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ARISTOLOCHIC ACID AS A RISK FACTOR FOR BALKAN ENDEMIC NEPHROPATHY

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Summary. *Background/Aims: Aristolochic acid nephropathy (AAN) is a unique type of rapidly progressive interstitial fibrosis, associated with urothelial cancer and related to the prolonged intake of Chinese herbal remedies (Aristolochia species) containing nephrotoxic and carcinogenic aristolochic acid (AA). On both clinical and morphological grounds, AAN is very similar to another fibrosing nephropathy, the Balkan endemic nephropathy (BEN), including the association with urothelial tumours. It has been suggested that the mycotoxin ochratoxin A (OTA) is associated with BEN, but also AA was considered as a possible causal factor in BEN already in the 1970s.*
Methods: We assessed AA exposure in endemic areas for BEN using the ^{32}P-postlabelling method. To this end we analysed renal tissue from three female farmers who lived in endemic areas and in two of whom an upper urinary tract cancer had developed, for OTA-related and AA-DNA adducts, described biomarkers of exposure for both genotoxins.
Results: In two of the three cases of whom one had urothelial malignancy, the major adenosine adduct of aristolochic acid I, 7-(deoxyadenosin-N^6-yl)-aristolactam I (dA-AAI), the most abundant AA-DNA adduct found in AAN patients, was identified by cochromatographic analyses on TLC and HPLC. In the same two patients OTA-related DNA adducts were detected. In the third patient, the tentatively assigned dA-AAI-DNA adduct spot was very faint and precluded complete identification. OTA-related DNA adducts were absent. Our data confirm the potential role of OTA in the development of urinary tract tumours in endemic regions and clearly demonstrate that people living in endemic areas for BEN have been exposed to AA, too.
Conclusions: Thus, AA should be considered as an additional potential risk factor. The epidemiology of AAN and AAN-associated urothelial malignancies might provide a clue to urothelial malignancies in endemic areas. Whether AA plays a role also in BEN remains to be further assessed in patients with definite diagnosis of BEN and in patients with other nephropathies but living in endemic areas for BEN.

Key words: *Balkan endemic nephropathy, DNA adducts, aristolochic acid, ^{32}P-postlabeling*

Abbreviations: *AA, aristolochic acid; AAI, aristolochic acid I (8-methoxy-6-nitrophenanthro[3,4-d]-1,3-dioxolo-5-carboxylic acid); dA-AAI, 7-(deoxyadenosin-N^6-yl)-aristolactam I; CHN, Chinese herbs nephropathy; AAN, aristolochic acid nephropathy; BEN, Balkan endemic nephropathy; OTA, ochratoxin A*

So-called Chinese herbs nephropathy (CHN), associated with the ingestion of herbal remedies containing aristolochic acid (AA), was first reported in young Belgian women who have been on a slimming regimen including the Chinese herb *Aristolochia fangchi* (1,2). CHN is a unique rapidly progressive nephropathy characterised by extensive renal interstitial fibrosis, tubular proteinuria, early and severe anemia and a high risk of urothelial cancer (3-6). AA is a mixture of structurally related nitrophenanthrene carboxylic acids with aristolochic acid I (AAI) being the major component. AA is nephrotoxic in several species, mutagenic in bacteria and carcinogenic in rodents (7-10). The detection of specific AA-DNA adducts by ^{32}P-postlabelling in kid-

ney and ureter tissue of CHN patients unambiguously demonstrated the exposure to AA in CHN (6,11-13).

So-called CHN and urothelial tumours have recently been reported in patients who were exposed to *Aristolochia* species and had no relationship with the Belgian slimming clinic (14-16). These reports demonstrate that the development of CHN lesions may be due to the toxicity of AA alone without requiring the other drugs prescribed in the slimming regimen. Moreover, the recent demonstration of renal interstitial fibrosis and urothelial malignancy in rabbits and rats treated with AA alone (9,10), removed any doubt on the causal role of AA in so-called CHN. Therefore, it has been proposed to designate the interstitial nephropathy in which the unequivocal role of AA has been fully documented as aristolochic acid nephropathy (AAN) (16,17).

