

# Biomonitoring and toxicological assessment of Ochratoxin A and Citrinin in a sex-gender perspective: The OTABioTox Project

Lucia Coppola<sup>1</sup>, Giuseppina Scialò<sup>2</sup>, Francesca Debegnach<sup>2</sup>, Gianluca Frustagli<sup>3</sup>, Viviana Iacovelli<sup>1</sup>, Gabriele Lori<sup>1</sup>, Martina Enza Grieco<sup>2</sup>, Francesca Maranghi<sup>1</sup>, Alessia Tamaro<sup>1</sup>, Roberta Tassinari<sup>1</sup>, Enrica Fabbrizi<sup>4</sup>, Patrizia Di Matteo<sup>4</sup>, Lorella Ciferri<sup>5</sup>, Marco Dolci<sup>6</sup>, Barbara Gigli<sup>6</sup>, Alessandra Di Stasio<sup>6</sup>, Beatrice Murri<sup>6</sup>, Giacomo Toffol<sup>7</sup>, Alessandro Ciccotosto<sup>7</sup>, Miriana Callegari<sup>7</sup>, Margherita Guarnieri<sup>8</sup>, Angela Pasinato<sup>7</sup>, Barbara De Santis<sup>2</sup> and Cinzia La Rocca<sup>1</sup>

<sup>1</sup>Centro di Riferimento per la Medicina di Genere, Istituto Superiore di Sanità, Rome, Italy

<sup>2</sup>Dipartimento Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria, Istituto Superiore di Sanità, Rome, Italy

<sup>3</sup>Servizio Grandi Strumentazioni e Core Facilities, Istituto Superiore di Sanità, Rome, Italy

<sup>4</sup>Ospedale di Civitanova Marche, Unità Operativa Semplice di Pediatria, Civitanova Marche (MC), Italy

<sup>5</sup>Azienda Sanitaria Territoriale Fermo, Porto San Giorgio (FM), Italy

<sup>6</sup>Azienda Sanitaria Territoriale Macerata, Civitanova Marche (MC), Italy

<sup>7</sup>Associazione Culturale Pediatri, Narbolia (OR), Italy

<sup>8</sup>Società Italiana Pediatria, Rome, Italy

## Abstract

**Introduction.** Ochratoxin A (OTA) and citrinin (CIT) are toxic compounds produced by fungi that may contaminate a variety of food, posing significant health risks, particularly following chronic exposure. OTA, a potent nephrotoxin, is widely present in cereals, dried fruits, coffee and animal-derived products; CIT, which often co-occurs with OTA, also exhibits nephrotoxic effects.

**Methodology.** This project aims to evaluate the exposure to OTA and CIT in adults and children of both sexes, and in breastfeeding women, and to investigate OTA toxicological effects using a juvenile animal model. The biomonitoring study analyzed OTA and CIT in urine, and OTA in breast milk. It also involved the evaluation of biomarkers of effect and the administration of food frequency questionnaires to assess dietary intake. The toxicological study employed an innovative animal model to identify early biomarkers of renal and endocrine effects and to elucidate OTA's mechanisms of action.

**Results.** The findings contribute to a deeper understanding of risk characterization and support the development of public health strategies aimed at minimizing OTA and CIT exposure.

## Key words

- mycotoxins
- children exposure
- human milk
- nephrotoxicity
- biomarkers

## INTRODUCTION

Mycotoxins are toxic chemical compounds produced by certain fungal species under specific environmental conditions [1]. The toxigenic fungi colonize agricultural commodities, spreading mycotoxin contamination into the food chain. These contaminants persist through food processing, and the contamination is also transferred to the derived food products posing significant risk to both humans and animals [2, 3].

Dietary exposure to mycotoxins represents a significant health concern, especially in cases of chronic exposure, which has been linked to a range of adverse health effects [4]. Due to cumulative exposure over a lifetime, mycotoxins can profoundly impact human health, causing a broad spectrum of toxic effects including carcinogenicity, nephrotoxicity, hepatotoxicity, estrogenic effects, neurotoxicity, and disruption to reproductive and immune system functions [1, 5].

Among the mycotoxins of greatest concern to risk assessors, ochratoxin A (OTA) is one of the most widely distributed. It is produced by ubiquitous fungal genera such as *Aspergillus* and *Penicillium* and several toxigenic species (e.g., *A. niger*, *A. ochraceus*, *A. carbonarius* and *P. verrucosum*) can occur in a broad range of plant derived foods products all entering human and animal diets. These commodities include cereals, dried nuts, spices, dried fruits, coffee, cocoa, licorice and food derivatives, including supplements (e.g., green coffee, goseberry, and licorice derived supplements). Furthermore, OTA contamination has also been detected in certain cured animal-derived products (such as ham, salami). In these cases, contamination can occur during meat maturation and curing process, where storage conditions may be viable to OTA production. Like many other mycotoxins, OTA is highly resistant to processing and heat treatments, contributing to its persistence in the food chain [6].

