Risk estimates of mycotoxin mixtures in the diets of our children

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Outline

1. Multiple mycotoxins in foodstuffs – a reality

2. Risk assessment - change of paradigm

3. Challenges in the risk assessment of multiple mycotoxins (mycotoxin mixtures) in food:
   - Hazard assessment
   - Exposure assessment
   - Risk characterization

4. Risk estimates in the diet of our children:
   - A Portuguese case study – MYCOMIX project
Ingestion of food is considered a major route of exposure to many contaminants, namely mycotoxins.

1. Multiple mycotoxins in food – a reality

- Natural co-occurrence of mycotoxins in foods
- Increasing concern
- Combined mycotoxins
- Expected to exert greater toxicity than exposure to single mycotoxins
2. Risk assessment – change of paradigm

Historically, the health risk from human exposure to mycotoxins has been evaluated on the basis of single-chemical and single-exposure pathway scenarios.

In general, exposures to mycotoxins through the food were assessed independently, and no concerted effort had been made to evaluate potential multiple exposures simultaneously.
Government and industry regulations are usually based on individual toxicities, and do not take into account the complex dynamics associated with interactions between co-occurring groups of mycotoxins.
Combined toxicity and interactive effects concerning multiple mycotoxins studies

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<th>Experimental system</th>
<th>Combined effect</th>
<th>Data analysis/modelling</th>
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<td>OTA-FA$_1$</td>
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<td>AFB$_1$-ZEA</td>
<td>Cytotoxicity: MTT</td>
<td>Raw 264.7</td>
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<td>AFB$_1$-DON</td>
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<td>AFB$_1$-ZEA or DON - Synergism</td>
<td>Central composite design; comparison between observed and expected dose-response curves</td>
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<td>AFB$_1$-ZEA or DON (high doses) - Synergism</td>
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<td>AFB$_1$-AFB$_2$</td>
<td>Cytotoxicity: MTT</td>
<td>J774A .1 cell line</td>
<td>Synergism</td>
<td>Statistical comparison of data from single and combined effects</td>
<td>Bianco et al., 2012</td>
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<td>AFM$_1$- AFM$_2$</td>
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</table>

Recent reports on worldwide exposure assessment to multiple mycotoxins in food

<table>
<thead>
<tr>
<th>Country</th>
<th>Population group (age, years old)</th>
<th>Samples</th>
<th>Number of analysed mycotoxins (toxin group)</th>
<th>Food consumption (data collection)</th>
<th>Analytical method: occurrence</th>
<th>Handling non-detects: substitution method</th>
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<th>References</th>
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<tr>
<td>China</td>
<td>children &amp; adults (&gt;7)</td>
<td>wheat &amp; maize foods</td>
<td>3 (trichotheccenes &amp; metabolites)</td>
<td>Shanghai Food Consumption Survey 2012-13 (24-h recall)</td>
<td>LC-MS/MS</td>
<td>LOD/2</td>
<td>deterministic &amp; probabilistic</td>
<td>Han et al., 2014</td>
</tr>
<tr>
<td>Portugal</td>
<td>infants (1-3)</td>
<td>breakfast cereals</td>
<td>12 (aflatoxins, trichotheccenes, fumonisins, ochratoxins)</td>
<td>Pilot survey 2014 (3-day food diary)</td>
<td>LC-FD; GC-MS, UPLC-MS/MS</td>
<td>0, LOD/2, LOD</td>
<td>deterministic &amp; probabilistic</td>
<td>Assunção et al., 2015b</td>
</tr>
<tr>
<td>Spain</td>
<td>adolescents &amp; adults (n.r.)</td>
<td>Coffee</td>
<td>21 (aflatoxins, trichotheccenes and metabolites, fumonisins, ochratoxins, enniatins, beauvericin, sterigmatocystin)</td>
<td>Spanish Agency for Food Safety Survey 2009</td>
<td>LC-MS/MS</td>
<td>0</td>
<td>deterministic</td>
<td>García-Moraleja et al., 2015</td>
</tr>
<tr>
<td>Belgium</td>
<td>adults (&gt;15)</td>
<td>nuts, dried fruits</td>
<td>2 (aflatoxins, ochratoxins)</td>
<td>Belgian National Consumption Survey 2004 (2x24h recall)</td>
<td>n.r.</td>
<td>0</td>
<td>probabilistic</td>
<td>Van de Perre et al., 2015</td>
</tr>
<tr>
<td>China</td>
<td>children &amp; adults (2-100)</td>
<td>wheat based foods</td>
<td>4 (Alternaria toxins)</td>
<td>China National Nutrient and Health Survey 2002 (3x24h recall)</td>
<td>UPLC-MS/MS</td>
<td>0, LOD/2, LOD</td>
<td>deterministic</td>
<td>Zhao et al., 2015</td>
</tr>
</tbody>
</table>

