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Review

OCHRATOXIN A AND ARISTOLOCHIC ACID INVOLVEMENT IN NEPHROPATHIES AND ASSOCIATED UROTHELIAL TRACT TUMOURS*

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This review addresses the unresolved aetiology of several nephropathies and associated upper tract tumours diagnosed all over the world, but especially in the Balkan regions. Studies conducted over the last 35 years point to mycotoxins, mainly ochratoxin A (OTA) as the main culprit. Recent theories however have implicated aristolochic acids (AA). The aim of this review is to put forward arguments in favour of the mycotoxin theory and to show the incoherence of the AA theory. It discusses the differences between the epidemiology of Balkan endemic nephropathy (BEN) and aristolochic acid nephropathy (AAN); OTA and AA carcinogenicity; clinical and pathological effects induced by OTA and AA; sources of OTA contamination (food, air, drinking water); OTA- and AA-DNA adduct formation; the role of genetic polymorphisms ; and the risk for young children.

Key words: *aetiology, contamination, DNA adduct, genetic polymorphism, pathological effects, urothelial cancer*

Balkan endemic nephropathy (BEN) is a familial chronic tubulointerstitial disease with insidious onset and slow progression to terminal renal failure unaccompanied by salt retention or early hypertension (1) (Table 1). It mainly affects people living along the tributaries of the Danube River in Bosnia, Bulgaria, Croatia, Romania and Serbia. BEN and the associated urothelial tract tumour (UTT) were first described in 1956 in Bulgaria, and then in Serbia in 1957 (2, 3).

BEN has a familial character. It may affect several generations in a single household. Affected and spared households live one next to another. This is why the incidence of BEN is uneven within and between affected villages. They are sometimes separated from unaffected villages by only a few kilometres. So far, the disease has been identified in 142 villages in Bosnia, Croatia, and Serbia, with local prevalence ranging between 0.5 % and 4 %, in 40 villages in Bulgaria with mean morbidity rate of 3 %, and in 40 villages and small towns of the Romanian districts of Mehedinți and Caraş-Severin, with prevalence of over 2 % (4). A high prevalence of tumours of the renal pelvis and ureter (upper urothelial cancer, UUC) was described in patients with BEN and in affected families. It was up to 100 times higher than in non-endemic areas (4-7). Studies conducted in villages around Slavonski Brod, Croatia, pointed to environmental factors as key determinants of individual susceptibility to BEN (8). Patients with UUC are at risk of developing bladder tumours, with an estimated

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occurrence of 15 % to 50 %. Bladder Transitional Cell Carcinoma (TCC) usually appears within five years. Some patients develop pelvis pain and bone metastases (9). The link between BEN and UTT can be explained by insult from an environmental contaminant (10).

In 1972, Akhmeteli (11) suggested that fungal toxins were involved in the aetiology of BEN, notably ochratoxin A (OTA) (12). Although a number of findings support the mycotoxin theory, the implication of aristolochic acid (AA) has emerged recently as an alternative (13). The aim of this paper is to review arguments in favour or against either theory of the development of nephropathies and associated urothelial tract tumours in the Balkan regions and other parts of the world.

Table 1 *Epidemiological, clinical, and functional characteristics of BEN* *

Epidemiological characteristics
Residence in an endemic settlement
Family history of renal disease and of renal deaths
Family history of urothelial tumours
Occupational history of farming
Clinical characteristics
Slowly progressive renal insufficiency
Anaemia - normochromic or slightly hypochromic
Oedema absent
Hypertension - rare in early, common in advanced renal failure
Urothelial tumours - common
Abnormalities on urinalysis
Reduced kidney size, normal size in early stages
Functional changes
Impaired concentrating capacity
Decreased glomerular filtration rate
Impaired urinary acidification
Glycosuria, aminoaciduria
Increased uric acid excretion
Renal salt wasting
Proteinuria of the tubular type

**Modified from Stefanovic (1)*

EPIDEMIOLOGICAL DIFFERENCES BETWEEN BEN AND AAN

BEN occurs exclusively in rural population. In 1957-1960, the average age of patients at death was 45.1 years while in 1991-2002 it was 69.2 years. Although increased life expectancy, similar to that of the general population in the area, is probably owed to lower exposure to the toxic compound, there are still new cases of the disease and it has not disappeared (7). BEN neither expands nor disappears from endemic areas (14). The sex ratio in BEN is approximately 1:1, while aristolochic acid nephropathy (AAN) is exclusively seen in women. The length of exposure before the appearance of clinical signs is quite different between BEN and AAN. BEN does not occur in children, but people who left the endemic area after over a decade of residence also develop BEN. This suggests that the causative agent is slow-acting, and that exposure had continued for many years. In contrast, patients with AAN often show a rapid deterioration in the renal function, with doubling of creatinine levels within about three months. The development of urinary tract malignancy is also much longer in BEN (6) than in AAN (15). The latency of malignancies is 20 to 27 years after the diagnosis of BEN, and 2 to 6 years after the diagnosis of AAN.

Until now, only one paper has implicated exposure to AA in a BEN area, based on a questionnaire about the presence of birthwort (*Aristolochia clematitis* L.) in local fields (13). Since birthwort was not mentioned before as a potential risk factor for the development of nephropathy, the importance of its presence in the field is not clear. The conclusion drawn in this paper implicating AA in BEN and associated UTT calls for scepticism for several reasons:

(i) wheat is typically harvested in mid-summer when seeds of *Aristolochia* spp. are immature and cannot contaminate wheat (16);

(ii) farmers from a BEN area usually bring sacks of wheat to the mill and exchange them for sacks of flour from wheat produced by other farmers (17);

(iii) Moreover, Hranjec et al. (13) made a miscalculation which exaggerated estimated exposure in Croatian endemic villages to AA by a factor of 7;

(iv) in some countries, flour contamination hypothesis does not match the hilly topography of the endemic and non-endemic villages. In Romania

for example, maize is the dominant cereal, and maize cannot be contaminated by AA. Exposure to AA in specific nephropathy households of Croatian (and other) endemic villages has yet to be demonstrated;

(v) a high dose of AA (50 mg kg^{-1} b.w. per day, for three days) elicited a toxic response only in female rats, but renal function recovered within a month. After 6 months, three renal carcinomas were found in the exposed group, but no TCC (19);

(vi) rabbits receiving i.p. AA dose of 0.1 mg kg^{-1} b.w. five days a week for 17 to 21 months showed anorexia and lower weight gain, but also doubled the kidney weight, which is in contradiction with kidney atrophy observed in BEN patients (Table 2);

(vii) an analysis of *p53* gene mutations revealed a statistically significant difference between the mutation spectra in both the kidney and the liver of AA-treated rats, with transversion A:T → T:A accounting for 78 % of all base substitutions. A study of samples taken from 90 Bulgarian patients with BEN showed only 10 % of transversions (20);

(viii) several *Aristolochia* spp. have been used in Chinese medicine at much higher doses for over 2000 years, and no case of nephropathy or urothelial cancer has been reported to correlate with the use of AA. In Germany, more than a thousand of patients had been using AA as immunomodulator for 25 years without catching acute or chronic interstitial nephritis. The doses were similar to those reported in a weight loss regimen claimed to be responsible for the development of AAN (equivalent to the daily dose of 1.2 mg of AA) (21);

