Manchester Institute of Biotechnology

From reference materials to reference methods – ways of harmonizing clinically-relevant allergen determination in food

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Declaration of interests

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UK Food Standards Agency

UK Biological and Biotechnological Sciences Research Council

UK Medical Research Council

European Union

European Food Safety Authority

NW Lung Centre Charity

Reacta Biotech Ltd

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Waters Corporation, Romer Laboratories Ltd, LGC, ManchesterBiogel

Spin-out company

ReactaBiotech Ltd

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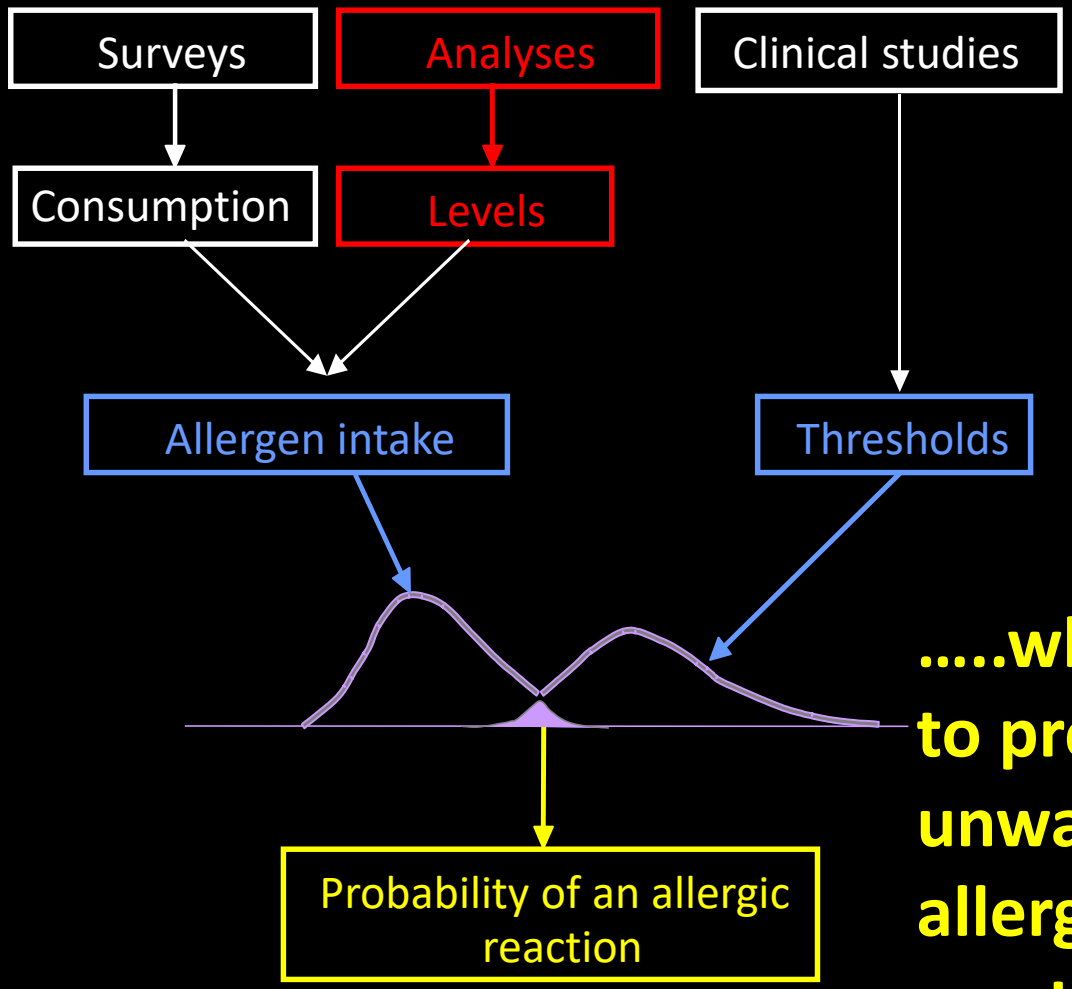
Food allergen analysis needs to sit within a risk assessment context…..



…..which seeks to prevent unwanted allergic reactions

Spanjersberg et al Food Chem Tox (2007) 45: 49-54; Madsen et al Food Chem Tox (2009) 47 480-489

What is the nature of the hazard?