The assessment of mycotoxin exposure is often based on calculations combining mycotoxin occurrence data in food with population data on food consumption. This indirect approach is associated with some limitations and uncertainties:

- the heterogeneous distribution of mycotoxins in food.
- the limited accuracy of food consumption data (can lead to an under- and overestimation of the exposure).
- the individual variation in absorption, distribution, metabolism and excretion (which is not integrated).

Human biomonitoring using biomarkers of exposure has been proposed as a suitable alternative to perform an accurate mycotoxin exposure assessment at individual level.
Human mycotoxin exposure assessment using a multibiomarker approach

<table>
<thead>
<tr>
<th>Country</th>
<th>N° of analytes</th>
<th>Analytes included</th>
<th>Sample preparation and cleanup</th>
<th>Methodology</th>
<th>Biological samples</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>4</td>
<td>DON, DON-3-GlcA, DON-15-GlcA, DOM-1, DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB1, FB3, AFB1, AFG1, AFB3, AFM1, ZEA, ZAN, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA, α/β-ZAL-14-GlcA, OTA, Oto, enniatin B and DH-CIT</td>
<td>&quot;Dilute and shoot&quot;</td>
<td>LC-MS/MS</td>
<td>Urine</td>
<td>Warth et al., 2012a</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>23</td>
<td>DON, DON-3-GlcA, DOM-1, T-2, HT-2, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL, CIT, AFB1, AFB3, AFG1, AFG3, AFM1, FB1, FB3, HFB1, OTA, Oto, 4-OH-OTA, FB1, HFB1, DON, DON-3-GlcA, DOM-1</td>
<td>&quot;Dilute and shoot&quot;</td>
<td>LC-MS/MS</td>
<td>Urine</td>
<td>Gerding et al., 2015</td>
</tr>
<tr>
<td>Cameroon</td>
<td>15</td>
<td>DON, DON-3-GlcA, DON-15-GlcA, DOM-1, T-2, HT-2, NIV, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL</td>
<td>&quot;Dilute and shoot&quot;</td>
<td>LC-MS/MS</td>
<td>Urine</td>
<td>Abia et al., 2013; Warth et al., 2012b</td>
</tr>
<tr>
<td>Germany</td>
<td>23</td>
<td>DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB1, FB3, AFB1, AFG1, AFB3, AFM1, ZEA, ZAN, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA, α/β-ZAL-14-GlcA, OTA, Oto, enniatin B and DH-CIT</td>
<td>&quot;Dilute and shoot&quot;</td>
<td>LC-MS/MS</td>
<td>Urine</td>
<td>Gerding et al., 2014, 2015</td>
</tr>
</tbody>
</table>
3. Challenges in the risk assessment of multiple mycotoxins in food

Risk Assessment
Science based

- Hazard identification
- Hazard characterization
- Exposure assessment
- Risk characterization

Holistic overview reflecting the interrelation between different steps of multiple mycotoxins health risk assessment and respective challenges

- Prioritization of mixtures for hazard assessment
- Harmonization of approaches/methodologies and data analysis/modelling
- Reinforcement of data on combined genotoxicity
- Exploration of mechanisms underlying toxicological interactions