*In vivo* studies showed that OTA has been associated with nephrotoxic, carcinogenic, teratogenic, immunotoxic and developmental effects, with kidney representing the critical target organ. OTA has been identified as a key causative agent in the development of porcine nephropathy, but the available epidemiological evidence remains insufficient to definitively classify it as a renal carcinogen in humans or directly link it to Balkan Endemic Nephropathy (BEN) or other unexplained endemic nephropathies [7-10]. Indeed, OTA is able to bind plasma-proteins, mainly albumin, with consequent reduction of excretion rate and accumulation in renal tissue. Albeit to a lesser extent, liver, muscle and fat are also involved in OTA toxicity in terms of signalling modulation of apoptosis, inflammation and stress responses [7, 11].

OTA showed also genotoxic effects both *in vitro* and *in vivo* studies, although the mechanisms are still unclear, and direct and indirect genotoxic and non-genotoxic modes of action may contribute to tumor development <https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efa.2020.6113>. The International Agency for Research on Cancer (IARC) classified OTA as “possibly carcinogenic for humans” (Group 2B), with carcinogenicity being the result of interactions among several epigenetic mechanisms [12, 5]. Because recent studies have raised uncertainty regarding the mode of action for kidney carcinogenicity, the European Food Safety Authority (EFSA) deemed it inappropriate to maintain the tolerable weekly intake (TWI) established in 2006 as the health-based guidance value (HBCV) [13]. Indeed, the Margin of Exposure (MOE) approach was considered most appropriate, providing a safety margin (10000 for carcinogenic compounds) below which the public health concern can be considered low, even considering overall uncertainties [7]. The use of the Benchmark Dose Lower Confidence Limit (BMDL<sub>10</sub>), the dose that causes a 10% increase in the incidence of a specific effect in an animal population, was proposed for neoplastic and non-neoplastic effects (BMDL10G 14.5 µg/kg body weight (bw) and BMDL10NG 4.75 µg/kg bw, respectively).

Citrinin (CIT), a mycotoxin with well-documented nephrotoxic properties, is commonly detected in food

in the co-occurrence with OTA, and it is known to have synergistic effects with OTA, potentially exacerbating kidney damage [6, 14, 15]. It is produced by several species of *Aspergillus*, *Penicillium*, and *Monascus*, and it is commonly found in stored grains. A No-Observed-Adverse-Effect Level (NOAEL) of 20 µg/kg bw per day was identified from a 90-day study in rats. However, due to a lack of data, EFSA did not establish a HBCV for CIT. Instead, a level of no concern for nephrotoxicity was set at 0.2 µg/kg bw per day, reflecting the limited understanding of its potential effects. Despite this provisional threshold, concerns about CIT possible genotoxic and carcinogenic properties remain unresolved. Risk characterization for CIT has therefore focused on limiting its concentrations in grains to levels that would keep dietary exposure levels below the no concern threshold for nephrotoxicity. EFSA emphasized the need for further research to better define the risk associated with CIT and since then several studies have expanded the understanding of CIT toxic effect confirming its impact on multiple organs and systems [16]. In fact, in addition to nephrotoxicity, which remains the major effect, also hepatotoxic, cardiotoxic, neurotoxic, and reproductive effects have been reported [17-21]. Studies reported CIT-induced liver and heart toxicity, including fatty liver disease and cardiac tissue damage, linked to mitochondrial impairment and oxidative stress confirming hepatotoxicity and cardiotoxicity [17-19], while the genotoxic and mutagenic potential of CIT remains controversial. Some *in vitro* studies show DNA damage, cell cycle arrest, and autophagy induction [17]. However, IARC classifies CIT as “not classifiable as to carcinogenicity in humans” (Group 3) due to insufficient evidence [21].

EFSA has emphasized the need for more comprehensive data on human exposure to OTA and CIT, particularly in vulnerable populations like children and adolescents, who are more susceptible to the toxic effects of these contaminants [7, 13]. In this respect, human biomonitoring studies (HBM) using biological fluids are useful for assessing internal doses of contaminants because of combined exposure. Moreover, HBM together with dietary exposure assessment represent an integrated approach for a more accurate and comprehensive evaluation of human exposure.

The Italian National Committee for Food Safety, which addresses urgent and critical topics public health issues, has raised concerns about the exposure of younger age groups to OTA through the consumption of certain foods, particularly cereals, cheeses and pork derived cured meats [22]. National official control and monitoring plans have consistently reported the presence of OTA in a variety of foodstuff, in particular coffee, cocoa, ham and more recently certain varieties of hard cheese. Among the different exposure scenarios, particular attention should be given to breastfed infants, who may be exposed to mycotoxins such as OTA through lactation. Breast milk can serve as a potential exposure pathway, transferring OTA from mothers to their children and thus representing an additional risk for this especially vulnerable population group [7]. This is particularly concerning since children have higher

metabolic rates and immature detoxification system, making them potentially more vulnerable to the accumulation of mycotoxins in their developing organs.

