

September 27-28, 2023

Emerging technologies for protein safety

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PROTEINS IN FOOD AND FEED – MANY DIMENSIONS

Nutrition



Enzymes in Food Biotechnology

Production, Applications, and Future Prospects

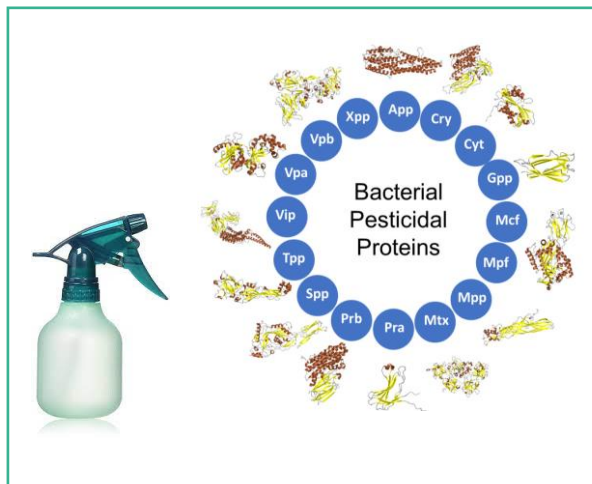


Edited by
Mohammed Kuddus



Food/feed technology

Others



Contaminants



WHY ASSESSING PROTEIN SAFETY?

Toxicity/Pathogenicity

Agglutinins
Neurotoxins
Haemolysins
Enterotoxins
etc.



IgE mediated

Non IgE mediated



**Immune-mediated reactions
(« Allergenicity »)**



HOW ARE PROTEINS ASSESSED IN THE EU

- Pre-market assessment (intentional use)
- Sectorial regulations
- EFSA guidance documents
- Consumers
- Operators/workers
- Environment

GMO

EFSA GMO Panel 2010, 2011, 2017, 2021

Food enzymes

EFSA CEP Panel 2009, 2019, 2021

Food additives

EFSA ANS/FAF Panel, 2012, 2021

Feed additives

EFSA FEEDAP Panel, 2017, 2018, 2019, 2021

Novel food

EFSA NDA Panel, 2016, 2021

Allergenic foods/food ingredients (labelling)

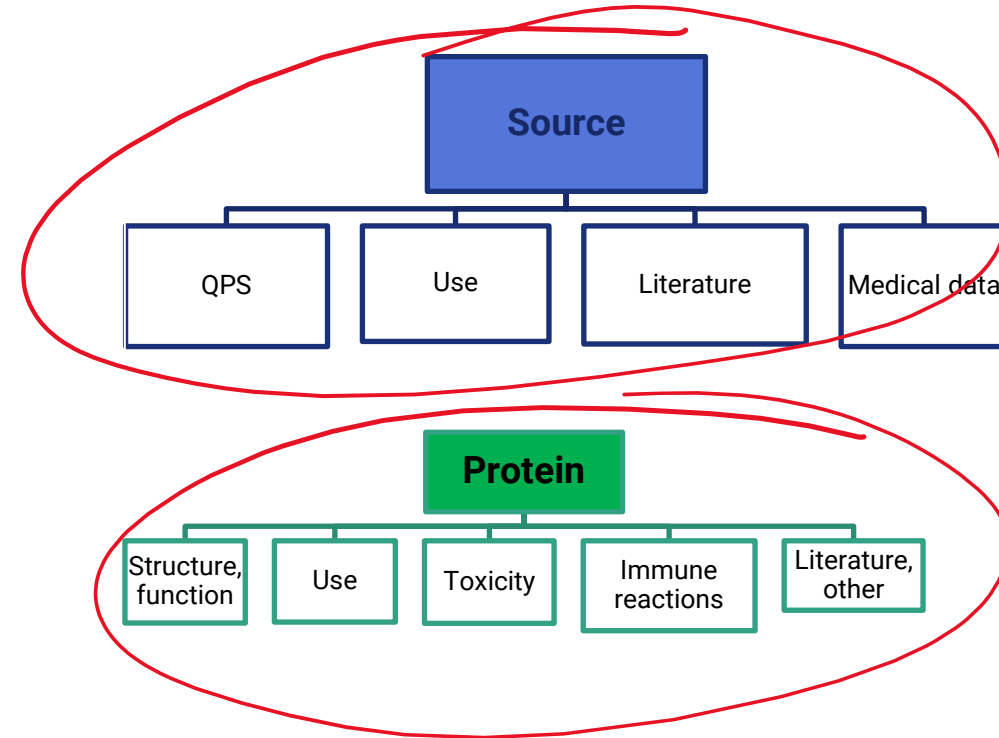
EFSA NDA Panel, 2014



WHY EXPLORING NEW WAYS? NEED TO EVOLVE

- **Current methodologies and tools**

- Bioinformatics (querying the genome, sequence alignment)
- Molecular biology (function, structure)
- *In vitro* studies (*in vitro* digestion)
- *In vivo* studies (toxicological studies)
- HoSU, other information



Recurrent issues

Assessment *adapted* from chemical RA

Possibilities

Evolution in protein science, new data, new tools, new approaches

Regulatory relevance



AND....NEW PROTEINS/USES OF PROTEINS



Novel food sources

Food 2030 - Innovative EU research ensures food system is future-ready

Nutrition for more sustainable and healthy diets
Climate resilience and environmental sustainability
Circularity and resource efficiency
Innovation and the empowerment of communities

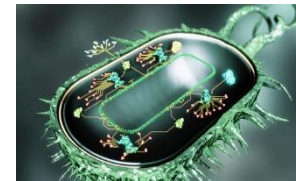
https://ec.europa.eu/knowledge4policy/publication/food-2030-innovative-eu-research-ensures-food-system-future-ready_en