- Availability of food consumption data
- Development of multianalyte methods
- Management of left-censored data
- Use of probabilistic models

- Availability of toxicological data
- Harmonization of terminology

Challenges on the hazard assessment

- Exploration of mechanisms underlying toxicological interactions
- Reinforcement of data on combined genotoxicity
- Harmonization of approaches/methodologies and data analysis/modelling
- Harmonization of methods to address bioavailability
- Prioritization of mixtures for hazard assessment
Bioavailability:

knowing the amount of an ingested mycotoxin may not be enough for exposure assessment. Only a certain amount of the contaminant will be available to reach the systemic circulation.

The amount of the contaminant that is available for the absorption in the gut after digestion corresponds to bioaccessibility (simulation by IVD models).

The fraction of the ingested contaminant that is present in the bloodstream and is available to exert its toxic effects on the target organs corresponds to the bioavailability.
Challenges on the exposure assessment

- Availability of food consumption data
- Development of multianalyte methods
- Management of left-censored data
- Use of probabilistic models
- Multibiomarker approach
Challenges on the risk characterization

- Availability of toxicological data
- Harmonization of terminology
Following the increasing interest of risk assessors, regulators and scientific community on the risk assessment of multiple mycotoxins in food, recent international meetings and research projects had pointed out the urgent need to address and discuss issues such as the co-occurrence of chemical mixtures including mycotoxins, their combined toxicity and cumulative risk assessment.

INSA repository
http://hdl.handle.net/10400.18/3214
4. Risk estimates of mycotoxin mixtures in the diets of our children

- **Children are constantly growing.** They breathe more air, consume more food, and drink more water than adults do, in proportion to their weight.
- **Children are still developing.** Exposure to toxicants (as mycotoxins) can lead to irreversible damage.

The **significance** and **potential health** risk of any **contaminant in foods** consumed by children is **increased** and diligent **attention** must be paid to this particular area.
A Portuguese Case Study – MYCOMIX Project

“Exploring the toxic effects of MIXtures of MYCOtoxins in infant food and potential health impact”

(PTDC/DTP-FTO/0417/2012)
Children are exposed to mycotoxin mixtures through their diet and this constitutes a health threat.

Could this exposure be a health threat to children?
Case Study
To assess the risk using a holistic approach

Portuguese children exposure to multiple mycotoxins in food

I. Co-occurrence
II. Assessment of children exposure
III. Bioaccessibility using a standardized IVD model
IV. Potential interactive toxic effects with different endpoints
I - Co-occurrence

**Aim:**
Quantification of multiple mycotoxins (10) in cereal-based products primarily marketed for children in Lisboa region, PT (2014-15; n=52: BC-26, PC-20, BIS-6; maize, wheat, rice, multigrain)

**Methods:**
- HPLC-FD (AFTs and OTA)
- GC-MS (DON, NIV, T-2, HT-2)
- UPLC-MS/MS (FBs, ZEA)

Analytical method performance according to Reg (CE) 401/2006 (linearity, recoveries, LOD, LOQ)

**Collaborations:**
Alessandra Jager, Carlos Oliveira - University of S. Paulo, BR
Sara Cunha, José Fernandes - University of Porto, PT

Carla Martins
PhD student
Public Health, PT
II - Assessment of children exposure

Aim:
Assess the children exposure to multiple mycotoxins through consumption of cereal-based foods primarily marketed for children in Lisboa region, PT

Methods:
- Consumption data: 3-days food diary, Primary Health Care Unit near Lisbon, to characterize of the consumption pattern of children from 1 to 3 years old (n=75), approved by Ethical Commission, Port. Data Prot Authority
- Food consumption data included in a plataforma OPEN Portugal
- Multiplying consumption data with occurrence data
- Deterministic and Probabilistic approaches for exposure assessment
- Different strategies to treat the left censored data