(ix) based on a study on mice which lasted for 28 days, Xue et al. (22) calculated that the no-observed effect level (NOEL) of exposure to *Aristolochia manshuriensis* Kom., *Akebia trifoliate* (Thunb.) Koidz., *Akebia quinata* (Thunb.) Decne and a traditional Chinese prescription called "Longdan Xieganwan" was 0.25 to 25 times the normal human dose in clinical prescription. Moreover, doses 400 times higher than used in the weight loss regimen did not induce any symptoms (22);

(x) rats with acute nephrotoxicity induced by *Aristolochia manshuriensis* (4 g kg^{-1} b.w. per day) did not show interstitial tubular damage (23, 24), contrary to findings in patients suffering from nephropathy after a slimming regimen;

(xi) in a Belgian study (25) only 100 of 1741 subjects who were taking slimming medication developed extensive interstitial fibrosis of the kidneys, the so-called Chinese herbs nephropathy. The disease did not correlate with the length of treatment. In an extensive review of Chinese herbs nephropathy, Meyer et al. (26) reported that slimming herbal preparations usually contained a mixture of ingredients of plant origin and other active substances, including fenfluramine (agonist of serotonine) and diethylpropion (sympathomimetic), which are powerful renal vasoconstrictors. They can induce renal ischemia, which in turn could amplify the nephrotoxicity of AA. The same authors noted that glomerular vasoconstriction in rats was observed in studies where serotonine induced ischemic nephritis without any effects on the medulla. These arguments suggest that fenfluramine could be responsible for interstitial nephritis evolving into interstitial fibrosis. Moreover, the slimming medicaments often contained acetazolamide, which also could increase nephrotoxicity (26).

CANCERS RISK: OTA VS. AA

OTA is one of the most potent renal carcinogens found to date (27, 28). Previous studies indicate that renal carcinoma metastases occurred mainly in the lungs and mammary glands of both male and female rats. An unusually high rate of 37 % was observed in male rats treated with 210 μg kg^{-1} b.w. of OTA. High-dosed female rats showed a significantly higher incidence of fibroadenomas of the mammary gland than controls (56 % vs. 34 %; N=50). They also had a higher incidence of multiple mammary fibroadenomas (two per animal) than controls (28 % vs. 8 %; N=50) (27). Higher incidence of mammary proliferative lesions was also found in another study of OTA-treated female Lewis rats (29).

These results were confirmed by a study of Lewis and Dark Agouti (DA) rats (30). Male DA rats were very sensitive to OTA-induced renal adenocarcinoma, whereas female DA rats were resistant. In addition, male DA rats were much more sensitive than either male or female Lewis rats. This difference in sensitivity may be due to differences in biotransformation capacity of OTA (31). In a recent study by Mantle et al. (32), OTA (100 μg per day) was administered to male Fisher-344 rats by gavage. The first tumour was recorded after 75 weeks of treatment. 20 % of rats developed renal carcinoma, mostly associated with metastatic nodules situated along the abdominal mesenteries, while some tumours spread to the lungs as well. The

authors also found other histopathological changes in the kidney such as karyomegalic nuclei in tubular epithelia, predominantly in the cortico-medullary region (32). The same authors reported that less than 10 months of dietary consumption of OTA in the first year of life sufficed for development of unilateral or bilateral renal tumours in some individuals after a latency of about a year (33). We also showed that OTA transplacental contamination of mice led to kidney tumours in male pups 9 months after birth (34).

Table 2 lists data about acute and subchronic toxicity of aristolochic acids I and II (35-40). Unlike OTA, AAs are mainly carcinogenic for the forestomach. Cosyns et al. (16) reported that Wistar rats receiving AA isolated from herbal slimming medication for three months developed tumour of the forestomach, but did not develop nephropathy. These data were confirmed by Chang et al. (41) who treated male Wistar rats with a mixture of aristolochic acids did not observe any impairment of the kidney function, interstitial nephropathy, or carcinoma.

CLINICAL CHARACTERISTICS AND PATHOLOGY OF NEPHROPATHY INDUCED BY OTA AND AA

OTA has been suspected to be involved in BEN, a human disease characterised by progressive renal fibrosis and associated with tumours of the urinary tract such as carcinoma of the renal pelvis, ureters, and bladder (42-44). Kidney fibroma, adenoma, or fibroadenoma have been reported in Bulgarian pigs with mycotoxic progressive nephropathy (MPN) (45-47). Stoev (48) found a positive correlation between the frequency of spontaneous porcine nephropathy and the rate of OTA contamination of feed samples which were not properly stored on farms. The frequency and duration of nephropathy in batches of slaughtered pigs depended on the duration of feeding on suspected feeds stored in poor conditions and at high humidity for a long time. Recently, Ceci et al. (49) showed that pigs from the Apulia region (Italy), fed on highly contaminated by OTA (149 $\mu g\ kg^{-1}$ to 327 $\mu g\ kg^{-1}$) accumulated a high amount of OTA in the kidney and urinary bladder, and developed macro- and microscopic lesions of the kidney (plurinucleate cells with hyperchromic nucleus and vacuolar degeneration of cytoplasm; karyomegaly and granular degeneration of the proximal tubule epithelium; hypercellularity and thickening of the capillary glomerular walls) and urinary bladder (mucosal hyperaemia and wall thickening; precancerous changes such as karyorrhexis, large nuclei, and hyperchromic nucleoli) (49). Likewise, the study on slaughtered pigs in Serbia confirmed the implication of accumulated OTA in porcine nephropathy. Histopathological analyses of porcine kidneys proved tubulopathies with oedema, cell vacuolisation and necrosis of the proximal tubules. The highest amount of OTA was found in the kidney, and the severity of nephritis highly correlated with the OTA level (50). It is important to stress that porcine kidney damages in spontaneous MPN in Bulgaria (48) and in Serbia (50) were more similar to those observed in human endemic nephropathy patients in the Balkan countries than in Danish MPN. As the OTA amount in feed was almost five times lower than the amount associated with Danish MPN, the authors (48, 50) concluded that kidney damage was caused by a synergistic effect of OTA and other mycotoxins. Pathomorphological changes in pig's kidney experimentally exposed to OTA and penicillic acid (PA) were more similar to spontaneous MPN than to Danish MPN (51). A similar comparison was made between spontaneous mycotoxic avian nephropathy (MAN) and kidney damage in chicks receiving both toxins through diet (52). In addition, analyses of feed given to pig which suffered from kidney lesions confirmed high levels of OTA and fumonisin B_1 (FB_1) (up to 40 $mg\ kg^{-1}$) (53).

Several experiments have clearly shown an additive or synergistic effect of OTA and other mycotoxins (54-57).

In a rodent study (58), we have demonstrated that citrinin (CTN) and OTA affected each other's toxicokinetics. CTN favoured the excretion of the toxins, resulting in a decrease of both CTN and OTA storages in the liver and kidney, but the decrease was not similar in all tissues. This could be explained by competition between the toxins for the transporters of organic anion (OAT). Moreover, co-exposure to OTA and CTN simultaneously modifies DNA adduct formation with increasing formation of the C-C8 dG-OTA adduct (58). In an analysis of OTA and CTN levels in a human double diet study in Serbia, we observed that contamination of CTN depleted OTA via urine (59).