In this context, exposure evaluation through biomonitoring studies in the general population and vulnerable groups, such as children, is essential to address existing gaps in risk assessment. In this framework, the project "Ochratoxin A: risk assessment in population groups through biomonitoring and toxicological characterization" (OTABioTox Project) aims to assess the exposure and toxicological effects of OTA and CIT through an integrated approach which includes: a) a biomonitoring study in adults and children of both sexes, as well as lactating mother-child pairs, including evaluation of selected kidney biomarkers; b) a dietary exposure evaluation, with a focus on foods that are particularly susceptible to OTA and CIT contamination, such as cereals, dried fruits, and cured meats; c) a toxicological study using male and female rats in the juvenile phase of life, corresponding to the childhood/peripubertal age in humans, treated at realistic dose levels of OTA derived from children exposure.

Upon the evidence of gender-based differences in the absorption and detoxification of contaminants, the project will possibly explore potential sex differences in human internal dose content and toxicological susceptibility [23, 24].

By combining biomonitoring, exposure assessment, and toxicological studies, this research seeks to bridge the knowledge gap in the exposure risks posed by OTA and CIT, considering adults and also vulnerable groups, such as young population and breastfeeding women.

## METHODOLOGY

### **Biomonitoring study**

The HBM in OTABioTox Project aims to measure the internal doses of OTA and CIT in urine and milk samples. The internal dose provides data at an individual level that helps to overcome the limitations associated with the heterogeneous contamination of food, sampling variability and constraints, and the availability of food and consumption data.

The elimination of OTA from the body is slow and incomplete, with variable rates of excretion through urine, blood, and breast milk collectively contributing to its persistence and potential for chronic health effects. OTA is typically excreted in urine with very low rates ranging approximately 2-3% of the ingested dose. In kidney, OTA is partially reabsorbed due to its binding with albumin [25]. Importantly, OTA has also an excretion route via breast milk, albeit at low concentrations (lower than in blood, comparable with urine) and it provides a route of exposure for nursing infants. The presence of OTA in breast milk underscores the risk of early-life exposure, especially in regions where dietary contamination is common [26].

CIT and its main metabolite dihydrocitrinone (DH-CIT) exhibit a high urinary excretion rate with about 40% of the ingested dose eliminated within 24 hours. This makes urine a particularly reliable matrix for CIT exposure assessment [20, 27, 28]. There is currently insufficient evidence to confirm significant transfer of

CIT to breast milk, so this pathway remains negligible.

To assess exposure to OTA and CIT, including their metabolites, a total of 350 subjects has been recruited across diverse demographic groups. The recruitment details are as follows:

- children aged 5-14 years: 70 boys and 70 girls;
- adults aged 18-65 years: 70 men and 70 women;
- breastfeeding women: 70 subjects.

In recent years, HBM studies have increasingly focused on evaluating biomarkers of effect, which can provide a link between internal exposure to contaminants and the related adverse health effects or diseases. They can increase the biological plausibility of the associations and indicate a possible mode of action. In this respect, the biomarkers of effect can improve the risk assessment of chemicals [29].

In OTABioTox project, albumin, creatinine and beta2-microglobulin are considered as kidney biomarkers associated to OTA and CIT exposure and measured in urine samples of the enrolled subjects. OTA is known binding serum albumin with a reduction of the clearance and a persistence of OTA in the body. However, a lower level of albumin increased OTA hepatotoxicity and nephrotoxicity [30]. Higher levels of beta2-microglobulin in the urine, affecting renal reabsorption function, was associated to higher OTA level in human [31]. Creatinine values will be also used to normalize OTA and CIT concentration values.

Family pediatricians in the National Health System and hospital pediatricians collaborated in recruiting patients. This physician network was established under the European LIFE PERSUADED biomonitoring project (Phthalates and bisphenol A biomonitoring in Italian mother-child pairs: link between exposure and juvenile diseases; ENV/IT/000482) and the Finalized Research program (Integrated Approach to Evaluate Children Agricultural Pesticide Exposure and Health Outcome; RF-2016-02364628) therefore, the healthcare professionals possess extensive experience in conducting such studies across the national territory.

Healthcare professionals were provided with informative materials to instruct themselves, and subsequently recruited subjects and their families for enrollment in the study:

- food frequency questionnaire;
- description of the study for the physicians;
- description of the study for enrolled adults and children;
- a fact sheet for children and mothers;
- instructions on how to complete the FFQ;
- instructions for collecting urine and breast milk samples;
- privacy information and legal basis for processing personal data;
- informed consent for parents.