Collaborations
Sonia Leal, Cidadela Primary Health Care Unit, Cascais, PT
Barbara Seljak, Josef Stephen Institute, SLO
Elsa Vasco and Baltazar Nunes, INSA, PT

Ricardo Assunção, PhD student Veterinary Sciences, PT
Case Study
To assess the risk using a holistic approach

Assessment of multiple mycotoxins in breakfast cereals available in the Portuguese market


- Breakfast cereals from Portuguese market revealed a high incidence of mycotoxins (below legislation levels)
- Different mycotoxins (two to seven) occurred simultaneously in breakfast cereals
- Twenty two different combinations of mycotoxins were detected in breakfast cereals
Case Study
To assess the risk using a holistic approach

- Daily exposure of children to ochratoxin A, fumonisins and trichothecenes showed no health risks to the children population considering individual mycotoxins.

- Exposure to aflatoxin B₁ (AFB₁) suggested a potential health concern for the high percentiles of intake (P90, P95 and P99).

- The combined margin of exposure (MoET) for the aflatoxins group could constitute a potential health concern and AFB₁ was the main contributor for MoET.
Case Study
To assess the risk using a holistic approach

Risk assessment of Portuguese children exposure to multiple mycotoxins through cereal-based products


Different mycotoxins (two to seven) occurred simultaneously in cereal-based products.

Estimated aflatoxins exposure suggested a potential adverse health effect for percentiles of intake above or equal to P50.

The obtained results suggested that future research actions should be set in motion in order to protect children health/ biomarkers.
OTA, ZEA and DON were the most commonly detected mycotoxins with 65%, 48% and 44% of analysed samples revealing values above the LOD, respectively.
• highest number of mycotoxins detected simultaneously was seven.

• combinations of two (OTA and DON; OTA and fumonisins) and four (aflatoxins, OTA and ZEA) mycotoxins were the most commonly detected.
<table>
<thead>
<tr>
<th>Toxins</th>
<th>Breakfast cereals</th>
<th>Processed cereal-based foods (flours)</th>
<th>Biscuits</th>
<th>Sum of estimated daily intake (ng/kg bw/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1</td>
<td>H2</td>
<td>H3</td>
<td>H1</td>
</tr>
<tr>
<td>AFM_{1}</td>
<td>0.005*</td>
<td>0.003*</td>
<td>0.001*</td>
<td>0.0064</td>
</tr>
<tr>
<td>AFB_{1}</td>
<td>0.012*</td>
<td>0.011*</td>
<td>0.011*</td>
<td>0.002</td>
</tr>
<tr>
<td>AFB_{2}</td>
<td>0.001*</td>
<td>0.001*</td>
<td>0.001*</td>
<td>0.002</td>
</tr>
<tr>
<td>AFG_{1}</td>
<td>0.003*</td>
<td>0.001*</td>
<td>0.000*</td>
<td>0.013</td>
</tr>
<tr>
<td>OTA</td>
<td>0.001*</td>
<td>0.010*</td>
<td>0.010*</td>
<td>0.0064</td>
</tr>
<tr>
<td>FB_{1}</td>
<td>60*</td>
<td>5.4*</td>
<td>53*</td>
<td>0.4</td>
</tr>
<tr>
<td>FB_{2}</td>
<td>10*</td>
<td>10*</td>
<td>8*</td>
<td>0.0</td>
</tr>
<tr>
<td>DON</td>
<td>2483*</td>
<td>2480*</td>
<td>2477*</td>
<td>1634</td>
</tr>
<tr>
<td>NIV</td>
<td>268*</td>
<td>156*</td>
<td>44*</td>
<td>0.0</td>
</tr>
<tr>
<td>ZEA</td>
<td>0.42</td>
<td>0.41</td>
<td>0.41</td>
<td>0.036</td>
</tr>
</tbody>
</table>

- H1 scenario (< LOD = LOD, worst case), the sum of daily intake through consumption of cereal-based products presented the highest value for DON (57.22 ng/kg bw/day), followed by FB_{1} (6.4 ng/kg bw/day), NIV (2.68 ng/kg bw/day), FB_{2} (1.0 ng/kg bw/day), ZEA (0.86 ng/kg bw/day), OTA (0.131 ng/kg bw/day) and AFM_{1} (0.069 ng/kg bw/day).
• results reinforced the outcomes obtained with the deterministic approach.
• When considered the simultaneous exposure to aflatoxins, MoET for percentiles P50 or higher revealed a potential health concern.