Sustainable use of pesticides

Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides

Low risk plant protection products

micro-organisms, botanicals and semiochemicals - e.g. pheromones with different characteristics than conventional chemical active substances used for plant protection



EFSA IS EXPLORING NEW WAYS FOR PROTEIN ASSESSMENT

Toxicity

Exploring *in silico* and *in vitro* tools to predict and investigate protein toxicity

NAM-based strategy for the toxicological assessment of (novel) proteins



“Allergenicity”

Developing novel approaches and tools to increase reliability of predictions in allergenicity assessment (IgE and non IgE)

Exploratory phase
Building the knowledge
Regulatory relevance



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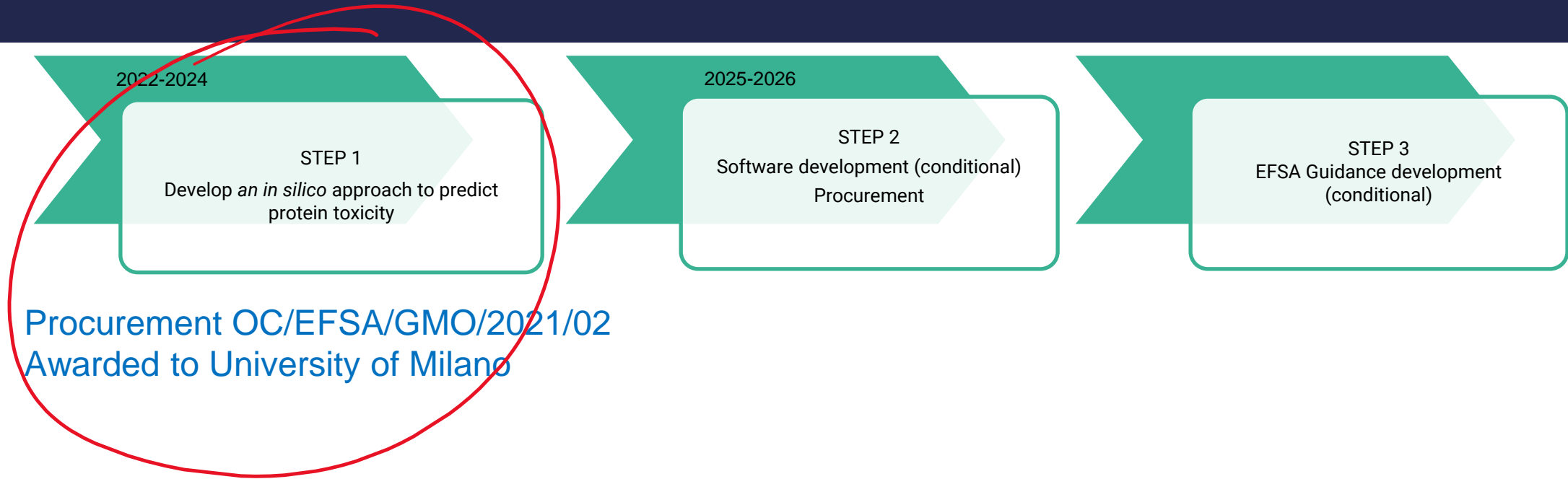
“Allergenicity”

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Exploratory phase
Building the knowledge
Regulatory relevance



TOXICITY - IN SILICO PREDICTION - PRELIMINARY !



Key steps

1. Literature search –prediction tools
2. Protein benchmark dataset
3. Tools testing phase
4. Pipeline setting

Uniprot KB (public available, curated)

Toxic proteins: identified in Uniprot KB and classified based on Geneontology (mechanisms of action at molecular level, biological processes evoked, and cellular component affected)

Palazzolo et al, 2020 <https://doi.org/10.2903/sp.efsa.2020.EN-1875>



TOXICITY – IN SILICO PREDICTION

Exploring the literature to identify protein toxicity prediction tools

Protein benchmark datasets

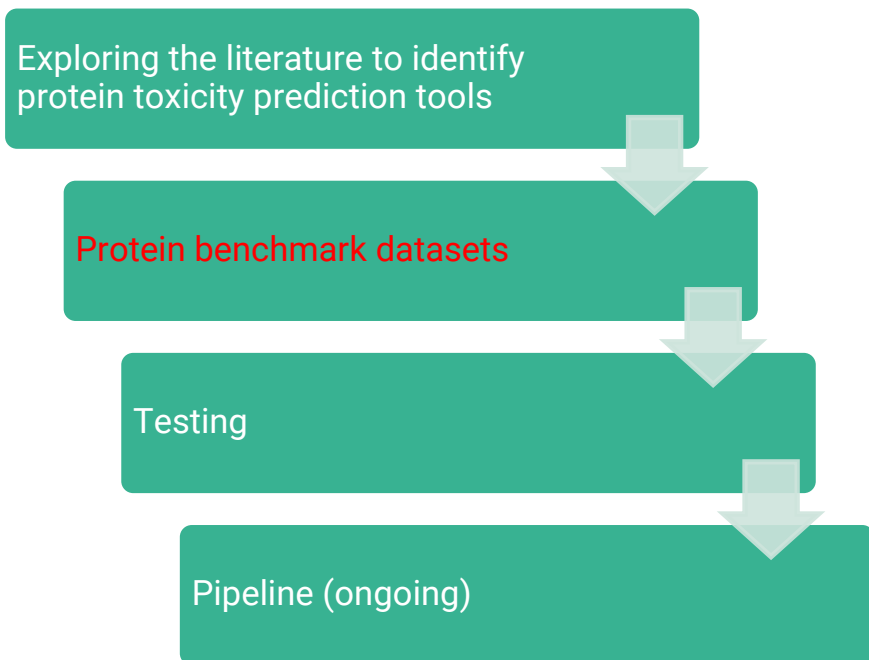
Testing

Pipeline (ongoing)