### **Food Frequency Questionnaire and dietary exposure**

A paper-based questionnaire was designed to collect personal, residential information, as well as dietary habits. The first section requested personal details such as age, body weight and physical activity level. The second section consisted of a specially tailored Food Frequen-

cy Questionnaire (FFQ) aimed at capturing participant dietary habits. The FFQ included tables for each food category covered in national and European control activities [13]. In the FFQ, each participant was asked to specify the food items consumed, indicating the frequency (daily, weekly, monthly) and usual portion size (small, medium, large, or specific quantity). Once validated, the final version of the FFQ was distributed to the pediatricians overseeing participant enrollment.

The completed FFQs were reviewed for consistency, and the data were entered into an online database. This database was designed to efficiently manage the volume of data and ensure consistency during data entry. Due to the extensive number of fields in each questionnaire (more than 130), general-purpose software such as spreadsheets was considered inadequate. Therefore, a custom web application was developed to provide an optimized and user-friendly interface, specifically designed to minimize input errors and allow full data editing capabilities. The application was built using the HTML-Javascript-PHP-MySQL-Apache stack, utilizing open-source software. Data entered into the application were stored in a relational database on a server and subsequently exported serialized via dedicated functions into Tab-Separated Values (TSV) files, which could then be processed further using scientific statistical analysis software.

#### **Analytical determination of OTA and CIT**

Urine and breast milk are valuable biological matrices for assessing exposure; however, it is essential to have appropriate, validated and accurate analytical methods to reliably measure the targeted biomarkers [25, 32]. The most important challenge in the analysis of mycotoxins in biological fluids is the extremely low concentrations of the analytes in the specimen. The choice of suitable analytical protocol shall be focused on the identification of appropriate limits of detection (LOD) and quantification (LOQ) values [33]. Liquid chromatography coupled with tandem mass spectrometry (e.g., LC-MS/MS) enable a sensitive and accurate detection of biomarkers in biological fluids also in condition of low excretion rate, such as the one of the OTA in urine samples. These capabilities of modern analytical techniques are essential for reliable biomonitoring studies [34].

In OTABioTox Project, urinary OTA and CIT, as well as OTA in breast milk, were analyzed in a two-step analytical approach. In the first step, a screening method was employed to detect the presence of OTA, CIT and/or other biomarkers in the biological matrices. Upon obtaining a positive result in the screening, a second step for specific quantification was performed applying sample dilution in phosphate-buffered saline (PBS) followed by immunoaffinity columns (IAC) cleanup prior injection.

The IAC cleanup employs monoclonal antibodies to selectively isolate and concentrate the mycotoxin while removing matrix interferences. This approach is particularly well-suited for targeting and quantify single or a limited number of analytes in complex biological matrices such as urine and milk [35].

The determination was performed by LC-MS/MS; the triple quadrupole, set in positive mode with MRM (Multiple Reaction Monitoring) acquisition mode, enables high accuracy, selectivity and sensitivity which is necessary as the concentration of mycotoxins in biological matrices is generally lower than the contamination levels found in food. The method was validated and met satisfactory performance of precision and trueness in both urine and breast milk matrices. The LOQ was established at 0.004 ng/g for OTA and 0.009 ng/g for CIT in urine and 0.008 ng/g for OTA in breast milk.

#### **Toxicological study**

An *in vivo* toxicological study is proposed, using an innovative juvenile animal model that corresponds to the pre- and peri-pubertal developmental stages in children [36]. This stage of the life cycle is particularly vulnerable and susceptible to the potential adverse effects of chemical substances, as body systems are still developing and maturing, while dietary exposure is higher compared to adults. This model enables the early detection of alterations, the study of underlying mechanisms and the identification of early effect biomarkers through the analysis of tissue, serum and molecular markers, and transcriptomics [37, 38]. The animal study was executed in accordance with Directive 2010/63/EU, the Italian Legislative Decree n. 26 of 4 March 2014, the OECD Principles of Good Laboratory Practice. The design and sample size of the animal studies were chosen according to 3R principles and ARRIVE guidelines 2.0 [39].

18 dams of CD1 mice (Envigo, Italy) with offspring will be kept under standard laboratory conditions (22±0.5 °C room temperature, 50%-60% relative humidity, 12 h of dark-light alternation with 12-14 air changes per hour) with water and food available *ad libitum*. Specific rodent diet was select to minimize the OTA e mycotoxin exposure (AIN-76A Purified Diet NOT RADIABLE purchased from Mucedola, Italy). After seven days of acclimatizing, weaning F1 mice were separated by the dams and housed in cages to obtain 4 groups of 18 mice/sex/group, treated with 3 dose levels of OTA dissolved in corn oil and a control group treated with vehicle only for 28 days (5 days/week) *per os* by gavage, from weaning (postnatal day, PND, 22 to sexual maturity PND60).