- Food allergic patients make **IgE to certain food proteins**
- These molecules are named using the Latin name of the species the food originally comes from

- The IgE binds to mast cells and basophils which are packed full of inflammatory mediators
- These are released when cells are re-exposed to intact or large fragments of allergen molecules
- They cause physiological changes which manifest as symptoms of a reaction

Bhari et al *JACI* 2018 142, 485-496. Sayers, Simpson, Mills et al unpublished

Reference doses for are represented by total allergenic food protein

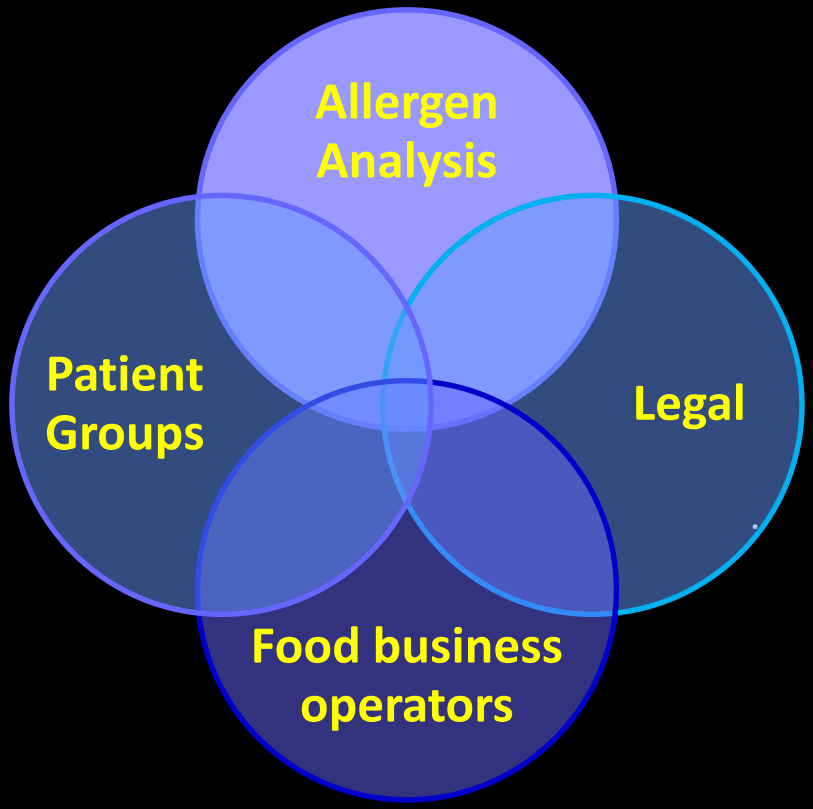
- Oral food challenges provide data for deriving reference doses and action levels for PAL which are expressed as mg food protein/Kg food
- It is also the unit used for assessing the potency of allergen products used to diagnose and treat food allergy

- Food regulatory authorities (e.g. Canada, USA) recognise the importance of protein in allergen risk assessment
- Ingredients with very low protein– e.g. highly refined soybean oil which are exempt from allergen labelling

Ballmer-Weber et al J Allergy Clin Immunol. 2015 135(4):964-71

Feedback and conclusions from joint JRC/SANTÉ workshop, JRC IRMM Geel, 16th- 17th June 2016

- Organised in the context of Regulation (EU) 1169 /2011 on the provision of food information to consumers and **the observed proliferation of precautionary allergen labelling by food producers.**
- It aimed to identify the **sequence of steps** required for **framing the current use of precautionary allergen information** and its enforcement across the EU.



Topic 3: The role of analysis in enforcing legislation

- Possible agreement on analytical marker(s) and their conversion to a common reporting unity should be encouraged.

- The most appropriate reporting unit for reporting analytical results is mg total allergenic ingredient protein per kg food.

- Establishing an expert group to facilitate the progression of all allergenic foods to report in this manner was thought beneficial. This group should be considerate of work done by CEN and other standardisation bodies in the area.

Only certain allergens seem to be associated with causing clinical reactions

- Of more than 14 peanut allergens only Ara h 1, 3, 2 and 6 appear to be important in activating effector cells
- Sensitisation to these allergens is associated with causing allergic reactions in peanut allergic patients

Nicolaou et al J Allergy Clin Immunol 2011 127(3):684-5; *Bhari et al JACI* 2018 142, 485-496.

Antibody targets in peanut ELISAs are usually clinically relevant allergens

ELISAs all recognise and determine the presence of major allergen molecules as markers of allergenic foods, although reporting units may be protein or whole peanut

Jayasena et al *J Agric Food Chem* 2015;**63**(6):1849-1855.