Tool	Application Scope	Technical limitations
<u>ToxClassifier</u>	Animal venoms	N.A.
<u>NNTox</u>	<u>All toxins</u>	N.A.
TOXIFY	Venom toxins	Sequences ≤ 500 amino acids*
<u>ToxDL</u>	<u>All toxins</u>	N.A.
<u>ToxIBTL</u>	<u>All toxins</u>	N.A.
ToxinPred2	<u>All toxins</u>	N.A.
KNOTTIN	Toxins belonging to the knottins family	Sequences ≤ 200 amino acids



TOXICITY – IN SILICO PREDICTION



True positives (TP)

known toxic proteins, non-redundant and falling within the application scope of the tool

alltox: All the reviewed UniProt entries matching the GO "toxin activity".

venom: All the reviewed UniProt entries matching the GO "toxin activity" and containing the general keyword "venom" in every field.

knottin: All the reviewed UniProt entries matching the GO "toxin activity" and the UniProt keyword "knottin"

True negatives (TN): known non-toxic proteins sharing high sequence similarity to the corresponding TPs.

allnontox: All the reviewed UniProt entries that *do not* match the GO "toxin activity".

Expected false negatives (EFN): where applicable, known toxins that are expected not to be correctly classified by the tool, as they fall outside of the tool field of application.

Dataset	query	#Entries
alltox	((go:0090729*) AND (reviewed:true))	7411
venom	((go:0090729) AND (reviewed:true)) AND venom	6265
knottin	((go:0090729) AND (reviewed:true)) AND (keyword:KW-0960**)	1292
allnontox	(NOT (go:0090729)) AND (reviewed:true)	560591

*: GO "Toxin activity";

**.: UniProt Keyword "knottin"



TOXICITY – IN SILICO PREDICTION

Exploring the literature to identify protein toxicity prediction tools

Protein benchmark datasets

Testing

Pipeline (ongoing)

Tools, Hidden Markov models,
Blasting → strategy

Tool	dataset TP	dataset TN	sens	spec
Deepfri *	alltox 80	allnontox	0.36	0.81
deepGO *	alltox_80	allnontox	0.16	0.99
	venom_80		0.16	
	knottin_80		0.15	
	alltox_not_venom80		0.12	
	alltox_not_knottin80		0.15	
NNTox	alltox 80	allnontox	0.95	1.00
ToxIBTL	alltox 80_50	allnontox_50	0.87	0.73
Toxify	venom80_500	tn_venom_500	0.90	0.58
	alltox_not_venom80_500		0.32	
ToxinPred2	alltox 80	allnontox	0.95	0.48
ToxClassifier	unreachable			
ToxDL	Unreachable / needs training			
KNOTTIN	Not working / unreachable			
DeepGraphGO *	undocumented			
FunfHMMER *	Not usable			
MetaGO *	Mandatory structure, web server only with reply via e-mail			
GOFGD *	unreachable			
ToxiTaxi	Not a predictive tool			
ConoServer	Not a predictive tool			
DBETH	Not a predictive tool			
T1Tadb	Not a predictive tool			
ClanTox	Unreachable			
BTXPred	Outdated (website, one query at a time; not usable)			
NTXPred	Outdated (website, one query at a time; not usable)			
ToxinPred	Outdated (superseded)			
PredCSF	Outdated (Website, results by e-mail, undocumented source code and missing installation instructions)			



TOXICITY – IN VITRO TESTING

In vitro toxicity testing strategy: 2022-2024 get an overview of in vitro systems to experimentally investigate protein toxicity and develop testing strategies for selected toxic proteins