The selected dose levels are 1 (low), 100 (medium) and 1000 (high) µg/kg bw per day. The low dose level was derived based on dietary exposure to OTA at the 95th percentile (i.e., among high consumers), which was estimated to reach up to 0.0517 µg/kg bw per day [7] that corresponds to 0.63 µg/kg bw per day in mice [40], rounded to 1 µg/kg bw per day as a practical value. The medium dose level derived from the LOAEL (Lowest Observed Adverse Effect Level) of 8 µg/kg bw day in pigs, corresponds to 93 µg/kg in mice, assuming interspecies extrapolation [40] and rounded to 100 µg/kg bw per day as a practical value. High dose level was obtained by a toxicological study on mice where OTA was administered orally for 28 days with toxicological effect in systems other than the renal one without clinical sign of toxicity [41].

Body weight, feed consumption, kidney, liver, thyroid, ovary, uterus, testis, hypothalamus absolute and relative weights will be measured. Serum clinical and biochemical parameters (albumin, globulin, total bilirubin, gamma-glutamyl transferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, creatine kinase, creatinine, glucose, triglycerides, calcium, phosphate, magnesium, potassium, and sodium) will be also assessed. In addition, a proteomic analysis of liver tissue will be conducted.

Specific effects of endocrine system will be analyzed by:

- hormones serum levels (e.g., thyroid-stimulating hormone, TSH, thyroxine, estradiol, testosterone);
- histopathological analysis (thyroid, reproductive organs and liver);
- gene expression of selected hypothalamus marker (TSH, luteinizing hormone, follicle-stimulating hormone).

Renal toxicity endpoints will be studied by:

- kidney DNA damage by comet assay;
- renal oxidative stress enzymes (e.g., catalase, superoxide dismutase, glutathione peroxidase 1);
- serum renal toxicity markers (e.g., proliferating cell nuclear antigen, vimentin);
- kidney expression of DNA repair and general toxicity genes (e.g., Ercc1, ERCC excision repair 1; Ogg1, 8-oxoguanine DNA-glycosylase 1; and Kim1, Kidney Injury Molecule 1; respectively);
- kidney histopathological analysis.

The sample size calculation is performed using the G\*Power 3.1.9.7 software on a comparison between two groups (treatment dose vs control) using the Mann-Whitney test, with a one-tailed significance level of  $\alpha=0.0167$  (corresponding to  $\alpha=0.05$  with Bonferroni correction applied to the three comparisons between each treatment dose and the control) and a power of  $1-\beta=0.80$ . The key endpoint used for the sample size estimation was the change in the activity of renal oxidative stress-related enzymes, as reported in a previous study involving oral administration of OTA to male mice for 45 days [42]. Table 1 shows the results of the sample size calculation (N) based on the selected enzymes.

**Table 1**

Calculation of sample size (N) according to decreased activity of renal oxidative stress enzymes

Endpoint	Control (0 mg/kg bw day) (means±SD)	OTA (1.5 mg/kg bw day) (means±SD)	N
Catalase activity (μmoles H <sub>2</sub> O <sub>2</sub> consumed/mg protein/min)	32.72±1.26	26.82±0.72	4
Superoxide dismutase activity (units/mg protein)	3.34±0.15	2.79±0.06	4
Glutathione peroxidase activity (μmoles NADPH consumed/mg protein/min)	0.38±0.09	0.25±0.03	8

OTA: ochratoxin A.

GSH activity is most conservative condition and a 34% reduction in the mean value is observed, corresponding to a Cohen's d effect size of 1.94 (very large effect). However, considering the selected dose levels and the short time of administration, it is important to detect smaller differences, starting from 20%, which are plausibly expected to occur in the groups treated with lower doses. For example, comparing  $0.38\pm0.09$  (control group) vs  $0.30\pm0.03$  (20% reduction) to GSH activity, the sample size is N=15. The final sample size per sex/group is increased to 18, taking into account that the expected mortality rate is no more than 5% and high the number of biomarkers to be measured in mouse serum.

### Data analysis plan

Statistical analyses will be performed using Stata 16.0 software (StataCorp), setting significance at  $p < 0.05$ . Descriptive statistics will be calculated for each group of population. Sex-stratified analysis will be performed both in adults and children using parametric or non-parametric tests, when non normal distribution is verified (Mann-Whitney test). The same tests will be used to compare the population groups. Confounders (e.g., age, breastfeeding frequency, renal function variability, diet reporting bias) will be considered in the analysis. Age will be considered as covariate to obtain age-adjusted estimates in all groups, whereas breast feeding frequency will be included in data from breastfeeding women. Urinary OTA and CIT concentrations will be normalized to creatinine concentration, measured in the corresponding urine sample.