- Peanut ELISA kits vary in performance BUT the iFAAM peanut ELISA ring trial showed variation in test results is a result of systematic bias (**ALL** ELISA test kit results lie on the diagonal)
- This type of bias can be corrected through the use of reference materials

Baricevic et al iFAAM ring trial (unpublished).

Reference materials and universal calibrators – a solution for gluten assay variation?

Study 1: Using a universal calibrator achieved a similar effect to removing an outlier kit (AllerTek) but did not improve precision

Study 2: Using an incurred reference helped to harmonise test results, improving performance statistics for some test kits only. It improved qualitative agreement of three test kit pairs only.

Rzychon M et al. Food Chem. 2017234:144-154.

Reference and QC materials can help to control for ELISA test kit batch-to-batch variation

- Peanut allergen QC materials LGCQC101-KT
- Used unreconstituted
- Identified batch-to-batch variation in ELISA test results across 24 months of analysis

Allergen incurred reference materials – what do we have so far?

MoniQA skimmed milk powder together with blank and incurred materials in gluten free cookies at either 3.54 or 17.7 mg/Kg milk protein

But what about all the other allergens???

Development of Quality Control Materials for Food Allergen Analysis FS 101206

Michael Walker, Gill Holcombe at LGC
Clare Mills, Chiara Nitride at The University of Manchester
Adrian Rogers, Romer Labs UK Ltd

Incurred reference materials for allergen analysis

- Five allergenic ingredients are being incurred into a chocolate spread matrix
  - Cow's milk , hen's egg, hazelnut, walnut and almond
- Following a MFAN stakeholder workshop it was agreed to provide the materials as
  - Allergenic ingredient alone
  - Blank chocolate paste
  - Incurred chocolate paste containing 10mg allergenic protein/Kg chocolate paste of each of the ingredients

Characterisation of allergenic ingredients

Allergenic ingredients are being characterized in terms of

- Protein content using Dumas total nitrogen determination
- 2D-PAGE profiling and immunoblotting analysis
- Allergen profiling using mass spectrometry

Detection and quantification of allergens in foods and minimum eliciting doses in food allergic individuals (ThRAll) GP/EFSA/AFSC, 2017/03

Clare Mills, Chiara Nitride, Rosa Pilloli, Christof van Pouke, Marc de Loose, Nathalie Gillard, Ann Catherine Huet, Olivier Tranquet, Karine Adel-Patient, Linda Monaci

[Mills et al J AOAC Int. 2019 Apr 2. doi: 10.5740/jaoacint.19-0063]

AFSCA-FAVV

European Food Safety Authority

Synergies with other projects

- UK FSA call FS101206 “Development of Quality Control Materials for Food Allergen Analysis” (LGC and UNIMAN)
- Building on EuroPrevall, iFAAM, Allersens (BE), Manoe (FR)
- To achieve the synergies LGC and the JRC will attend the scientific KO meeting and stakeholder meetings of ThRAll.

EuroPrevall

iFAAM

manoe
MAITRISE ALLERGENE NUTRITION ENFANT

ThRAll Objective 1 (lead Monaci, CNR-ISPA)

To develop a harmonised quantitative MS-based prototype reference method for the detection of multiple food allergens in standardised incurred food matrices

Allergenic ingredients and incurred food matrices (Lead Van Poucke, ILVO)

Egg

Peanut

Hazelnut

[shared with FSA project]

Soybean

Almond

[shared with FSA project]

Milk

Incurred matrices

Matrices are chose to avoid duplication (no more cookies….) building on ILVO food processing expertise:

- Chocolate bar
- Broth powder - a matrix which has undergone extensive food processing including cooking, boiling down and drying to a powder and includes a variety of animal and plant-derived ingredients.

Allergenic ingredients incurred into broth and a chocolate matrix at 0, 2, 4, 10, 40 mg protein/Kg

PEPTIDE MARKER DETECTION

- Optimization of Instrumental settings
- Extraction conditions
- Trypsin digestion

PROTOTYPE MULTI-ALLERGEN MS METHOD

Inter-laboratory comparison of analysis of ThRALL incurred matrices for all allergens

ELISA ANALYSIS

ELISA optimization

Development of calibrants

ELISA analysis of ThRAll incurred matrices for all allergens

DIGITAL DROPLET PCR ANALYSIS

PCR optimization

PCR analysis of ThRAll incurred matrices [peanut, soya, hazelnut and almond]

Development of conversion factors to allow comparison of allergen analysis by MS, ELISA and PCR

Selection of published peptide markers for ThRAll foods (led by Linda Monaci and Rosa Pilloli)