Table 2 *Acute and subchronic toxicity of aristolochic acids*

Reference Year	Species Sex Number of animals	Route of administration Doses Duration of treatment	Parameters analysed	Main findings
Mengs (35) 1988	Mice, NMRI strain Females 39 treated, 11 controls	Oral 5 mg kg^{-1} b. w. of AAs mixture per animal (77.2% AA I and 21.2% AA II in NaCl), Treatment: 3 weeks	Follow up for 56 weeks. Animals were killed at weeks W3, W9, W18, W26, W37, W48 and W56 Histological examination.	At W18 and W26, low to middle-grade papillomatosis in the forestomach of all animals, but no signs of malignancy. W37 and W48; 1/5 of mice had squamous cell carcinoma. Adenocarcinoma of glandular stomach observed in 1 mouse at W37. Carcinoma of the forestomach observed in all mice killed at W56; adenoma of the kidney cortex (6/8), lung carcinoma (8/8), malignant lymphoma (4/8), and uterine haemangioma (3/8) were also observed. No tumour in control mice at W56.
EMEA (36) 2005	Wistar rats Male 14 animals per dose level	Oral Daily doses of AA extract in water: 0.2 mg kg^{-1} b. w.; 1.0 mg kg^{-1} b. w.; 5.0 mg kg^{-1} b. w. and 25 mg kg^{-1} b. w. Treatment: 4 weeks	Body weight Haematological and clinical chemical parameters Histopathological examination.	At the highest dose 25 mg kg^{-1} b. w. per day: decrease in weight and death of 2 animals. Decrease in mean corpuscular volume, number of reticulocytes, total serum protein, and glycaemia. Increased proteinuria and glycosuria. Atrophy of the thymus and spleen, hepatocellular basophila, inflammation of the forestomach hyperplasia, nephritis, and testicular degeneration. In the bladder; mild urothelial hyperplasia and slight cystitis. At 5 mg kg^{-1} b. w. per day: Changes similar to the higher dose, but lower intensity. At 1 mg kg^{-1} b. w. per day: mild changes At 0.2 mg kg^{-1} b. w. per day : no modifications.
Debelle et al. (37) 2002	Wistar rats at low-salt diet Male 48 treated animals, 18 controls	Percutaneous injection of AA mixture per animal (40 % AA I and 60 % AA II dissolved in polyethylene glycol and then diluted in water) Low-dose group: 1.0 mg kg^{-1} b. w. per day High-dose group: 10 mg kg^{-1} b. w. per day Control: vehicle only Treatment: 5 weeks	Body weight Evaluation of kidney function, urinary excretion of glucose, creatinine level, and leucine aminopeptidase activity. Blood analysis. Histolopathogical analysis (kidney, lung, skin, liver) On days 10 and 35.	At 10 mg kg^{-1} b. w. per day: decreased body weight; glycosuria, proteinuria, increased creatinine level and decreased urinary leucine aminopeptidase activity on days 10 and 35. Tubular necrosis associated with infiltrations of lymphocytes on day 10 and tubular atrophy with interstitial fibrosis on day 35. At 1 mg kg^{-1} b. w. per day : no significant effects in biochemical parameters compared to control. In both groups: urothelial lesions and malignant fibrohistiocytic sarcoma at injection site after day 35.
Mengs et al. (38) 1982	Wistar rats 30 male and 30 female	Oral 0.1 mg kg^{-1}, 1.0 mg kg^{-1} and 10 mg kg^{-1} b. w. of AA extract (77.2% AA I and 21.2% AA II) in form of its sodium salt. Treatment: 3 months, with 1.0 mg kg^{-1} b. w. and 10 mg kg^{-1} b. w., with a 3- and 6-month follow-up; 3 and 12 months with 0.1 mg kg^{-1} b. w., with a 6-, 12, and 16-month follow-up	Histolopathogical analysis	At 10 mg kg^{-1} b. w. :carcinoma of forestomach in 13/18 males and 8/13 females treated for 3 months and killed at 6 months. Carcinoma of the renal pelvis (8/18) in males; carcinoma of forestomach in 4/4 females treated for 3 months and killed at 9 months. At 1 mg kg^{-1} b. w.: carcinoma of the forestomach only in 3/11 males treated for 3 months and killed at 6 months. Carcinoma of the forestomach in 6/9 males and 2/11 females treated for 3 months and killed at 9 months. At 0.1 mg kg^{-1} b. w.: no abnormality detected at the end of the treatment. Carcinoma of the forestomach only in 2/7 males treated for 3 months and killed at 12 months. Carcinoma of the forestomach in 4/4 males and 1/5 females treated for 12 months and killed at 16 months. Control: 1 tumour (spontaneous polyp of endometrium) in a female.

Table 2 *(cont.)*

Mengs (39) 1983	Wistar rats Males 108 treated rats, 37 control rats	Oral 10 mg kg^{-1} b. w. of AA (77.2 % AAI and 21.2 % AAII) dissolved in ultrapure water. Treatment: 90 days	Histopathogical analysis.	Treated group: extensive necrosis of squamous cells of fore stomach followed by regeneration and hyperplasia, formation of papilloma and, invasive carcinoma of squamous cells. Control group: no effect.
Cosyns et al. (40) 2001	New Zealand white rabbits Female 12 treated animals and 10 controls	Intraperioneal Injection 0.1 mg kg^{-1} b. w. of AA dissolved in saline Treatment: 5 per week for 17 to 21 months	Carcinogen study. Body weight, kidney function, urinary excretion of glucose and low molecular proteins recorded before killing. Histopathogical analysis.	11 surviving animals. Growth decrease, increased serum creatinine, glucosuria, tubular proteinuria, and anaemia. Kidney fibrosis in all animals: interstitial fibrosis. Two animals developed kidney tumours (carcinoma of renal cells and tubulo-papillar adenoma). 1 case of cellular carcinoma of ureter and 1 peritoneal mesothelioma.

OTA-DNA VS. AA-DNA ADDUCT FORMATION

As early as 1993, we detected specific OTA-DNA adduct in kidney and bladder tumours of Bulgarian patients with BEN and associated UTT (60). Interestingly, we observed the same DNA adduct patterns in pig, chicken and human kidney, the last from affected households in Bulgaria (Figure 1). Detection of DNA adducts by postlabelling allows us to get an insight into the metabolic pathways involved in the genotoxicity of OTA (Figure 2; 43, 62). OTA-covalent-DNA adducts are formed after biotransformation into quinone derivatives (63). The main adduct, C-C8dG OTA, has recently been identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the kidney of a patient with BEN and UTT and in an animal fed with OTA (64-66). Increasing intake of OTA increases OTA-DNA adducts formation, notably in pig (Figure 3A) Several OTA derivatives have been identified in blood, urine, and tissues (Figure 3B). Metabolites found in blood and urine of Serbian men and women from BEN families were similar to OTA derivatives found in rats and pigs (Figure 4). Men and male rat have more metabolites in blood than female rat and women, who in contrast eliminate them more efficiently. A positive correlation is observed between the intake of OTA and these metabolites (data not shown).

Simultaneous ingestion of OTA and fumonisin B_1 (FB_1) further enhances OTA-DNA adduction in pigs. The DNA adduct pattern is similar to that observed in some Croatian patients with BEN and UTT (Figure 5). In a study on rats, we demonstrated the genotoxicity of OTA even with a dose that was considered safe (5 ng kg^{-1} b.w. per day). OTA genotoxicity dramatically increased when rat are exposed simultaneously at 200 ng kg^{-1} b.w. of FB_1 per day and even more at 50 µg kg^{-1} b.w. of FB_1 per day (Figure 6). Exposure to a higher OTA dose (50 µmol L^{-1}) resulted in a lower number of DNA adducts than exposure to lower doses. This is because at 50 µmol L^{-1} OTA is cytotoxic, and thus DNA adduction is indirectly lowered.