If data are missing from the subject's questionnaire, the operators responsible for data quality control will identify the pediatrician who enrolled the subject through the alphanumeric code of questionnaire and will request the completion of the unanswered questions. The pediatrician will then contact the patient to obtain the missing information. This process ensures that all fields are completed, thus avoiding the exclusion of data. If no response is received, the incomplete data will be excluded. Correlations between OTA/CIT levels and renal biomarkers will be assessed using Pearson/Spearman coefficients and multiple regression models. The association between dietary intake and OTA/CIT levels will be analyzed through linear/logistic regression and multivariate analysis (PCA). Data from toxicological study will be analyzed using ANOVA with post-hoc tests. Risk assessment will be conducted by calculating the Margin of Exposure (MOE) for OTA and CIT comparing the estimated provisional dietary intake of OTA and CIT with established safety thresholds.

### State of the art

Ethical approval was obtained by the National ethics committee for clinical trials of public research bodies (EPR) and other national public institutions at the Istituto Superiore di Sanità (AOO-ISS-30/06/2023-0030990 Class: PRE BIO CE 01.00). To date, the enrollment of the subjects was completed, obtaining samples and FFQs filled in. The FFQs were reviewed and, in case of missing data, the pediatricians involved in the en-

rolment asked the subjects. All the data has now been transferred to the online database.

The animal study protocol was approved by the Italian Ministry of Health (879/2023-PR). The study has been completed, and general toxicological data are summarized in the Excel file. Statistical analysis is currently ongoing. Animal serum and tissues have been stored.

Analytical tests of human biological fluids and serum and tissues of mice are currently in progress.

## EXPECTED RESULTS

The main outcome of the OTABioTox Project will be the development of a model to evaluate the exposure and toxicological effects of OTA and CIT through an integrated approach. The integration of the scientific results will provide an estimate of the predictive risk of the potential effects of exposure to OTA and CIT on the health of different population groups, taking into account age and sex.

Relevant results will be achieved for OTA and CIT in the frame of risk assessment:

As for *human exposure*:

evaluation of CIT and OTA urinary levels in children and adults of both sexes, as well as in breastfeeding women;

- association of the internal levels with the food consumed according to data acquired from the FFQ to ascertain if group of foods are triggers for internal levels;
- estimation of OTA and CIT provisional dietary exposure upon standard daily urine volumes, excretion data and body weights values;
- association between the observed exposure levels and effect biomarkers of renal function to highlight potential health impacts in children and adults, considering sex differences;
- evaluation of the OTA levels in breast milk of the enrolled breastfeeding women;
- estimation of exposure to OTA and CIT in breastfed children, who have breast milk as the sole food;
- correlation between OTA and CIT levels in breast milk with the corresponding values measured in the urine of the same breastfeeding woman to identify

possible associations of critical ratios of contamination levels in the two biological matrices;

- calculation of the Margin of Exposure (MOE) for OTA, based on the comparison of BMDL10 for non-neoplastic and neoplastic effects with the calculated exposure values; whereas for CIT, comparison of the tolerable dose for nephrotoxicity with the calculated exposures.

As for *toxicological characterisation*:

- evaluation of OTA effects in a juvenile rodent model at dose levels compatible with estimated exposure levels in children;
- identification of early effect biomarkers for the renal and endocrine systems;
- evaluation of sex-related differences in susceptibility;
- study of the mechanisms by specific omics technologies.

The selected endpoints will primarily focus on the endocrine system. Additionally, the mechanisms of action and DNA damage induction by OTA at the renal level will be investigated to identify and characterize early markers of damage associated with carcinogenesis processes. Finally, the use of both male and female mice will allow the identification of sex-specific effects on the endocrine system and renal damage.

In conclusion, these findings will provide new insights into OTA and CIT exposure in adults and children, increasing the understanding of their toxicological effects and supporting the development of evidence-based strategies for risk mitigation and public health protection.

## Funding sources

The project OTABioTox was funded by Italian Ministry of Health (ID Fasc. 8S04, CUP: I85E22000710005, 2022-2025).

## Conflicts of interest statement

The Authors declare no conflict of interest.

Received on 31 October 2025.

Accepted on 9 December 2025.