On both clinical and morphological grounds, AAN is very similar to another fibrosing nephropathy, the Balkan endemic nephropathy (BEN) (3), which is found in certain rural areas of Rumania, Croatia, Bosnia, Serbia and Bulgaria along the Danube river basin (18-20). Both diseases share normal blood pressure, aseptic leukocyturia, early and severe anemia on clinical ground and morphologically extensive hypocellular interstitial sclerosis, tubular atrophy, global sclerosis of glomeruli, cellular atypia and malignant transformation of the urothelium (3-6, 18-20). These similarities have led to the hypothesis of a common etiological agent for both diseases. In this context it is noteworthy that food contamination by AA has been suggested as a possible causal factor in BEN already in 1970 (21). Alternatively evidence has accumulated that BEN is an environmentally induced disease strongly associated with the oral intake of food from plant origin contaminated with the mycotoxin ochratoxin A (OTA) (19,20). Like AA, OTA is nephrotoxic and carcinogenic (22).

Similarly to DNA adduct formation by AA found in AAN, high levels of OTA-related DNA adducts have been found in urinary tract tumours of Bulgarian patients suffering from BEN supporting the hypothesis that OTA is a causal factor in the development of BEN (23). Thus, OTA-related and AA-DNA adducts are suitable biomarkers for elucidating the molecular epidemiology of both diseases.

Recently, we investigated the potential role of OTA exposure in AAN (6,13). Together with findings from previous studies (2, 24) a major role of OTA in AAN was excluded. Here we assessed exposure to AA in endemic areas for BEN. Therefore, we analysed kidney tissue for both OTA-related and AA-DNA adducts by ^{32}P-postlabelling from three female patients from endemic regions. A unilateral nephroureterectomy had been performed for pyeloureteral urothelial malignancy (cases 1 and 2) and for ureteral stenosis (case 3). The unavailability of sufficient clinical and renal morphological data did not allow us to classify these patients as clearly suffering from BEN (25). However, it is of interest to note that they lived in endemic villages near Slavonski Brod (Croatia) where they had a farming activity, and that two of them had developed an upper urinary tract malignancy. Tissues were stored at -80°C until analysis.

For the detection of AA-DNA adducts we used the ^{32}P-postlabelling assay as described previously (12). We detected one major adduct spot in patients 1 and 3, chromatographically indistinguishable from the adenosine adduct of AAI, dA-AAI [7-(deoxyadenosin-N^6-yl)-aristolactam I], the most abundant AA-DNA adduct found in AAN patients (11-13,15,16). In renal tissue of patient 2 the tentatively assigned dA-AAI-DNA adduct spot was very faint and precluded complete identification. As a second, independent chromatographic procedure to confirm the identity of the adduct spots we employed reversed-phase HPLC analysis (12). In order to obtain a reference compound for the identification of the adduct dA-AAI was prepared by *in vitro* incubation, as reported before (12). Cochromatographic analysis on HPLC with the reference compound revealed that the adduct spot found in patients 1 and 3 were chromatographically indistinguishable from dA-AAI and eluted with a retention time of 23.45 min, identical to the dA-AAI standard. Thus, the spot was assigned as 3',5'-bisphospho-7-(deoxyadenosin-N^6-yl)-aristolactam I. The dA-AAI adduct is also the major AA-DNA adduct observed in rats treated orally with the plant extract AA (26). Moreover, the dA-AAI adduct showed an apparently life-long persistence in various organs in rats (12, 27) and it was still detectable in renal tissue of Belgian AAN patients more than 7 years after the patients stopped taking the herbal slimming regimen (6). Since the renal tissue samples were collected between 1987-1990 our results confirm that the dA-AAI adduct is a suitable biomarker for exposure to AA even years later.