Procurement
OC/EFSA/NIF/2022/01

Awarded to ANSES-LIST
Consortium

ONGOING

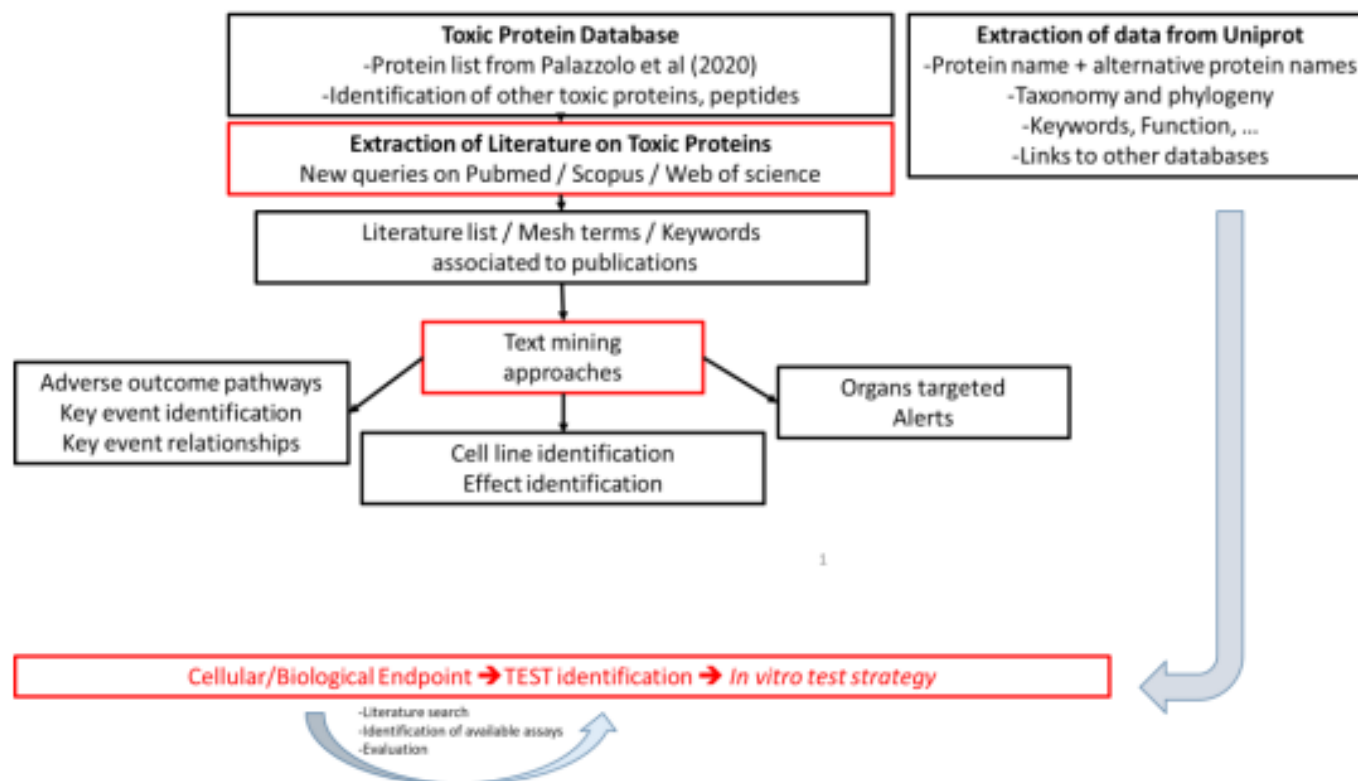


Figure 2: Identification of key mechanisms of toxicity and cellular endpoints for in vitro evaluation of protein toxicity

EFSA IS EXPLORING NEW WAYS FOR PROTEIN ASSESSMENT

Toxicity



“Allergenicity”

EFSA GMO Panel et al 2022. Scientific Opinion on development needs for the allergenicity and protein safety assessment of food and feed products derived from biotechnology

<https://doi.org/10.2903/j.efsa.2022.7044>

ease
ment



ALLERGENICITY ASSESSMENT

EFSA procurement:

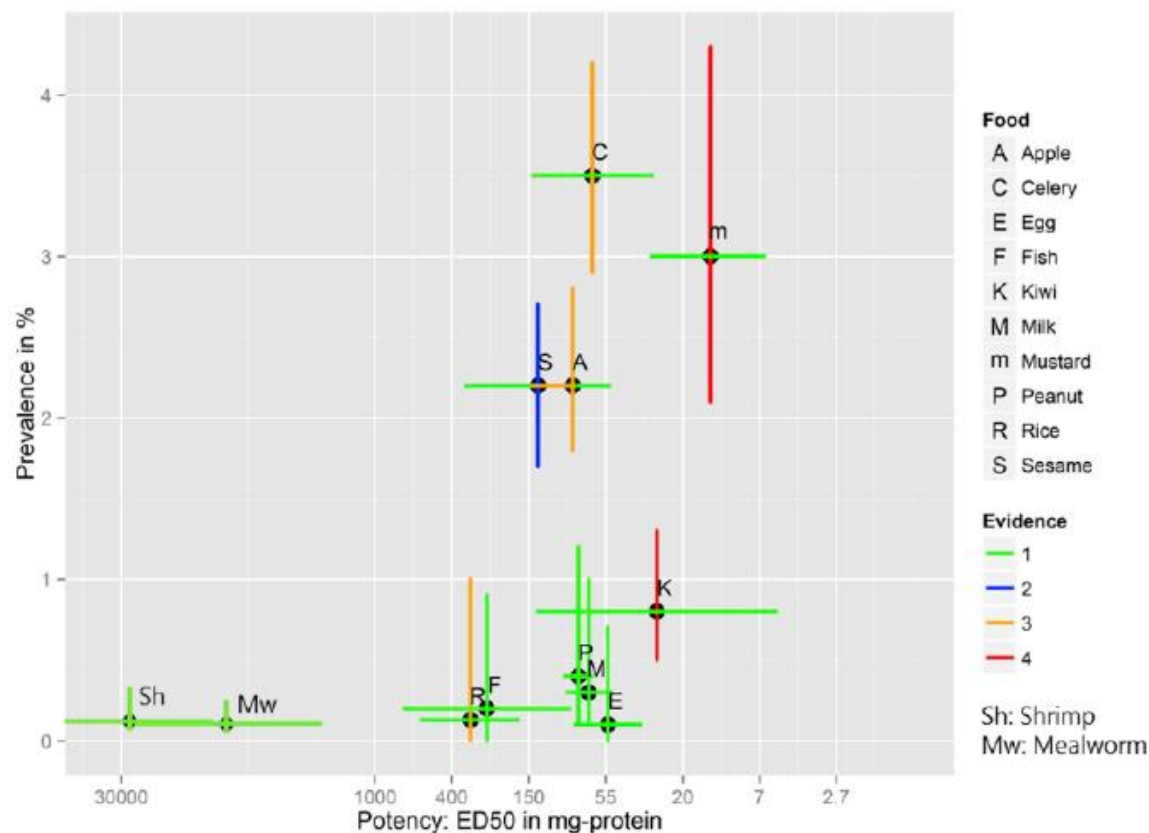
[OC/EFSA/GMO/2021/04](#) - Novel strategies for predicting allergenicity: development of a ranking method and screening tools to assess the allergy risk of innovative proteins

Purpose:

- To improve the current prediction (*in silico* methods and tools)
- To propose a ranking strategy (database)
- To develop novel strategies better integrating *in silico* analysis and follow up actions (in vitro/in vivo) in the RA process