## REFERENCES

1. Mafe AN, Büsselberg D. Mycotoxins in food: cancer risks and strategies for control. *Foods*. 2024;13(21):3502. doi: 10.3390/foods13213502
2. World Health Organization. Mycotoxins fact sheet. Geneva: WHO; 2023. Available from: <https://www.who.int/news-room/fact-sheets/detail/mycotoxins>.
3. Rahim K, Farooq A, Farinazleen MG. A comprehensive review of mycotoxins: toxicology, detection, and effective mitigation approaches. *Heliyon*. 2024;10(8):e28361. doi: 10.1016/j.heliyon.2024.e28361
4. Goessens T, Mouchtaris-Michailidis T, Tesfamariam K, et al. Dietary mycotoxin exposure and human health risks: a protocol for a systematic review. *Environ Int*. 2024;184:108456. doi: 10.1016/j.envint.2024.108456
5. Awuchi CG, Ondari EN, Nwozo S, et al. Mycotoxins' toxicological mechanisms involving humans, livestock and their associated health concerns: a review. *Toxins (Basel)*. 2022;14(3):167. doi: 10.3390/toxins14030167
6. Ostry V, Malir F, Ruprich J. Producers and important dietary sources of ochratoxin A and citrinin. *Toxins (Basel)*. 2013;5(9):1574-86. doi: 10.3390/toxins5091574
7. Schrenk D, Bodin L, Chipman JK, del Mazo J, Grasl-Kraupp B, et al. Scientific opinion on the risk assessment of ochratoxin A in food. *EFSA J*. 2020;18(5):6113. doi: 10.2903/j.efsa.2020.6113
8. Castegnaro M, Canadas D, Vrabcheva T, Petkova-Bocharova T, Chernozemsky IN, Pfohl-Leskowicz A. Balkan endemic nephropathy: role of ochratoxins A through biomarkers. *Mol Nutr Food Res*. 2006;50(6):519-29. doi: 10.1002/mnfr.200500182