Therefore, chronic exposure low OTA doses could be much more damaging than acute exposure to a high dose. Analyses of several DNA adducts in patients with transitional cell carcinomas from different European countries show that 30 % of the kidney carcinoma could be attributed to OTA intake (59).

In 1994, Wiessler (61) suggested that AA could not cause BEN and that AA-DNA adduct was never found in humans, even though AA had been used in therapy for decades. Surprisingly, two years later Schmeiser et al. (67) claim that AA-DNA adduct was detected in Belgian women suffering from Chinese herbs nephropathy after a slimming regimen even though AA was not found in the pills

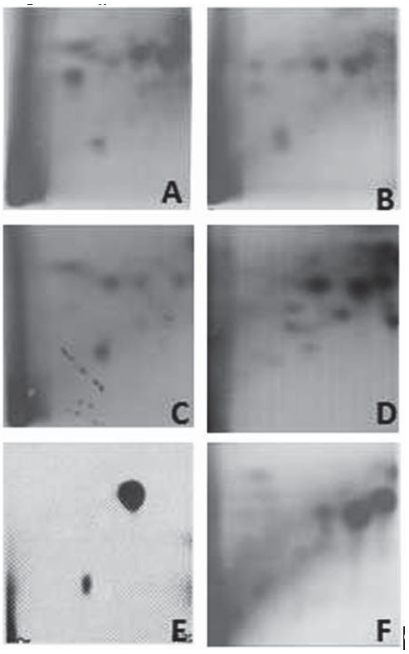

Figure 1 *Kidney DNA adduct pattern of a pig (A, C); chicken (B, D); a Bulgarian farmer (E); and the DNA adduct of the bladder of a Bulgarian farmer (F) suffering from BEN and UTT. (A) and (B) correspond to pig and chicken from a Bulgarian farm; (C) and (D) correspond to a pig and chicken fed with OTA in feed for 6 months. DNA adducts were separated with the contact transfer method using OTA solvents: D1 - 2.3 mol L^{-1} sodium phosphate, pH 5.7; D2 - 4.77 mol L^{-1} lithium formate, 7.65 mol L^{-1} urea, pH 3.5; D3 - 0.6 mol L^{-1} sodium phosphate, 5.95 mol L^{-1} urea, pH 6.4; D4 - 1.7 mol L^{-1} sodium phosphate pH 6*

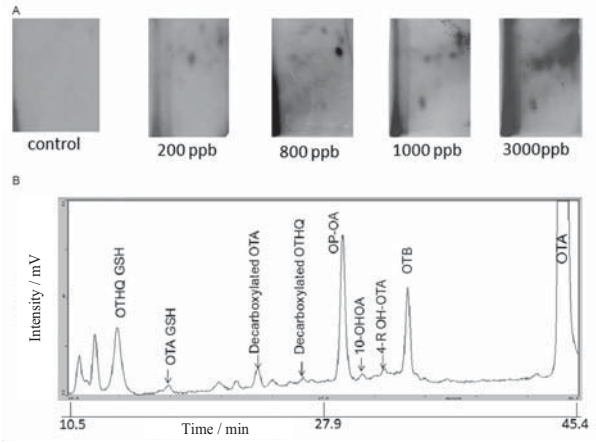

Figure 3 *Kidney DNA adduct pattern of a pig fed with increasing amounts of OTA for 6 months (A); OTA derivatives excreted in pig urine (B). DNA adducts were separated using the contact transfer method and OTA solvents: D1 - 2.3 mol L^{-1} sodium phosphate, pH 5.7; D2 - 4.77 mol L^{-1} lithium formate, 7.65 mol L^{-1} urea, pH 3.5; D3 - 0.6 mol L^{-1} sodium phosphate, 5.95 mol L^{-1} urea, pH 6.4; D4 1.7 mol L^{-1} sodium phosphate, pH 6*

they had been taking and they stopped the treatment several months or years ago (67). DNA adducts have been described in only eight of 100 Belgian patients who developed nephropathies following a slimming regimen. These data were published three times and were the basis to declare AA

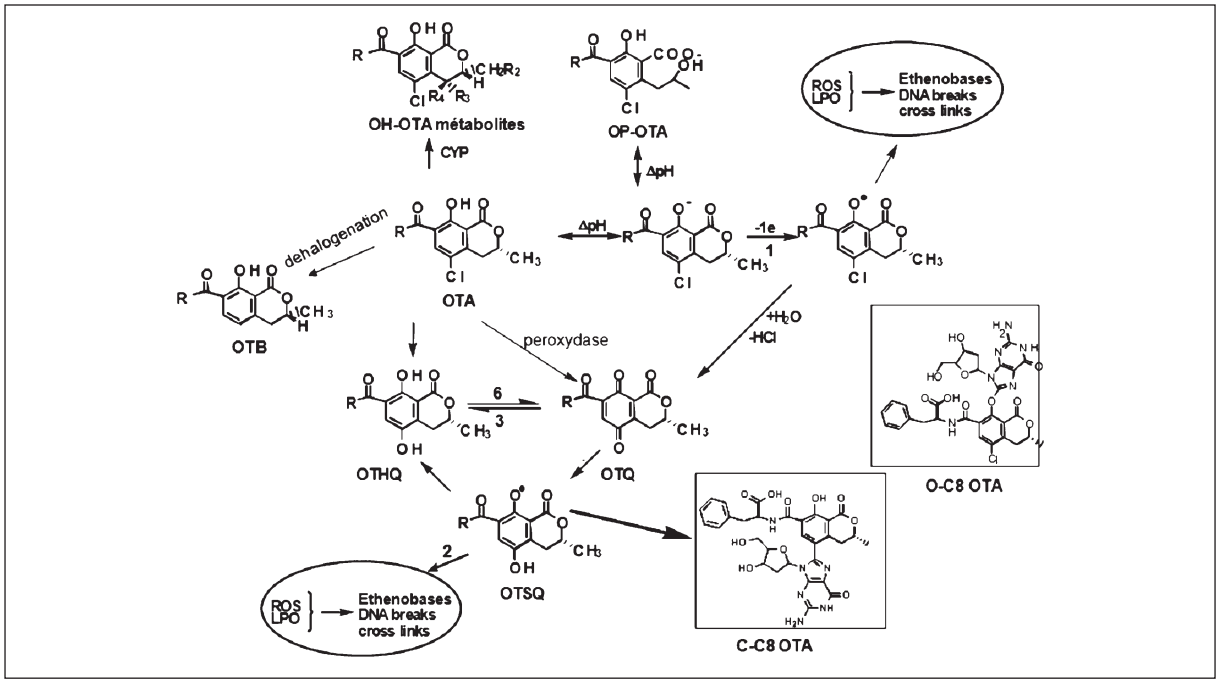

Figure 2 *Metabolic pathway of OTA.*
OTA - ochratoxin A; OTB - dechlorinated ochratoxin; OP-OTA - open ring ochratoxin; OTHQ - ochratoxin hydroxyquinone; OTSQ - ochratoxin semiquinone, OTQ - ochratoxin quinone; ROS - reactive oxygen species; LPO - lipoperoxydes; C-C8 dG OTA - deoxyguanosine ochratoxin adduct