SCALING ALLERGY RISKS OF FOODS



Strategy for ranking the allergic potential of known proteins as a way forward
(FAO/WHO, 2001; EFSA GMO Panel, 2017; Remington et al 2018; Verhoeckx et al 2020; Fernandez et al 2021)

Houben et al 2019. Food and chemical toxicology, 127, pp 61-69

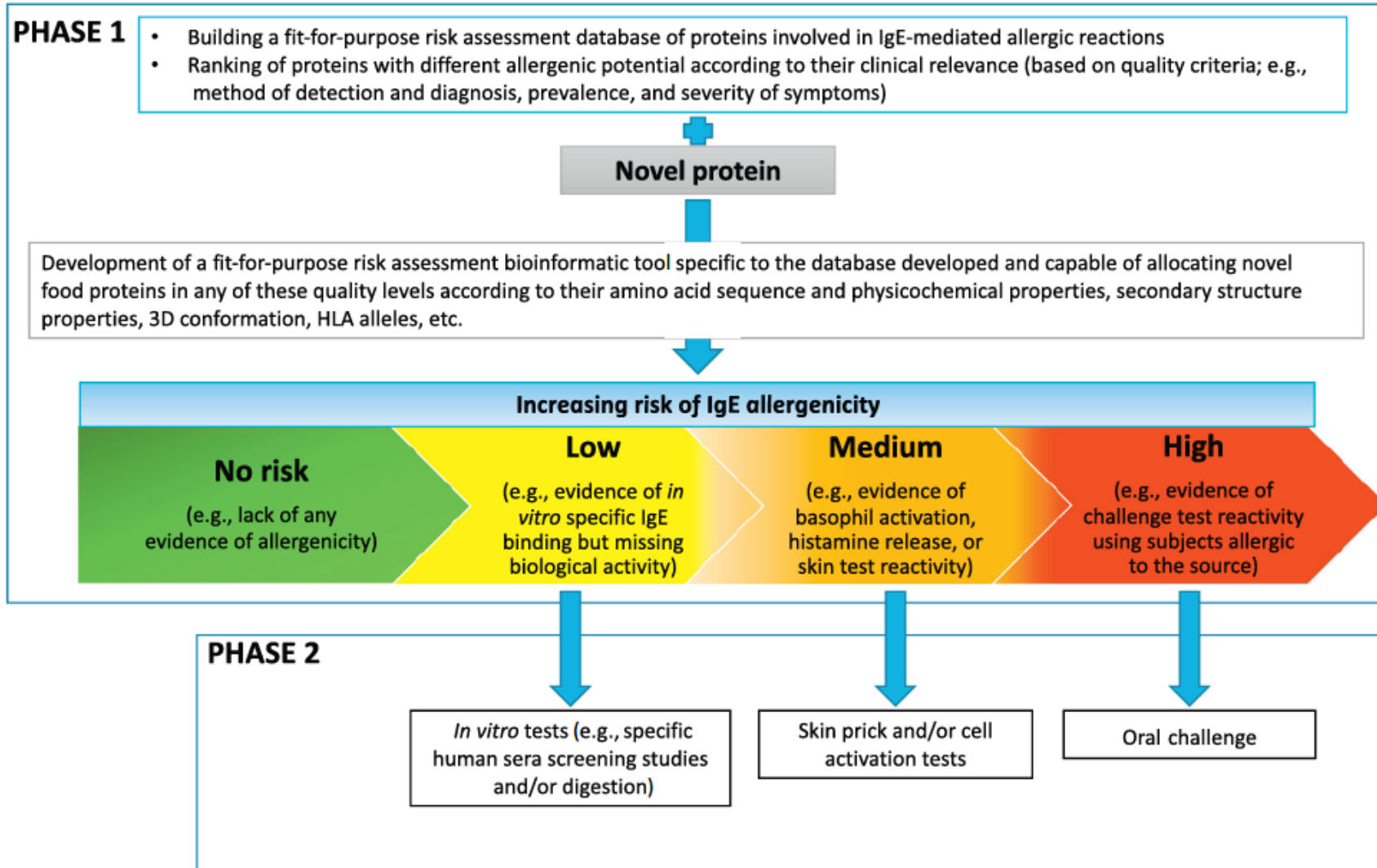
Whole foods



Individual allergens?



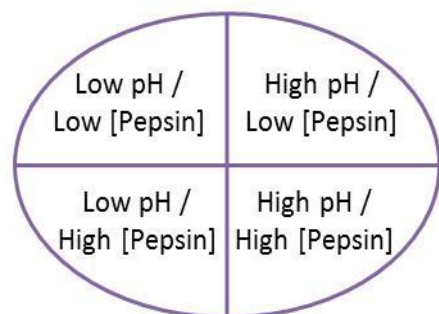
ALLERGIES - DEVELOPMENT OF A RANKING METHOD



IN VITRO PROTEIN DIGESTION

Examples for test conditions – digestion conditions

Possible gastric conditions:



Elderly/adults in fasted state
Elderly/adults in fed state
People with impaired gastric function
People taking antacids
Infants