9. Pfohl-Leszkowicz A, Manderville RA. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. *Mol Nutr Food Res.* 2007;51:61-99. doi: 10.1002/mnfr.200600137
10. Habschied K, Kanižai Šarić G, Krstanović V, Mastanjević K. Mycotoxins-biomonitoring and human exposure. *Toxins (Basel).* 2021;13(2):113. doi: 10.3390/toxins13020113
11. Marin-Kuan M, Cavin C, Delatour T, Schilter B. Ochratoxin A carcinogenicity involves a complex network of epigenetic mechanisms. *Toxicol.* 2008;52(2):195-202. doi: 10.1016/j.toxicol.2008.04.166
12. International Agency for Research on Cancer. Ochratoxin A. In: *IARC Monographs on the evaluation of carcinogenic risks to humans.* Vol. 56. Lyon: IARC; 1993. p. 26-32.
13. EFSA Panel on Contaminants in the Food Chain. Opinion of the Scientific Panel on Contaminants in the Food Chain (CONTAM) related to ochratoxin A in food. *EFSA J.* 2006;4(6):365. doi: 10.2903/j.efsa.2006.365
14. Awuchi CG, Ondari EN, Ogbonna CU, et al. Mycotoxins affecting animals, foods, humans, and plants: types, occurrence, toxicities, action mechanisms, prevention, and detoxification strategies – a revisit. *Foods.* 2021;10(6):1279. doi: 10.3390/foods10061279
15. Ali N. Co-occurrence of citrinin and ochratoxin A in rice in Asia and its implications for human health. *J Sci Food Agric.* 2018;98(6):2055-9. doi: 10.1002/jsfa.8667
16. EFSA Panel on Contaminants in the Food Chain. Scientific opinion on the risks for public and animal health related to the presence of citrinin in food and feed. *EFSA J.* 2012;10(3):2605. doi: 10.2903/j.efsa.2012.2605
17. de Oliveira Filho JWG, Islam MT, Ali ES, et al. A comprehensive review on biological properties of citrinin. *Food Chem Toxicol.* 2017;110:130-41. doi: 10.1016/j.fct.2017.10.002
18. Zargar S, Wani TA. Food toxicity of mycotoxin citrinin and molecular mechanisms of its potential toxicity effects through the implicated targets predicted by computer-aided multidimensional data analysis. *Life.* 2023;13(4):880. doi: 10.3390/life13040880
19. Zhang H, Ahima J, Yang Q, Zhao L, Zhang X, Zheng X. A review on citrinin: its occurrence, risk implications, analytical techniques, biosynthesis, physiochemical properties and control. *Food Res Int.* 2021;141:110075. doi: 10.1016/j.foodres.2020.110075
20. Narváez A, Izzo L, Rodríguez-Carrasco Y, Ritieni A. Citrinin dietary exposure assessment approach through human biomonitoring high-resolution mass spectrometry-based data. *J Agric Food Chem.* 2021;69(22):6330-8. doi: 10.1021/acs.jafc.1c01776
21. International Agency for Research on Cancer. Citrinin. In: *IARC Monographs on the evaluation of carcinogenic risks to humans.* Vol. 40. Lyon: IARC; 1986. p. 67
22. Comitato Nazionale per la Sicurezza Alimentare. Unregulated mycotoxins: Ochratoxin A in cheeses and pork products. *Zenodo.* 2021. doi: <https://doi.org/10.5281/zenodo.5537074>
23. Gochfeld M. Framework for gender differences in human and animal toxicology. *Environ Res.* 2007;104(1):4-21. doi: 10.1016/j.envres.2005.12.005
24. La Rocca C, Tassinari R, Tait S, Carè A, Maranghi F. Sex and gender differences in toxicological studies. In: Wexler P (Ed). *Encyclopedia of toxicology.* 4th ed. Cambridge (MA): Academic Press; 2024. p. 483-6. doi: 10.1016/B978-0-12-824315-2.00731-4
25. Kőszegi T, Poór M. Ochratoxin A: molecular interactions, mechanisms of toxicity and prevention at the molecular level. *Toxins.* 2016;8(4):111. doi: 10.3390/toxins8040111
26. Das TA, Hafsa JM, Hasan A, et al. Occurrence of ochratoxin A in breast milk and urine samples of nursing mothers in Bangladesh. *Mycotoxin Res.* 2024;40(1):135-46. doi: 10.1007/s12550-023-00510-5
27. Degen GH, Ali N, Gundert-Remy U. Preliminary data on citrinin kinetics in humans and their use to estimate citrinin exposure based on biomarkers. *Toxicol Lett.* 2018;282:43-8. doi: 10.1016/j.toxlet.2017.10.006
28. Kamle M, Mahato DK, Gupta A, Pandhi S, Sharma N, Sharma B, Mishra S, Arora S, Selvakumar R, Saurabh V, et al. Citrinin mycotoxin contamination in food and feed: impact on agriculture, human health, and detection and management strategies. *Toxins (Basel).* 2022;14(2):85. doi: 10.3390/toxins14020085
29. Rodríguez-Carrillo A, Mustieles V, Salamanca-Fernández E, et al. Implementation of effect biomarkers in human biomonitoring studies: a systematic approach synergizing toxicological and epidemiological knowledge. *Int J Hyg Environ Health.* 2023;249:114140. doi: 10.1016/j.ijheh.2023.114140
30. Kuhn M, Hassan R, González D, et al. Role of albumin in the metabolism and excretion of ochratoxin A. *Mycotoxin Res.* 2024;40:433-45. doi: 10.1007/s12550-024-00538-1
31. Yordanova P, Wilfried K, Tsolova S, Dimitrov P. Ochratoxin A and  $\beta$ 2-microglobulin in BEN patients and controls. *Toxins (Basel).* 2010;2(4):780-92. doi: 10.3390/toxins2040780
32. Chen M, Liu W, Xiang P, et al. Development of an LC-MS/MS method for the determination of multiple mycotoxins in human urine. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess.* 2023;40(7):917-27. doi: 10.1080/19440049.2023.2222842
33. Escrivá L, Manyes L, Font G, Berrada H. Mycotoxin analysis of human urine by LC-MS/MS: a comparative extraction study. *Toxins (Basel).* 2017;9(10):330. doi: 10.3390/toxins9100330
34. Cramer B, Visintin L, Maris E, Kuhn M, Degen GH, Turner PC, Humpf HU, De Saeger S. Human biomonitoring of mycotoxins: key challenges and future directions. *Mycotoxin Research.* 2026;42:13. doi: <https://doi.org/10.1007/s12550-025-00612-2>
35. Senyuva HZ, Gilbert J. Immunoaffinity column clean-up techniques in food analysis: a review. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2010;878(2):115-32. doi: 10.1016/j.jchromb.2009.05.042
36. Narciso L, Catone T, Aquilina G, et al. The juvenile toxicity study as a tool for a science-based risk assessment in the children population group. *Reprod Toxicol.* 2017;72:136-41. doi: 10.1016/j.reprotox.2017.06.188
37. Tassinari R, Narciso L, Tait S, et al. Juvenile toxicity rodent model to study toxicological effects of bisphenol A (BPA) at dose levels derived from Italian children biomonitoring study. *Toxicol Sci.* 2020;173(2):387-401. doi: 10.1093/toxsci/kfz226
38. Tassinari R, Tait S, Busani L, et al. Metabolic, reproductive and thyroid effects of bis(2-ethylhexyl) phthalate (DEHP) orally administered to male and female juvenile rats at dose levels derived from children biomonitoring study. *Toxicology.* 2021;449:152653. doi: 10.1016/j.tox.2020.152653
39. Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, et al. Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0. *PLoS Biol.* 2020;18(7):e3000411. doi: <https://doi.org/10.1371/journal.pbio.3000411>
40. Janhavi P, Divyashree S, Sanjaiall KP, Muthukumar SP. DoseCal: a virtual calculator for dosage conversion be-

- tween human and different animal species. Archives of Physiology and Biochemistry. 2022;128(2):426-30. doi: <https://doi.org/10.1080/13813455.2019.1687523>
41. Izco M, Vettorazzi A, de Toro M, Sáenz Y, Alvarez-Erviti L. Oral sub-chronic ochratoxin A exposure induces gut microbiota alterations in mice. Toxins (Basel). 2021;13(2):106. doi: 10.3390/toxins13020106
42. Chakraborty D, Verma R. Ameliorative effect of *Emblca officinalis* aqueous extract on ochratoxin-induced lipid peroxidation in the kidney and liver of mice. Int J Occup Med Environ Health. 2010;23(1):63-73. doi: 10.2478/v10001-010-0009-4