Balkan Endemic Nephropathy and the Causative Role of Aristolochic Acid

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Summary: Balkan endemic nephropathy is a chronic tubulointerstitial disease with insidious onset, slowly progressing to end-stage renal disease and frequently associated with urothelial carcinoma of the upper urinary tract (UTUC). It was described in South-East Europe at the Balkan peninsula in rural areas around tributaries of the Danube River. After decades of intensive investigation, the causative factor was identified as the environmental phytotoxin aristolochic acid (AA) contained in *Aristolochia clematitis*, a common plant growing in wheat fields that was ingested through home-baked bread. AA initially was involved in the outbreak of cases of rapidly progressive renal fibrosis reported in Belgium after intake of root extracts of *Aristolochia fangchi* imported from China. A high prevalence of UTUC was found in these patients. The common molecular link between Balkan and Belgian nephropathy cases was the detection of aristolactam-DNA adducts in renal tissue and UTUC. These adducts are not only biomarkers of prior exposure to AA, but they also trigger urothelial malignancy by inducing specific mutations (A:T to T:A transversion) in critical genes of carcinogenesis, including the tumor-suppressor *TP53*. Such mutational signatures are found in other cases worldwide, particularly in Taiwan, highlighting the general public health issue of AA exposure by traditional phytotherapies.
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In 1993, the occurrence of a rapidly progressive form of renal interstitial fibrosis associated with a weight loss diet that included the ingestion of pulverized plant extracts used in traditional Chinese medicine led to the description of a new toxic nephropathy.[1] The identification of aristolochic acid (AA) in these powders brought to attention the severe toxicity of certain species of *Aristolochia*.[2] Since then, secondary nephropathies resulting from the toxicity of plants containing AA have been described worldwide.[3] The similarity between the histologic aspects of this particular nephropathy and the so-called Balkan endemic nephropathy (BEN) proved instrumental in reviving an old hypothesis of the etiology of BEN.[1,4] In 1969, Ivić[5] suggested that the latter, occurring in certain villages throughout the Danube Valley, might be caused by the chronic ingestion of the seeds of *Aristolochia clematitis*, a common plant growing in the wheat fields of these endemic regions. This hypothesis now has been confirmed by the discovery of specific DNA adducts that are formed by the metabolites of AA (aristolactams) in the renal tissue and the urothelial tumors of those patients suffering from BEN, as well as in the initial cohort of Belgian patients.[6-9]

Today, the term *aristolochic acid nephropathy* (AAN) is used to include any form of toxic interstitial nephropathy that is caused either by the ingestion of plants containing AA as part of traditional phytotherapies (formerly known as *Chinese herb nephropathy*), or by the environmental contaminants in food (BEN).[10]

Although the initial Belgian cohort included more than 100 patients, it is estimated that exposure to AA affects 100,000 people in the Balkans (where the total

number of patients with kidney disease amounts to approximately 25,000), 8 million people in Taiwan, and more than 100 million in mainland China.[3,11] Given the fact that the nephrotoxic effects of AA are irreversible and that its carcinogenic effects may be very slow in manifesting itself after the patient's initial exposure, AAN and associated cancers are likely to become a major public health issue in the years to come.[12]

The *Aristolochia* species is a genus of herbaceous, perennial plants that include more than 500 species. They are widespread in the warm regions of the Mediterranean, Africa, and Asia. In France, *A clematitis* or birthwort, *Aristolochia rotunda*, and *Aristolochia pistolochia* grow mainly in limestone soil and can be found on roadsides, in coppices, vineyards, and other agricultural areas. Furthermore, *A clematitis* is a parasitic plant that grows alongside wheat in the local wheat fields in the warm and humid regions of the Danube Valley.[13]

The accidental ingestion of these species of *Aristolochia* not only explains the existence of BEN, but also clarifies incidents of severe livestock poisoning, occurring among horses in the Balkans and goats in Africa, respectively.[5,14] In Spain, moreover, the regular consumption of an infusion with *A pistolochia* led to a case of chronic interstitial nephritis.[15]

In the past, *Aristolochia* were widely used in Western medicine.[13] In fact, their first use to stimulate the expulsion of the placenta during childbirth was responsible for coining the name "Aristos lokos" or "excellent delivery."[16] Because the plants also were recommended for treating snake bites, *Aristolochia* were included in the preparation of theriac. As a result, they were prescribed for treating gout (Dutchman's pipe).