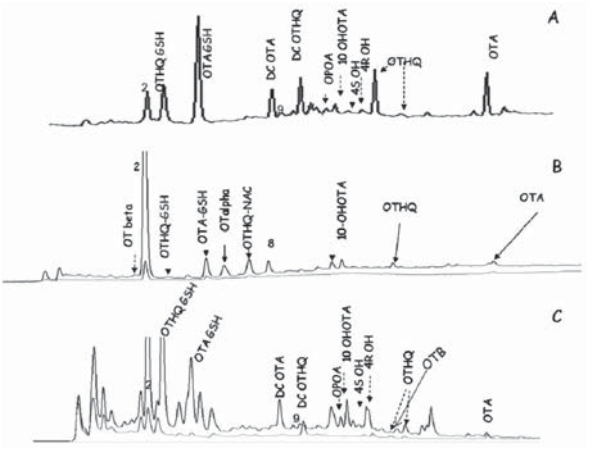

Figure 4 *Blood (B) and urine (C) OTA derivatives in men and women belonging to BEN families compared to rat urine (A).OTA ochratoxin A; OTHQ hydroxyquinone ochratoxin ;OTB dechlorinated ochratoxin; OP-OTA open ring ochratoxin; GSH glutathione conjugate; NAC N-acetylcystein; DC OTA decarboxylated ochratoxin; DCOTHQ decarboxylated ochratoxin quinone*

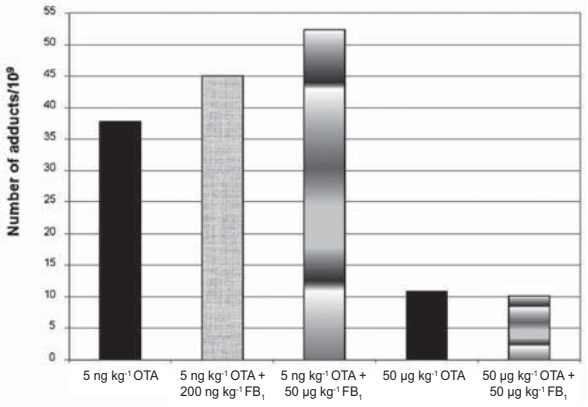

Figure 6 *Kidney DNA adduct pattern in a rat fed with OTA alone (black) or OTA and FB_1 (hatched grey) for four weeks. OTA intake was 5 ng kg^{-1} b.w. per day or 50 µg kg^{-1} b.w. per day. FB_1 intake was 200 ng kg^{-1} b.w. per day or 50 µg kg^{-1} b.w. per day. DNA adducts were analysed using the method described in Figures 1, 3, and 5.*

responsible of Chinese herb nephropathies (67-69). Furthermore, questionable is the presence of DNA adducts at a level as high as five adducts per 10^7 nucleotides several years after the intake stopped. Most of the cells would have been replaced after several months even in slowly renewing tissues such as the kidney and even if no repair occurred. A simple calculation can show that if these hypotheses were true all the molecules of DNA would be adducted! Surprised by this finding, we compared the formation and repair of DNA adduct in human renal cells treated with either OTA or AA. Neither pure AA I nor a mixture of AA I and AA II induce DNA adducts to a higher extent than OTA. These DNA adducts are repaired as quick as OTA DNA adducts, and thus they could not be found several months after AA intake had stopped (59). To investigate the strange results published by Schmeiser et al. we analysed their publications in depth. For better understanding, Figure 7 describes different methods of DNA adduct separation. Figure 8 shows a drawing of different DNA adduct patterns. In the first publication, Schmeiser et al. noted only one DNA adduct attributed to dA-AA I (7-deoxyadenosin-N^6-yl)-aristolactam I in the kidney of Belgian woman who followed a slimming regimen (Figure 8A) whereas Arlt et al. (69) observed a number of DNA adduct patterns in the same Belgian patient, either using the contact transfer method and OTA separation solvents (figure 8D) or the multidirectional method and AA separation condition (Figure 8B). Both types of DNA adducts (OTA and AA) were observed using OTA solvents (Figures 8D, 8E, and 9F). In

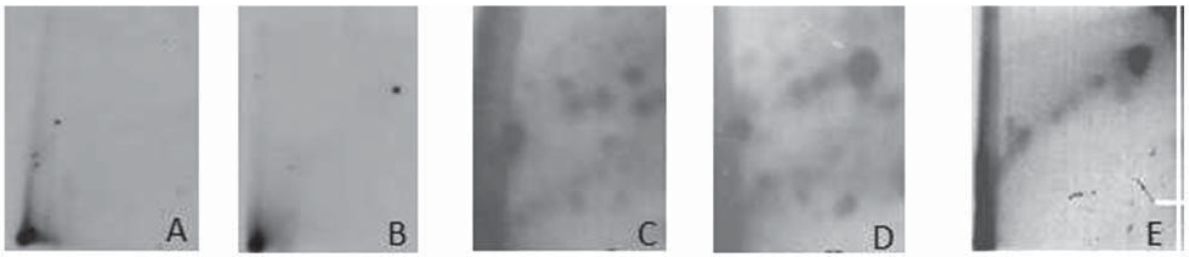

Figure 5 *Kidney DNA adduct pattern of a pig fed with FB_1 and/or OTA and the kidney DNA adduct of a Croatian farmer suffering from BEN: A - control; B - pig fed with FB_1; C - pig fed with OTA; D - pig fed with FB_1 and OTA; and E - Croatian farmer. DNA adducts were separated using the contact transfer method and OTA solvents: D1 - 2.3 mol L^{-1} sodium phosphate, pH 5.7; D2 - 4.77 mol L^{-1} lithium formate, 7.65 mol L^{-1} urea, pH 3.5; D3 - 0.6 mol L^{-1} sodium phosphate, 5.95 mol L^{-1} urea, pH 6.4; D4 - 1.7 mol L^{-1} sodium phosphate, pH 6*

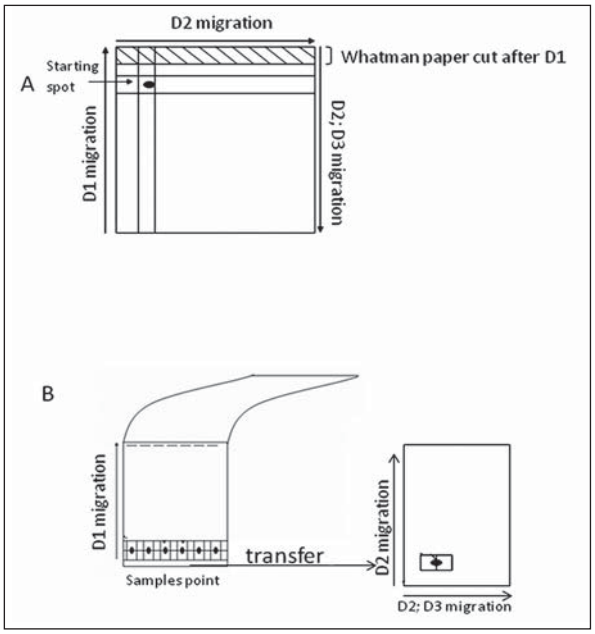

Figure 7 *DNA adduct detection using the multidirectional method (A) or contact transfer method (B). The solvents migration systems used for AA separation were the following: D1 - 1 mol L^{-1} sodium phosphate ; D2 - 3.5 mol L^{-1} lithium formate, 8.5 mol L^{-1} urea, pH 3.5; D3 - 0.8 mol L^{-1} lithium chloride, 0.5 mol L^{-1} Tris-HCl, pH 9.1; D4 - 1.7 mol L^{-1} sodium phosphate, pH 6. OTA solvents were: D1 - 2.3 mol L^{-1} sodium phosphate, pH 5.7; D2 - 4.77 mol L^{-1} lithium formate, 7.65 mol L^{-1} urea, pH 3.5; D3 - 0.6 mol L^{-1} sodium phosphate, 5.95 mol L^{-1} urea, pH 6.4; D4 - 1.7 mol L^{-1} sodium phosphate, pH 6*