Selection criteria have included peptides

- > 6 amino acids long
- Stable to chemical modification after food processing
- Peptides validated in different papers and evaluated in food matrices similar to those used in ThRAll
- Specific for each food at a species level checked by BLAST searching against UniProt Knoweldgebase (unreviewed), and the International Nucleotide Sequence Database Collaboration (INSDC) resources

This has been undertaken through an analysis of the published literature

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Experimental identification of peptide markers (led by Linda Monaci and Rosa Pilloli)

- Proteins extracted from
  - ThRAll incurred matrices (chocolate, broth powder)
  - Ingredients spiked into blank ThRAll matrices
- Extracts reduced, alkylated and digested with trypsin and analysed by untargeted high resolution MS/MS analysis
- Peptides identified common to spiked and incurred matrices which are
  - > 6 amino acids long
  - Have no missed tryptic cleavage sites
  - Specific for each food at a species level checked by BLAST searching against Uniprot (unreviewed) INSDC) resources

Aim: to identify at least three peptides (one quantifier and two qualifiers) for each allergenic ingredient with relevant selective reaction monitoring transitions

Harmonising reporting units

ELISA

- Detects protein markers
- Assay readouts depend on units assigned to calibrant in a given kit
- Conversion maybe required from commodity to protein

Mass Spectrometry

- Detects peptide marker of protein
- Requires conversion to protein but there is no agreed process

mg allergenic protein/Kg food

PCR

- Detects DNA **NOT** protein
- Assay readouts are in copy number
- Conversion is always required to get to protein but there is no agreed process

Wikimedia Commons

Many challenges remain for allergen analysis

- Reference materials are being produced – but we need to start using them!!
- Ways of calculating and reporting allergen which is meaningful for everyone – including patients – need to be agreed! **THESE NEED TO BE IN PROTEIN!**
- Black box for immunoassays with batch-to-batch variations in performance
  - Unknown composition of calibrators in ELISA kits
  - Antibody quality and cross-reactivity not defined
- Mass spectrometry has a way to go –
  - Lack of sequenced genomes makes development of MS methods for food allergens more difficult
  - Issues of specificity could also affect MS
  - Variability of results due to processing effects and matrix effects (and other?) effects means no single ideal extraction method for all food matrices is likely
  - **Peptide calibrators MUST be verified**

ThRAll Objective 2 (Lead Mills, UNIMAN)

To develop consensus approaches on quality assessment of data will be developed to support consistent definition of lowest observed adverse effect levels. These will be applied to collate publicly available data to provide "cleaned" analysis-ready data sets

Development of harmonised protocols for collection of threshold data in food allergic individuals

- Developing harmonised clinical protocols for undertaking oral food challenge studies that provide data to underpin calculation of threshold doses, building on those used in studies such as iFAAM, EuroPrevall, TRACE and MANOE
- Develop a consensus on how to curate and classify such data
- Undertaken with an expert group
  - Addenbrooks (UK): Shelly Dower
  - Charité (DE): Kirsten Beyer
  - Hospital Clinico San Carlos (ES): Montserrat Fernandez-Rivas
  - Centre Hospitalier (FR): Martine Morisset
  - UMC Utrecht (NL): Andre Knulst
  - DAAB (DE): Sabina Schnadt

Population and curation of database with historic and published data

- A web-based data collection interface is being developed usng REDCap
- This will be piloted using anonymised data sets from projects such as EuroPrevall, iFAAM, MANOE, TRACE amongst others
- Data sets will be reviewed and transformed into the common format, cleaned using pre-agreed set of criteria and classified;
- Where necessary they will be reviewed at a second expert panel review meeting.
- For those foods for which threshold data are collected which exceed 30 subjects responding with objective symptoms, dose distributions will be modelled.
- Data will be made publicly available

The Team

Manchester University: Rebekah Sayers, Chiara Nitride, Justin Marsh, Anuradha Balasundaram, Aida Semic-Jusafagic, Angela Simpson, Adnan Custovic, Marina Themis, Ivona Baricevic-Jones, Victoria Lee, Rosemary Adaba, Huan Rao, Daniel Schäffer, Angela Simpson, Phil Couch, Bushra Javed, Hadeer Mattar, Matt Sperrin, Rene Crevel

ThRAll partners and collaborators: Rosa Pilloli, Christof van Pouke, Marc de Loose, Nathalie Gillard, Ann Catherine Huet, Olivier Tranquet, Karine Adel-Patient, Hervé Bernard, Linda Monaci, Gavin O'Conner, Michael Walker, Gill Holcomb

Ring-trial participants

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Thank You!

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