2
Analytical data market surveillance: (Max. value of n = 104)	90 µg/g (0.009%)	0.2 µg/cm^2 ****

* Mixture of isomers, not specifically 1-OOH-isomer
** Dose used in study on specific Limonene-1-OOH isomer by Christensson, Contact Dermatitis 2014
*** Different dose depending on product type (e.g. Cream 10 mg/cm^2, fine fragrance, 2.2 mg/cm^2)
**** Based on the typical application dose of fine fragrance per area

- Even the single 'positive' sample leads to a dose per area exposure which is 400-fold below the inducing level in the LLNA
- Level is 1000-fold below the patch test dose when calculated as dose per area
- Reporting limit is clearly below induction doses and even below or close to the elicitation level, based on current knowledge*

* Recent publication by Bennike et al Contact Dermatitis 2019 requires detailed analysis

Conclusion

- The study has significantly extended the knowledge on HP occurrence in Consumer Products
- This is the first study analyzing multiple products from patients
- We could not detect and confirm HPs above reporting limit in the vast majority of the samples analyzed – meaning HP of Linalool and Limonene above 50 ppm are not widespread in consumer products
- These (negative) results were validated by ‘standard addition’
- Aging of Products has little to no impact on the HP levels found
- Results from the IDEA market surveillance on Limonene and Linalool HPs do not explain the cause leading to high frequency of positive patch tests seen in clinical studies because all samples assessed were well below induction thresholds.
- The cause of frequent positive patch test reactions remains unclear and needs to be investigated further.

Next steps
Pre- and pro- haptens

Moving forward, IDEA is considering the following additional work to better understand the clinical phenomena, specifically:

- An assessment of aggregate exposure to Limonene and Linalool hydro-peroxides using the Creme-RIFM model.
- A verification of induction thresholds for Limonene and Linalool hydro-peroxides.
- Critical reanalysis of existing clinical data with specific reference to the validity of patch test diagnosis for Limonene and Linalool hydroperoxides.
- A detailed understanding of the clinical observations at individual patient level, including patient history, outcome of diagnosis of allergy and specificity of the reaction, analysis of exposure, clinical relevance, etc.

Thank you for your attention