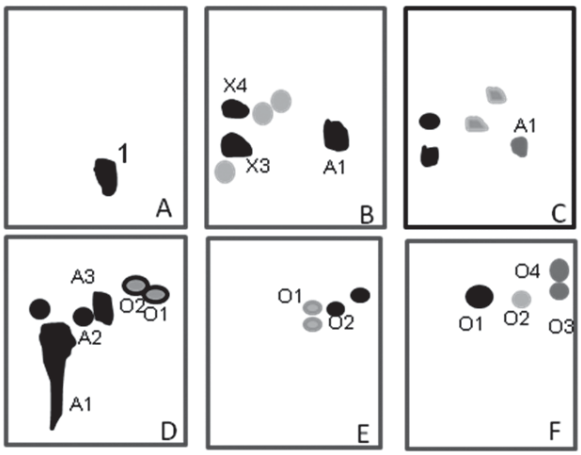

Figure 8 *DNA adduct patterns described in papers referenced under 68-71. Schemes A to C correspond to the DNA adduct pattern obtained using the multidirectional method and aristolochic acid solvents: (A) Belgian kidney published in references 68, 69 (B) Belgian kidney published in reference 70 (C) Croatian kidney published in reference 71. Schemes D-F correspond to DNA adduct patterns from the same samples as A-C, using transfer contact method and OTA solvents: (D) Belgian kidney published in reference 70 (E); Belgian kidney published in reference 59 (F); and Croatian kidney published in reference 71*

contrast, specific OTA adducts were lost using AA solvents and the contact transfer method (Figure 9D). The absence of AA-related DNA adducts in Figure 8E and 8F is not possible, as they are observed in the other migration systems (Figure 8B and 8C). In contrast, OTA-related adducts were not lost when multidirectional method was used whatever, regardless of the migration solvent (Figure 10). In the AA solvent migration system using the multidirectional method we observed two to four adducts OTA-related adducts (called X1 to X4). The main adducts X3 and X4 were found in the Belgian samples (Figure 8B), but also in a Croatian patient (Figure 8C) and in a French patient described in Arlt's et al. (70). Nevertheless, the authors did not make any comments about the presence of these OTA-related adducts. To our even greater surprise, using the contact transfer method and OTA solvent they observed only OTA-related DNA adducts, and we wonder how they then found AA related adducts! A careful analysis of the DNA adduct pattern using different methods of separation is essential to avoid any misinterpretation. Grollman et al. (71) concluded that EN was caused by AA based on the DNA adduct pattern in four patients detected using *Polyacrylamide* Gel Electrophoresis (PAGE). However, this method is questionable for two reasons: (i) DNA was extracted using a commercial Qiagen® kit, and we have shown that this type of extraction usually gives low DNA purity (34, 66) and (ii) detection was based on the comparison of bands, but the standard AA adduct bands were so wide that the faint band seen in the sample coincided with them.

The data published by Arlt et al. (72) are doubtful and contain misinformation about the women who have followed a slimming regimen. In addition to herbal pills, these women were taking several other drugs including amfepramone and mesotherapies (but they did not describe their composition). This information was also lacking in a paper by Stengel and Jones (73). We analysed DNA samples of both women using two different chromatographic separation (OTA solvents versus AA solvent). One patient clearly showed OTA-DNA adducts but no AA-DNA adduct, and the other showed no adduct whatsoever (59). Moreover, only small quantities of AA, if any, could be detected in the pills, and calculations based on equivalent human dose indicate that to reach the toxic dose the women should have been taking 150 mg of

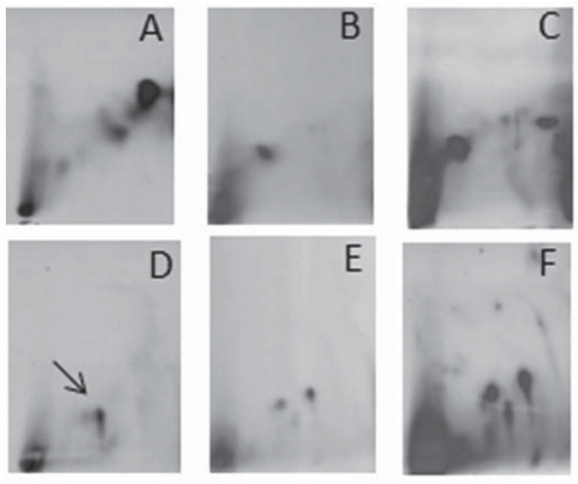

Figure 9 *Kidney DNA adduct pattern of a rat fed with OTA (A, D); AA (B, E); and co-migration of OTA and AA (C). DNA separation using the transfer contact method either with OTA solvents (D1 - 2.3 mol L^{-1} sodium phosphate, pH 5.7; D2 - 4.77 mol L^{-1} lithium formate, 7.65 mol L^{-1} urea, pH 3.5; D3 - 0.6 mol L^{-1} sodium phosphate, 5.95 mol L^{-1} urea, pH 6.4; D4 - 1.7 mol L^{-1} sodium phosphate, pH 6) (A-C) or with AA-solvents (D1 - 1 mol L^{-1} sodium phosphate; D2 - 3.5 mol L^{-1} lithium formate, 8.5 mol L^{-1} urea, pH 3.5; D3 - 0.8 mol L^{-1} lithium chloride, 0.5 mol L^{-1} Tris-HCl, pH 9.1; D4 - 1.7 mol L^{-1} sodium phosphate, pH 6) (D-F).*

AA, that is, 416 pills a day! The persistence of AA-DNA adduct eight years after the treatment is also hard to believe (74). In addition, it has clearly been demonstrated that either Belgian women or French women were not taking herbal pills alone, but also many other substances (75)

GENETIC POLYMORPHISMS AND OTA

Since recently, genetic polymorphisms of some xenobiotic-metabolising enzymes have been associated with BEN (76). A significantly higher risk of BEN (OR 2.41, 95% CI 1.09 to 5.33) was observed in individuals carrying the *CYP 3A5*1* allele . CYP 3A are the most common enzymes in the liver. The prevailing form of CYP 3A in the kidney is CYP 3A5, especially in the proximal tubule. A link between *CYP 3A5* expression and renal metabolism of endogenous factors has been also suggested (77-79). Its expression is polymorphic in the liver, intestine, and kidney. *CYP 3A5*1* (adenine at position 6986) is related to an enhanced protein expression (so-called expressor allele), while guanine at the same position (*CYP 3A5*3*) leads to absence or defect of this CYP protein (non-expressor allele). The carrier of the *CYP 3A5*1* allele may have the ability to more efficiently convert OTA into genotoxic metabolites that provoke BEN and create an individual predisposition to the disease.