• A simulation considering a quarter (1/4) of the aflatoxins daily intake reduce the aflatoxins MoET values revealing that just percentiles of intake above P90 could be under health concern.

Carcinogenic: Values of MoE (ratio BMDL_{10}/exposure) or MoET below 10000 signifies that continuous exposure to such cereal-based products could pose serious adverse health effect to such susceptible groups of individuals, as young children.
all HQs were below one, *i.e.*, indicating no cause for concern for individuals exposed to mycotoxins through consumption of cereal-based products.

non-carcinogenic:
For the HQ (hazard quotients; exposure/reference dose=PTWI), a tolerable or a non-tolerable exposure level was considered if HQ was below or above one, respectively.

*Figure 2.* Characterization of risk associated with the exposure to ochratoxin A (OTA), fumonisins (FB1 and FB2), trichothecenes (DON and NIV) and zearalenone (ZEA) through the consumption of the three food products considered (breakfast cereals, processed cereal-based foods and biscuits). HQ and HI were derived from estimates obtained by the probabilistic approach [percentiles P50, P75, P90, P95 and P99 (P99)]. Data for HI scenario (H1: < LOD = LOD), as the worst case scenario, is presented.
III. Bioaccessibility using a standardized IVD model

**Aim:**
Determine the bioaccessibility of mycotoxins in cereal-based foods

**Methods:**
Standardized *in vitro* digestion model

- Simulated digestion fluids composition (saliva, gastric and intestinal)
- Enzyme activities in each fluid
- pH and incubation time at different digestion stages

**Collaboration:**
Didier Dupont, INRA, Cost Action INFOGEST (WG2, Short term scientific mission)

Didier Dupont,
French National Institute for Agricultural Research, INRA, FR

Standardized *in vitro* digestion method:

**Oral phase**

- 2 g of sample + 2 mL of oral fluid with:
  - 1.5 mM CaCl₂
  - Amylase (75 U/mL)
  - At pH 7.0

  2 min incubation in a rotation wheel, at 37°C

**Gastric phase**

- Oral sample + 4 mL of gastric fluid with:
  - 2000 U/mL Pepsin, 0.15 mM CaCl₂
  - At pH 3.0

  2 h incubation in a rotation wheel, at 37°C

**Intestinal phase**

- Gastric sample + 8 mL of intestinal fluid with:
  - 100 U/mL of pancreatin, 0.6 mM CaCl₂, 10 mM Bile.
  - At pH 7.0

  2 h incubation in a rotation wheel, at 37°C
  
  Digestion arrest with 1 mM of Pefabloc and liquid N₂.
  
  Samples kept at -80°C until analysis.

Minekus et al (2014) Food and Function 5:1113-1124
Harmonization of IVD models:

- Simulated digestion fluids composition (saliva, gastric and intestinal)
- Enzyme activities in each fluid
- pH and incubation time at different digestion stages
Case Study
To assess the risk using a holistic approach

Patulin and ochratoxin A co-occurrence and their bioaccessibility in processed cereal-based foods: A contribution for Portuguese children risk assessment

Ricardo Assunção A,b,c, Carla Martins A,b,d, Didier Dupont e,f, Paula Alvito A,b,*

- PAT and OTA were detected in 75% and 50% of the processed cereal-based food samples
- PAT and OTA were present simultaneously in 40% of analyzed samples
- A significant portion of PAT (52%) and especially of OTA (100%) can reach the small intestine
- Considering bioaccessibility and exposure results, PAT and OTA exposures are not expected to be of health concern.
PAT concentrations are significantly reduced during digestion process until reach intestine (internal exposure overestimated if not using bioaccessibility values).