In addition to the dramatic history of AA environmental exposure in the Balkans that will be further described in this review article, it should be underlined that regarding global public health issues, *Aristolochia* are considered an integral part of the herbology used in traditional Chinese medicine (TCM),[12] Japanese Kampo,[17] and Ayurvedic medicine.[18] They are found within the same therapeutic family as the *Akebia*, *Asarum*, *Cocculus*, and *Stephania* plants. Referred to by common names such as Mu Tong, Mokutsu, and Fang ji, they are used in a multitude of herbal mixtures for therapeutic use.[3] Because of the ambiguity surrounding the nomenclature of medicinal plants used in traditional medicine, the detection of AA by means of the phytochemical analysis of plant extracts is the only way to certify their potential toxicity.

EPIDEMIOLOGY AND ETIOLOGY OF BEN

BEN is a chronic tubulointerstitial nephropathy associated with urothelial carcinoma of the upper urinary tract (UTUC).[19,20] It was described in South East Europe at the Balkan peninsula in rural areas located in the valleys of great tributaries of the Danube River. The first cases were recognized in the middle of the 20th century. Disease was observed only in harvesting farmers and affected only certain villages. Family, or, more precisely, household aggregation, was reported in all BEN countries (Bosnia and Herzegovina, Bulgaria, Croatia, Romania, and Serbia) (Fig. 1A). An inherited pattern of the disease was ruled out by the fact that BEN often affected several members of the same household who were not necessarily blood-related. At the World Health Organization meeting that was held in 1964 in Dubrovnik, Croatia, the disease was named *endemic nephropathy*. The average prevalence of diseased subjects in the past years has ranged between 2% and 5%. However, the prevalence of farmers suspected to have BEN is much higher and was reported to be 10% to 15%. There were no gender differences, although a slight insignificant female predominance was found (1:1.2). Importantly, it was never reported in children, indicating that a long period of exposure to the environmental agent is needed. In past decades, the age when BEN patients started to receive dialysis was shifted to older ages, raising the question of whether the etiologic agent is still present or active. This is in concordance with the results obtained in Croatian field surveys conducted between 2005 and 2015, when neither new BEN nor new UTUC patients were detected in some previously established BEN villages.[21] Similar trends were observed in Serbia.[22,23]

An early hallmark of BEN is proximal tubule damage, which is manifested as low-molecular-weight (tubular) proteinuria and enzymuria that are in line with the pathologic findings: good preservation of glomeruli and a gradient of tubular atrophy with severe interstitial fibrosis that decreases in severity from outer-to-inner cortex. Two target tissues were identified in BEN patients: proximal renal tubular cells, leading to interstitial fibrosis and eventually chronic renal failure, and urothelial (transitional) cells, leading to a high prevalence of UTUC. The specific mortality of UTUC was reported to be 55 times higher in Croatian BEN county versus other parts of Croatia and similar findings were found in Serbia and other countries where BEN has been observed.[19,20,23,24] Epidemiologic findings and striking geographic correlation of two otherwise very rare diseases (chronic interstitial nephropathy and UTUC) pointed to a common etiologic agent. In 1985, Čeović et al[25] reported firm evidence on the importance of environment and lifestyle, observing that Ukrainians who settled the Croatian-endemic villages and lived in this area for more than 20 years had the same risk for BEN as local autochthonous farmers. On the contrary, Ukrainians who settled at the same time in the Croatian-nonendemic villages did not develop BEN, and, finally, BEN has never been described in the Ukraine.

For more than 50 years extensive research has focused on the etiology of BEN. Various hypotheses were investigated and rejected (heavy metals, microelements, Pliocene lignite, bacteria, viruses, immunologic and