OTA induces mutation on the lacZ gene in NIH/3T3 cells transfected with distinct human CYP450

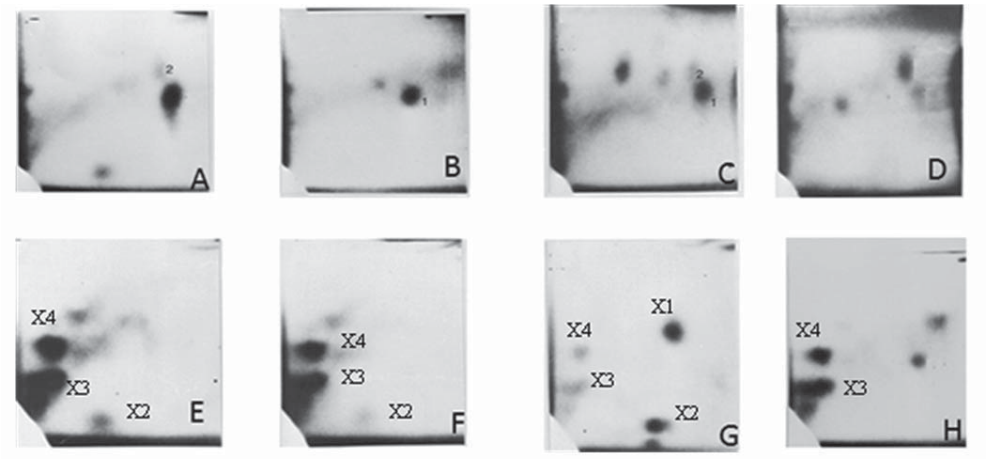

Figure 10 *Kidney DNA adduct pattern in a pig (A, E), chicken (B, F), and a rat (C, G) fed with OTA; and in a human with UTT and having OTA in blood, urine, and tissue (D, H). DNA adducts were separated using the multidirectional method and OTA solvents (D1 - 2.3 mol L^{-1} sodium phosphate, pH 5.7; D2 - 4.77 mol L^{-1} lithium formate, 7.65 mol L^{-1} urea, pH 3.5; D3 - 0.6 mol L^{-1} sodium phosphate, 5.95 mol L^{-1} urea, pH 6.4; D4 - 1.7 mol L^{-1} sodium phosphate, pH 6) (A-D); or AA solvents (D1 - 1 mol L^{-1} sodium phosphate; D2 - 3.5 mol L^{-1} lithium formate, 8.5 mol L^{-1} urea, pH 3.5; D3 - 0.8 mol L^{-1} lithium chloride, 0.5 mol L^{-1} Tris-HCl, pH 9.1; D4 - 1.7 mol L^{-1} sodium phosphate, pH 6) (E-H)*

enzymes. This mutation is more pronounced in the presence of CYP 2C9 (81). Implication of CYP 2C9 in OTA genotoxicity has been shown in cell culture (82) and in rats (31), and is related to the ability to metabolize debrisoquine. It has been observed that individuals having high capacity to metabolized debrisoquine are more frequent in BEN patient compared to individuals non affected by BEN (80)

Many studies have assessed the risk of different diseases, and bladder cancer in particular, in relation to glutathione S-transferase (GST) (83-86).

In a Bulgarian study (87) carriers of at least one GSTM1 wild-type allele (positive conjugators) were more prevalent among BEN patients than among controls (chi-square=7.92, p=0.005). In general, conjugation of xenobiotics with glutathione (GSH) leads to detoxification. However, some data show that certain GSH conjugates could be nephrotoxic such as halogenated derivatives, hydroquinones (88, 89) or OTA. (43). We have demonstrated that both oxidative stress and covalent binding are involved in OTA nephrotoxicity and carcinogenicity (90) while Faucet-Marquis et al. (91) established a correlation between DNA adducts and OTA derivatives in an opossum kidney cell culture (91).When applied to renal tubular cells, OTA led to GSH depletion (92). Using primary human urothelial cells from several donors, Lebrun et al. (93) pointed out the role of genetic GST expression and the extent of DNA damage induced by OTA. *GSTT1* positive genotype was more frequently observed in the subgroup with DNA damage induced by OTA compared to subgroup without DNA damage. The effect of GSH on OTA toxicity is dual: GSH favour detoxification rate of ROS and thus depletion will be deleterious for the kidney; in contrast GSH will not decrease carcinogenicity as some GSH-conjugates are genotoxic (90, 94).

Excessive coffee consumption (more than three cups a day) was implicated in upper tract transitional cell carcinoma and bladder cancer (92, 95, 96). The risk is higher for heavy coffee drinkers carrying the genotype GSTP1 105-104 val (96). This is important to stress, as coffee is one main source of OTA intake.

SOURCES OF CONTAMINATION WITH OTA

In all countries, farmers who consume their own food products are more affected by nephropathies and associated urothelial tract tumour (43, 97) than the general population. In the Balkan region, residents of BEN-affected households often live in close proximity of poultry and pig husbandry. As the feed is stored without any precaution, both the residents and the animals are exposed to airborne dust. Therefore, inhalation of mycotoxins is an additional risk source (98).

Exposure through food

Barley, wheat, maize, rice (99), nuts, beers, cocoa, spices, milk, and pork (43) could be contaminated by OTA and other mycotoxins. There is a large body of papers documenting OTA contamination of green and processed coffee and reporting OTA-producing fungal isolates from coffee. OTA contamination of commercial, packaged coffee is very common, despite the fact that over half of OTA is destroyed by roasting (100-102). OTA frequently co-occurs with other mycotoxins. Multi-mycotoxin aetiology of MPN/MAN was recently confirmed in Bulgaria (52). High contamination with PA [(838.6±223.9) μg kg^{-1}; 88 % samples positive] and FB1 [(5564.1±584) μg kg^{-1}; 96 % positive samples] was found in feed samples from Bulgarian farms with MPN associated with a relatively low level of OTA (188.8±27.3) μg kg^{-1}. A similar multi-mycotoxin aetiology was also found for South African MPN (52). The following levels were found in feed samples from pig farms with nephropathy problems: OTA (67.8±39.2) μg kg^{-1} (80 % samples positive); PA (149.2±64.1) μg kg^{-1} (41.7 % samples positive); and FB_1 (5046.7±1301) μg kg^{-1} (80 % samples positive).

Co-occurrence of OTA and fumonisins was reported in Croatia (103) and several countries in the Balkan region (104). Co-occurrence of OTA and CTN was reported in Bulgaria (105-107). Vrabcheva et al. (105) observed co-occurrence of OTA and CTN in BEN families. The amount of CTN was about five times higher than OTA. We also observed that BEN families in Serbia were co-exposed to OTA and CTN, but CTN exposure was not found in non-BEN families (59).

Exposure through inhalation

Historically, airborne OTA poisoning is related to the "old book disease" and the "curse of Tutankhamun". Several archaeologists who opened ancient Egyptian tombs died suddenly and mysteriously of unexplained causes. It has been suggested that the cause of death was acute renal failure due to inhalation of spores (*Aspergillus ochraceus*) which contained ochratoxins (108).

Agricultural workers are often exposed to airborne OTA when they manipulate with stored wheat, especially if it is kept in a closed compartment for longer time. Farm workers are exposed to contaminated airborne aerosols when they tend cows or work with mouldy feed and bedding materials.

It has recently been demonstrated that OTA is present in spores and airborne dust (109-110). A study conducted of three dairy farms in Norway (110) showed OTA in airborne dust ranging between 0.2 μg kg^{-1} and 70 μg kg^{-1}, with an average level of 27.5 μg kg^{-1}. Two Italian studies (111, 112) have demonstrated human exposure to OTA from dust at workplace and reported a relation between airborne OTA and OTA levels in workers' blood. The levels of airborne OTA in the handling and processing areas were higher than in office air. Serum OTA at the end of the work shift was significantly higher (0.94 ng mL^{-1} to 3.28 ng mL^{-1}) than in the control group of workers (0.03 ng mL^{-1} to 0.95 ng mL^{-1}) (111). Another study reported that serum OTA in malt factory workers increased with exposure to dusts and were higher in the autumn, after grain delivery, than in the summer (113).