Proposed gastrointestinal conditions:

pH 5.5

[Pepsin] = ~1,000 U/mL¹
of gastric juice
(biosurfactants)²

pH 1.2-2.0

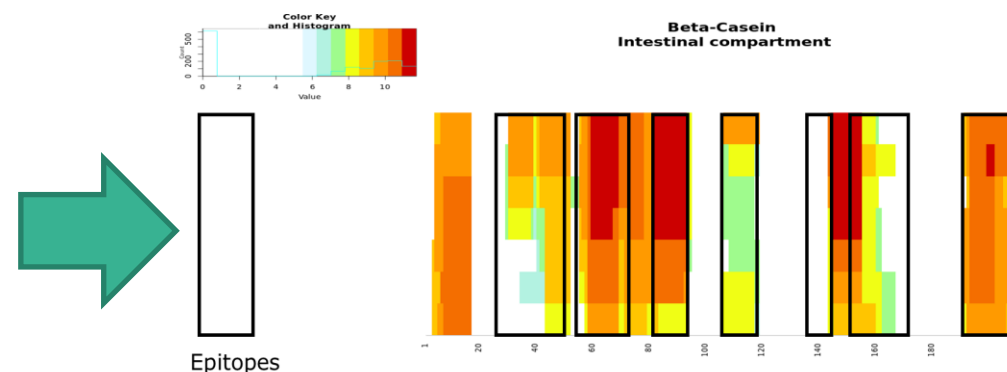
[Pepsin] = Classical pepsin-
resistance test³
(biosurfactants)²

**Intestinal
digestion**

pH 6.5

[Trypsin] = ~1,600 U/mL¹
[Chymotrypsin] = ~800 U/mL¹
of intestinal juice
(biosurfactants)²

<https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2019.EN-1765>



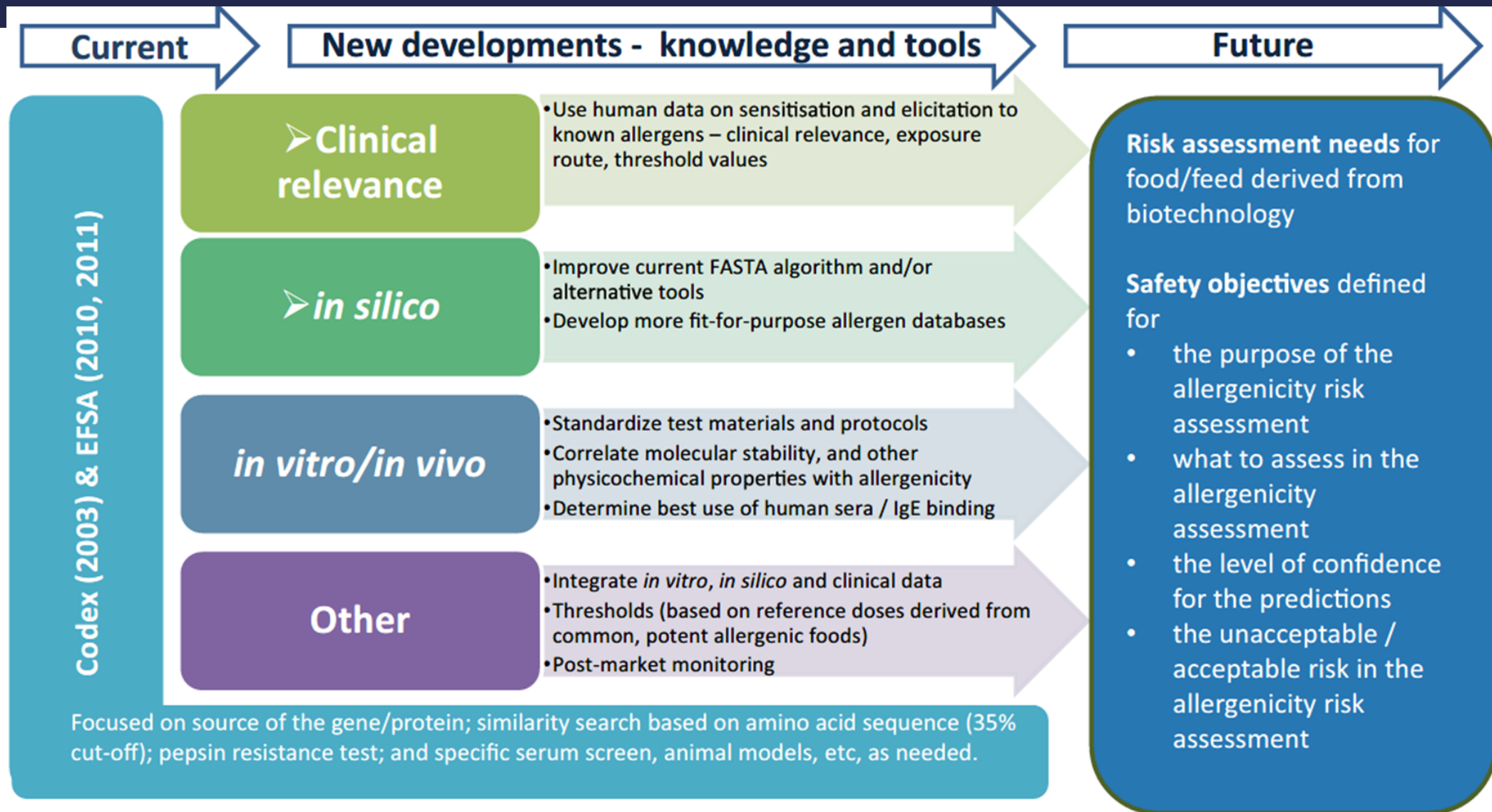
Mackie et al 2019

There is a need
for more control
proteins

¹Dependent on the used substrate and enzyme activity assay; ²for further details please see Annex B-3; ³Pepsin : protein ratio of 10 U : 1 µg (Thomas et al., 2004).