OTA concentrations reach intestine almost unchanged.

### Table 3 - Bioaccessibility (%) results of PAT and OTA in processed cereal-based food samples (n=6), artificially contaminated.

<table>
<thead>
<tr>
<th></th>
<th>Patulin (μg/g)</th>
<th>Ochratoxin A (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>70 ± 3.2</td>
<td>95 ± 0.3</td>
</tr>
<tr>
<td>F2</td>
<td>42 ± 1.2</td>
<td>105 ± 1.5</td>
</tr>
<tr>
<td>F3</td>
<td>56 ± 1.8</td>
<td>97 ± 1.8</td>
</tr>
<tr>
<td>W/o 1</td>
<td>77 ± 1.9</td>
<td>98 ± 1.5</td>
</tr>
<tr>
<td>W/o 2</td>
<td>39 ± 0.7</td>
<td>102 ± 0.3</td>
</tr>
<tr>
<td>W/o 3</td>
<td>30 ± 2.5</td>
<td>102 ± 3.9</td>
</tr>
<tr>
<td>Mean</td>
<td>52 ± 4.2</td>
<td>100 ± 1.1</td>
</tr>
</tbody>
</table>

“F” and “W/o” samples represent samples with and without fruit in their content, respectively.
IV - Potential interactive toxic effects with different endpoints

Aims:
Determine the potential interactive effects of mycotoxins in cells, using different endpoints: cytotoxicity and intestinal membrane integrity.

Methods:
MTT using Caco-2 cells
Transepithelial electrical resistance (TEER)

Collaborations:
Susana Loureiro, Aveiro University, PT
Tor Lea, Charlotte Kleiveland, NMBU, NO
MJ Silva, H Louro, A Tavares, INSA, PT

Intestinal mucosa is the first biological barrier encountered by natural toxins in food - Caco 2 cells
Case Study
To assess the risk using a holistic approach

HOLISTIC APPROACH

- PAT affected Caco-2 barrier function by perturbation of ZO-1 levels.
- Phosphorylation of MLC2 accompanied PAT barrier function perturbation.
- Low doses of PAT inhibited T cell proliferation induced by a polyclonal activator.
- Epithelium and immune cells of the intestinal mucosa are important targets for the toxic effects of mycotoxins.
Case Study
To assess the risk using a holistic approach

Insights into individual and combined toxic effects of patulin and ochratoxin A on human intestinal cells (in preparation)

PAT (IC$_{50}$=16 uM) is significantly more toxic to Caco-2 cells, compared to OTA (IC$_{50}$=145 uM).

Combined effect to these two mycotoxins suggests an additive effect according to citotoxicity data, however synergism at low doses by TEER data (health concern).
Case Study
To assess the risk using a holistic approach

- Existence of antagonistic toxic effects between OTA and AFM₁ in a human cell line representative of the primary site of contact of both toxins, i.e., the intestine;

- Both components, food monitoring and interactions characterization in in vitro models are complimentary and contribute to prevent mycotoxins-associated diseases, particularly, on the long-term (e.g., cancer).
Case Study
To assess the risk using a holistic approach

Multi-mycotoxin determination in baby foods and *in vitro* combined cytotoxic effects of aflatoxin M₁ and ochratoxin A

A.M. Tavares¹,², P. Alvito³, S. Loureiro⁴, H. Louro⁴ and M.J. Silva⁴

- Existence of antagonistic toxic effects between OTA and AFM₁ in a human cell line representative of the primary site of contact of both toxins, i.e., the intestine;

- Both components, food monitoring and interactions characterization in *in vitro* models are complimentary and contribute to prevent mycotoxins-associated diseases, particularly, on the long-term (e.g., cancer).
The present work underlines the need to adopt a holistic approach for multiple mycotoxins risk assessment integrating data from exposure, bioaccessibility and toxicity domains, in order to contribute to a more accurate risk assessment.

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Thank you for your attention!