A recent air analysis of a poultry house revealed not only OTA, but also aflatoxins and zearalenone (ZEA) (114). The authors calculated that a poultry worker breathed in up to 600 ng of mycotoxins in a working day, of which OTA accounted for 68.24 ng. In another study, air analysis of grain storage showed 2 pg m^{-3} of OTA, 2 ng m^{-3} of DON, and 1 ng m^{-3} of ZEA (98).

OTA in combination with either sterigmatocystin or citrinin has often been found in stored wheat, barley, oat, and corn (115). Analyses of different commodities including raw foodstuffs and processed food in Belgium have shown that 40 % of samples contained up to 25 μg kg^{-1} of OTA (116). In settled dust collected ingrain storages, 104 ng m^{-3} of OTA and 244 ng m^{-3} were detected (117). In Europe, cereals are frequently stored at the farm for economic reasons (low price, use as feed). This increases the risk of exposure to mycotoxins because harvested grains are not always low in moisture to prevent fungal growth during the storage. Puissemier et al. (118) have reviewed data from different countries and concluded that atypical severe contaminations of cereals by OTA (up to 1000 μg kg^{-1} measured in Poland on one occasion) could be related to bad storage conditions in individual farms. We also observed the highest contamination with OTA and CTN in wheat stored at farms in France (59). During milling, dust particles may be dispersed in the environment and increase exposure to mycotoxins by inhalation among grain workers (119). As storage work generates five times more dust through threshing, it is considered an important exposure factor (120). Dust of contaminated products usually contains mycotoxins in greater quantities that the products themselves (113). In a hyper-endemic former Yugoslav community, nephrotoxic species of *Penicillium* were also isolated from air samples (121).

Exposure through water

Filamentous fungi have recently been reported to contaminate drinking water (122, 123) *Aspergillus* spp. (notably *A. fumigatus*), *Cladosporium* spp., *Fusarium* spp. and *Penicillium* spp. (mainly *P. citrinum, P. glabrum, P. restrictum, P. expansum*) were the prevalent species. Many of the identified fungal species are known to produce toxic secondary metabolites in different matrices. For example 35 of 61 of *Fusarium* species produced fumonisins and zearalenone (122). Aflatoxins produced by *Aspergillus flavus* were also detected in water sampled from a cold water storage tank (123). This kind of contamination is low in normal domestic tap water. Problems arise when water is stored in cisterns or wells, where mycotoxin concentration is much higher (123) This could explain the reported correlation between BEN and the use of wells (97).

CONCLUSION

Thirty years of research clearly show that mycotoxins, and OTA in particular, play a major role in some nephropathies and associated urothelial tract tumours in rural population all over the world.

In rural economies, one tends to eat what one grows, including the pigs that feed on the same grain. This may result in high chronic exposure to OTA (124). Average European daily intake of OTA has been estimated to 0.7 ng kg^{-1} to 4.6 ng kg^{-1} of body weight, with over half of the OTA coming from cereals (125). However, in the Balkan endemic areas the intake is around 100 μg kg^{-1} b.w. per day. This explains the prevalence of EN in these areas.

Individuals from other countries all over the world are often exposed to low OTA doses, which still exceed the putative safety limit of 1.5 ng kg^{-1} b. w. per day, established by Kuiper-Goodman et al. (126), and which may lead to urothelial tract tumours. Particularly vulnerable are people sensitive to OTA due to genetic polymorphisms, but also those who have been exposed to OTA during prime childhood. Because of their lower body weight, young children could be exposed to OTA doses three times as high as adults, sufficient to cause kidney damage, which could go unnoticed for a while, as the kidney has a large functional reserve. When the accumulated damage reaches a threshold, the kidney function may deteriorate to the end-stage renal failure (125).

Human exposure to OTA is mainly through food, but also through inhalation and drinking water, the last mainly in rural areas, and mainly to well water, which has been corroborated by the correlation between BEN and wells used.

OTA seems to be also a critical agent in Canada where 4,300 new cases of kidney cancer appeared per year (National cancer Institute Canada, 2004). The affect mainly male (63%).

The highest kidney cancer incidence rate was found in Manitoba (127), and it correlated with male individuals who had the highest blood plasma levels of OTA (128).

Moreover, co-exposure to OTA and other mycotoxins such as fumonisins and citrinin increase the risk, as it has been demonstrated in Croatia and Bulgaria. Interestingly, the kidney DNA adduct pattern of BEN patients is similar to the kidney DNA adduct pattern of pigs living in the same farm and of pigs co-exposed to OTA and fumonisins or citrinin. These promote biotransformation of OTA into quinone derivatives, which are responsible for a specific OTA covalent DNA adduct, C-C8dG OTA, recently identified by ms/ms (66). The correlation between OTA intake and the levels of OTA derivatives in the urine of on Serbian people and complete absence of AA (18, 59) or its derivatives corroborate the association between OTA and not AA and EN.

Despite clear evidence of implication of OTA and other mycotoxins in the development of nephropathy and urothelial tract tumour, IARC and FDA are reluctant to consider OTA as a human carcinogen and the main cause of BEN and associated UTT. Until OTA is publicly denounced as the culprit, food industry will be saving on detoxification from mycotoxins. On the other hand, redirecting attention to AA as responsible for Belgian nephropathy gives the pharmaceutical industry an opportunity to increase sales of amphetamines in weight loss programmes.

However, economic reasons should not blur facts. Mycotoxins are a major health problem everywhere in the world, and it is urgent to develop preventing measures.

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Sažetak

SAZNANJA O ULOZI MIKOTOKSINA I ARISTOLOHIČNE KISELINE U NEFROPATIJAMA I PRIDRUŽENIM TUMORIMA MOKRAĆNOG SUSTAVA

Etiologija dijela nefropatija i srodnih im tumora gornjeg dijela mokraćnog sustava koji se dijagnosticiraju diljem svijeta, a posebice na prostoru Balkana, još nije razjašnjena. Rezultati istraživanja provedenih zadnjih 35 godina upućuju na mikotoksine, a posebice okratoksin A (OTA), kao glavne uzročnike. U posljednje vrijeme raspravlja se i o mogućoj ulozi aristolohičnih kiselina (AA). Svrha ovoga preglednog rada jest obrazložiti argumente koji govore u prilog uključenosti mikotoksina kao čimbenika odgovornih za nastanak navedenih bolesti te rasvijetliti zablude oko teze koja govori u prilog uključenosti AA kao mogućeg uzročnika. U članku se raspravlja o razlici između (i) epidemiologije endemske (balkanske) nefropatije (BEN) i nefropatije uzrokovane pod utjecajem aristolohične kiseline (AAN), (ii) karcinogenosti OTA i AA, (iii) kliničkim i patološkim učincima izazvanim pod utjecajem OTA i AA, (iv) izvorima kontaminacije s OTA (hrana, zrak, pitka voda), (v) nastanku DNA-adukata pod utjecajem OTA ili AA, (vi) ulozi genskog polimorfizma i (vii) riziku za malu djecu.

KLJUČNE RIJEČI: *bubreg, DNA-adukt, etiologija, genski polimorfizam, karcinom mokraćnog sustava, kontaminacija, patološki učinci*

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