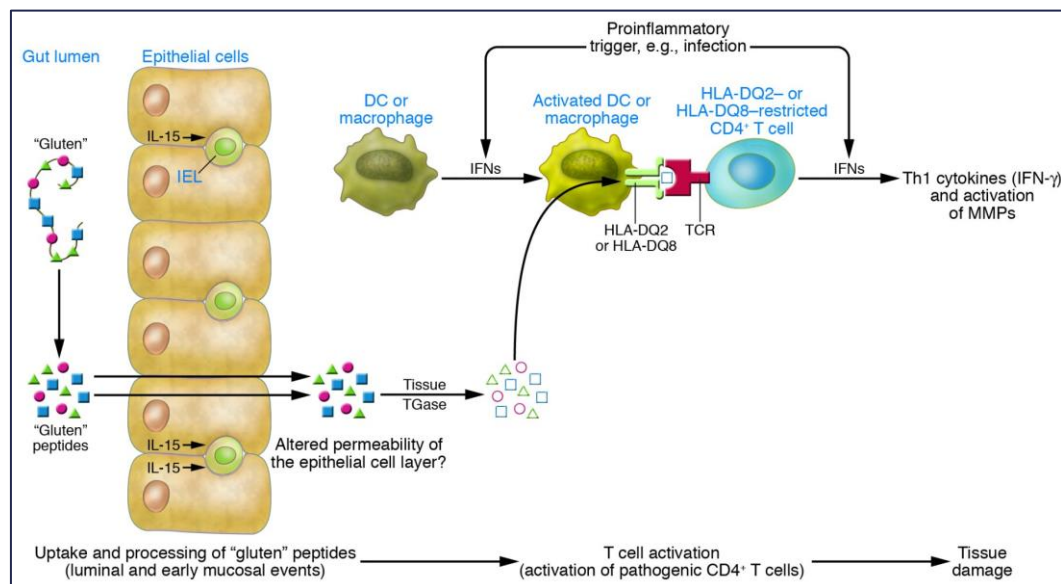
ROADMAP TO IMPROVED “WEIGHT OF EVIDENCE” RA



CELIAC DISEASE – THE STRATEGY AND DEVELOPMENTS



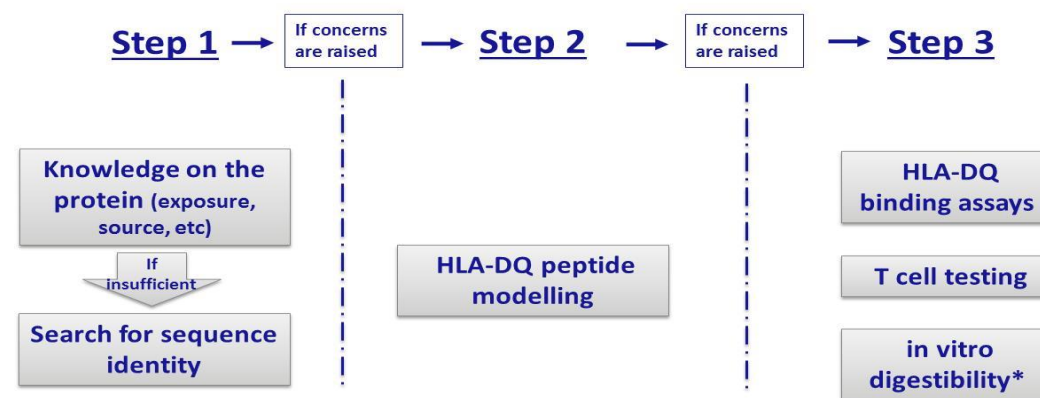
Uncontrolled intestinal immune response to gluten proteins in wheat and gluten-like proteins in barley and rye



Kagnoff, *J Clin Invest.* 2007;117(1):41-49. <https://doi.org/10.1172/JCI30253>.

The EFSA strategy

Fig 1. Stepwise approach for risk assessment



* for details, please see chapter on *in vitro* digestibility

EFSA GMO Panel et al, 2017. Guidance on allergenicity assessment of genetically modified plants. *EFSA Journal* 2017;15(5):4862, 49 pp. <https://doi.org/10.2903/j.efsa.2017.4862>



CELIAC DISEASE DEVELOPMENTS - SOFTWARE

EFSA procurement
OC/EFSA/GMO/2019/02 – HLA-DQ
peptide modelling software

Software tool available at
<https://r4eu.efsa.europa.eu/app/predq>

[Home](#) | [Method Description](#) | [Contact](#)

preDQ

a tool for peptide binding prediction to HLA-DQ2 and/or HLA-DQ8



1. Insert a single sequence in one CAPITAL letter code.

2. Choose an allele:

HLA-DQ2.5 (A1*05:01/B1*02:01) ▼

For prediction of multiple sequences upload a file in fasta format.

No file selected.

3. Choose deamidation position

☐ p1 (recommended for HLA-DQ8.1)
☐ p4 (recommended for HLA-DQ2.5 and HLA-DQ8.1)
☐ p6 (recommended for HLA-DQ2.5 and HLA-DQ8.1)
☐ p7 (recommended for HLA-DQ2.5)
☐ p9 (recommended for HLA-DQ2.5 and HLA-DQ8.1)