The role of AA in the genesis of BEN was under debate, since AA was found in flour obtained from wheat contaminated with seeds of *Aristolochia clematitis* in endemic regions for BEN (21). However, this hypothesis has not yet received sufficient support (20). Our findings here provide the first evidence that people living in endemic areas for BEN have been exposed to AA. Since many herbal remedies are used locally in these areas (20), the cases reported here raises major concerns for public health.

DNA adducts can be considered both as markers of the biologically effective dose and as markers of cancer risk (28). As pointed out previously (5,6,11,12,29) AA-DNA adducts may trigger the carcinogenic process in AAN patients. Indeed, the dA-AAI adduct is a premutagenic lesion and is associated with mutations in genes involved in carcinogenesis, such as the H-*ras* protooncogene and the p53 gene (5, 30-31). Therefore, our results may provide a molecular link to the cause of urothelial tumours observed in patients living in BEN areas. Whether AA-DNA adducts play a causal role also in BEN awaits further elucidation.

On the other hand some clinical and morphological features of the two diseases still differ. For instance by the rapidity of evolution from a few months to years in

AAN versus several years in BEN and also the age of the patients which is generally higher in BEN than for AAN (3). As mentioned before, exposure to the mycotoxin OTA was strongly associated with BEN. We therefore also looked for OTA-related DNA adducts in these patients. The detection of OTA-related DNA adducts by ^{32}P-postlabelling requires chromatographic conditions different to those routinely used for lipophilic adducts, like AA-DNA adducts (13). Several adduct spots were present in all three renal tissues analysed. Patient 1 and 3 showed a similar adduct pattern consisting of one major adduct spot and a few minor adducts similar to the adduct pattern observed in renal tissue of rat and mouse treated with OTA which served as reference (22, 32). These findings are in line with the detection of high levels of OTA-related DNA adducts in urinary tract tumours of BEN patients (23), but the low adduct levels prevented cochromatographic identification. These results clearly show that two of the Croatian patients living in high-risk areas for BEN have been exposed to OTA. Thus, our findings confirm a potential role of OTA in the development of upper urinary tract tumours in these areas. Whether OTA plays also a role in the interstitial nephropathy characterising BEN remains to be determined.

Interestingly the same two patients exposed to OTA have been exposed to AA, too. The detection of both OTA-related and AA-DNA adducts in our patients may suggest interactive toxicologic effects of both toxins in endemic areas in the development of urothelial malignancy. Given the absence of evidence that these patients clearly suffered from BEN, the effects of these toxins in the development of BEN remain to be assessed. AAN patients have been exposed to high amounts of AA within a few months (approximately 15 to 20 months) providing a reason for the rapid development of AAN and AAN-associated urothelial tumours (approximately 2 to 6 years) (3, 5-6, 19-20). In contrast, people in endemic areas for BEN might have been exposed chronically to low amounts of AA over years, which might explain the slow development of BEN and BEN-associated urothelial malignancy. Specific p53 mutation profiles are one way to proof a direct link between carcinogen exposure and the development of tumours, as has been shown for example for p53 mutations in liver cancers associated with exposure to aflatoxin B1 (33) and in lung tumours associated with tobacco smoking (34). The comparison of the p53 mutational spectra from urothelial tumours of AAN patients and BEN patients might provide new insights in the epidemiology of both diseases.

In summary, we found evidence of AA exposure in a few farmers coming from endemic areas for BEN with tumoural or stenotic urinary tract obstruction. This provides evidence that in addition to OTA, AA may be a potential risk factor in the development of urothelial cancer in endemic areas. Whether AA plays a role also in BEN awaits further investigation in patients with definite diagnosis of BEN. The respective role of AA and OTA in this entity should be evaluated by the detection of both OTA-related and AA-DNA adducts in urinary tract tissue from patients with definite diagnosis of BEN and patients with other nephropathies but living in endemic areas for BEN.

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