4. Search only peptides containing Q/E-X1-P-X2 motif

☒ Recommended for celiac peptides and HLA-DQ2.5 allele

5. Choose the output format

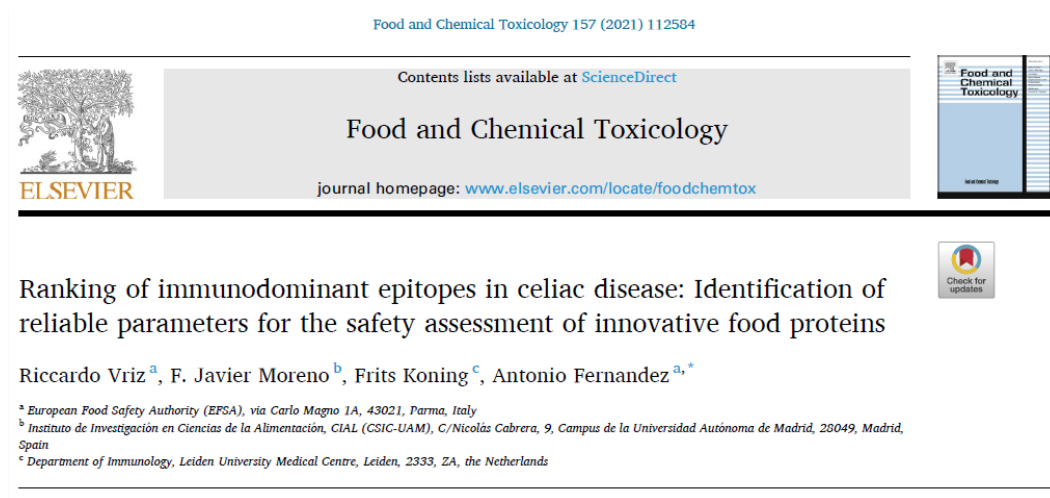
☒ Majority voting (at least 3 models out of 5 with positive prediction)

6. Non-binders

☐ include
☒ exclude

[Help](#) [Example in plain format](#)

CELIAC DISEASE – DEVELOPMENTS - AOP



- Enzymatic digestion of proteins involved in CD – Delivery of immunologically active fragments to gut mucosal segments
- Epitope binding to HLA-DQ surface receptors of APC – CD4⁺ T-cells recognise gluten peptides only when presented by HLA-DQ molecules
- Activation of pro-inflammatory gluten-specific CD4⁺ T-cells



CELIAC DISEASE – DEVELOPMENTS

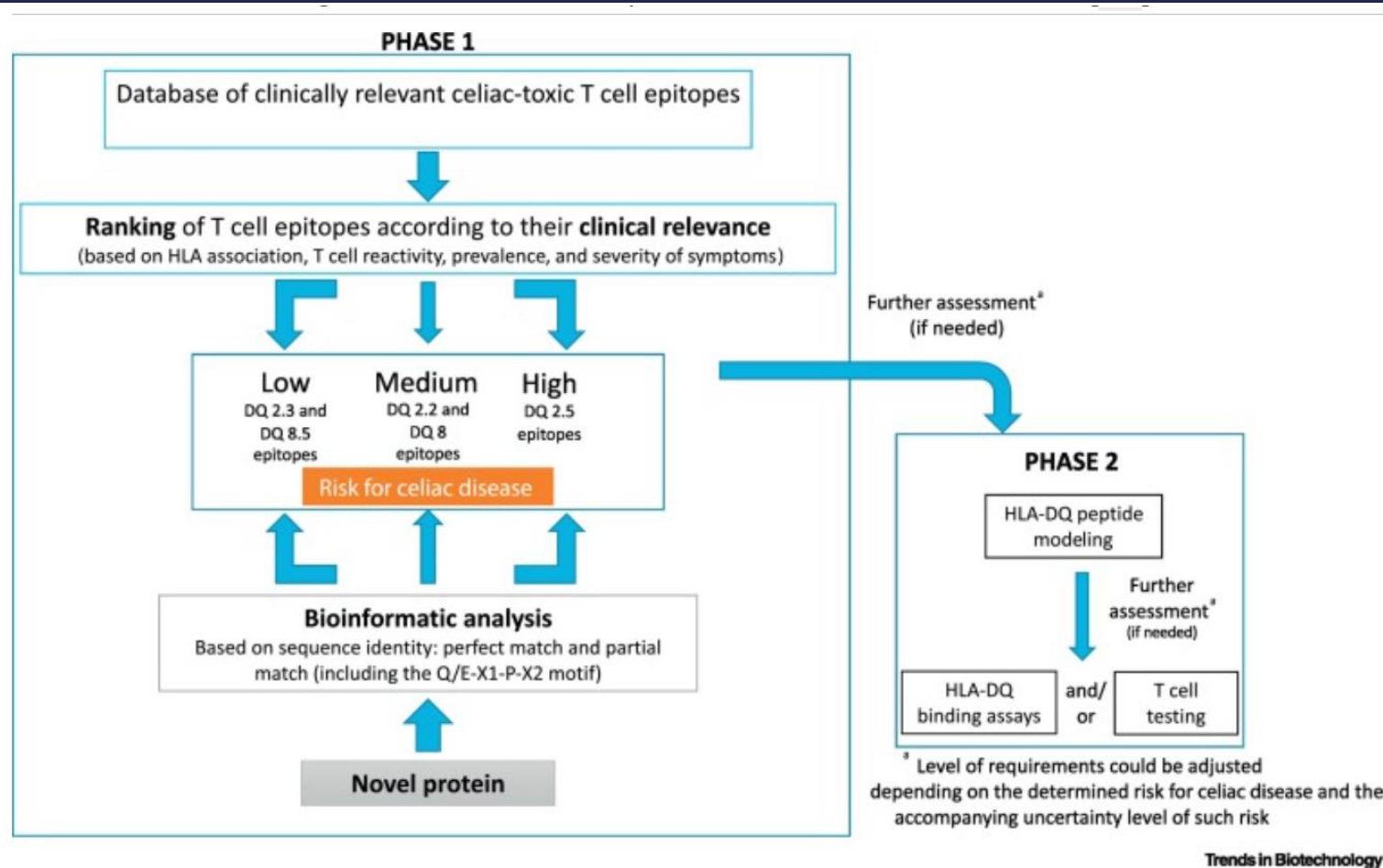


Figure 1 Strategy for the Risk Assessment of Novel Food Proteins Potentially Causing Celiac Disease.



THANKS

EFSA

- Antonio Fernandez Dumont NIF Unit
- Giovanni Iacono IDATA
- Luca Belmonte IDATA
- George Kass NIF Unit

All contractors

Experts (GMO panel)



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