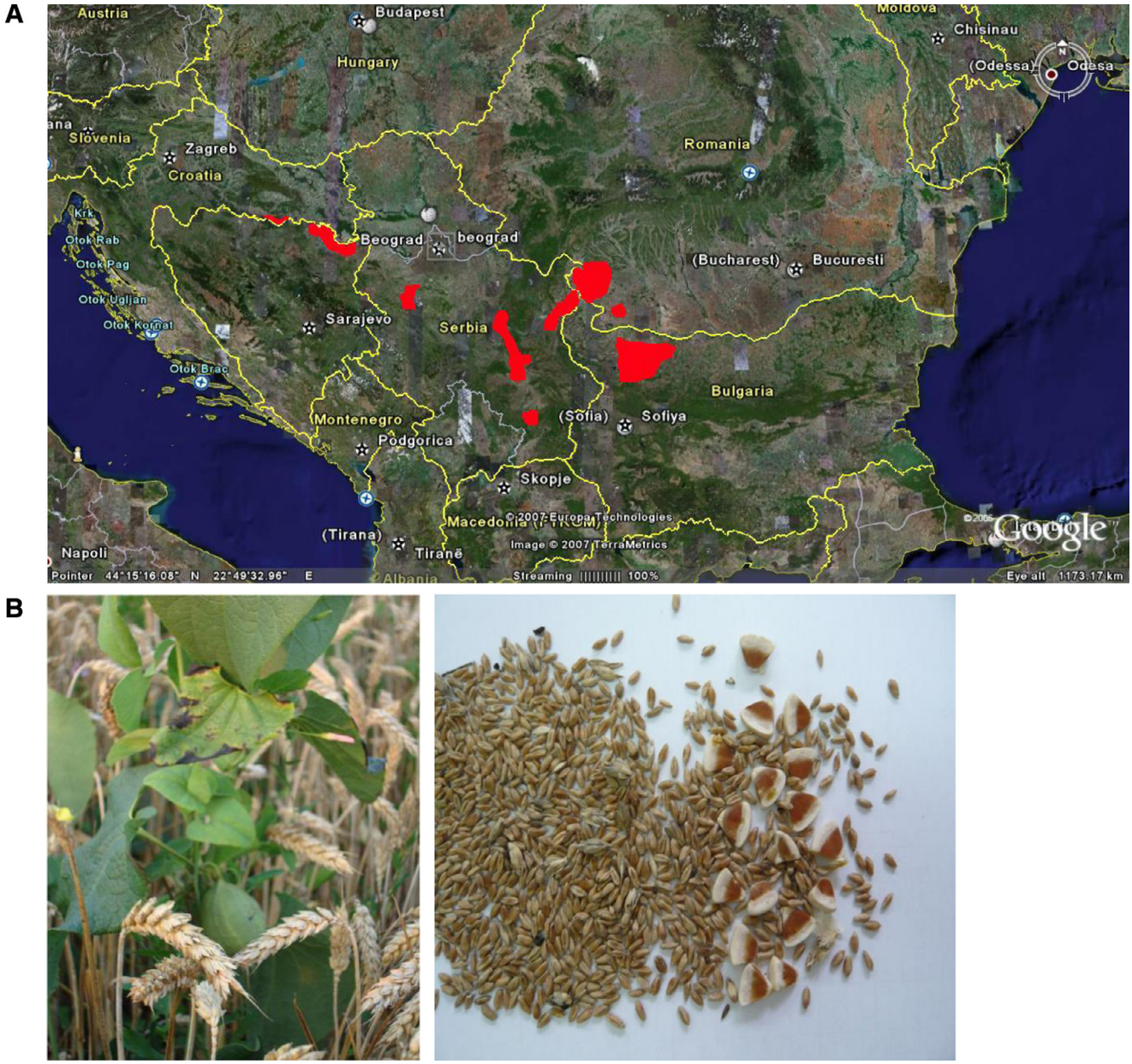

Figure 1. (A) BEN areas in Croatia, Bosnia and Herzegovina, Serbia, Romania, and Bulgaria (in red). (B) *A clematitis* growing in the wheat fields and providing seeds in harvesting time (Croatian-endemic area near the endemic village of Kaniža, August 2013; photograph by B. Jelaković), and seeds of *A clematitis* among wheat seeds (Serbian endemic village of Vreoci, August 2015; photograph by J. Nikolić). (C) Macroscopic finding of a Croatian BEN patient from the endemic village of Kaniža (data on positive aristolactam-DNA adducts and p53 signature mutation were published by Karanović et al[48]; small kidney: length 7 cm, weight 26 gram, smooth surface) (first row, left). Photomicrograph of gradual decrease in fibrosis severity and tubular atrophy from outer-to-inner cortex (hematoxylin-eosin, ×40) (first row, right). Photomicrograph of interstitial fibrosis and tubular atrophy with spared glomeruli (Hematoxylin-eosin, ×100) (second row, left). Photomicrograph of extensive interstitial fibrosis (Mallory's trichrome stain × 100) (second row, right). Macroscopic finding of a Croatian patient with pyelon cancer from the endemic village of Slavonski Kobaš (data on positive aristolactam-DNA adducts and p53 signature mutation were published by Grollman et al[31]: kidney of normal length and weight with pyelon cancer; high-grade transitional cell cancer (third row, left). Hematoxylin-eosin, ×100 (third row, right). Hematoxylin-eosin, ×400 (fourth row). Photograph courtesy of Karla Tomić, MD, PhD, pathologist (General Hospital Slavonski Brod). Used with permission.

metabolic alterations, and heredity and environmental toxins).[19,20] In the critical evaluation of environmental exposure agents suspected in the etiology of BEN, Voice et al[26] in 2006 concluded that mycotoxins and AA are the primary targets. Ochratoxin A (OTA) was mostly investigated but in 2006 the EU Committee on Food Safety reported that there was no convincing evidence from human epidemiology to confirm the association between OTA exposure and the prevalence of BEN or UTUC.[27] This conclusion was based on several important facts: (1) OTA is present in food worldwide; (2) significant overlap was found in the average OTA contamination levels of relevant food categories between BEN and non-BEN areas; (3) higher average blood OTA concentrations

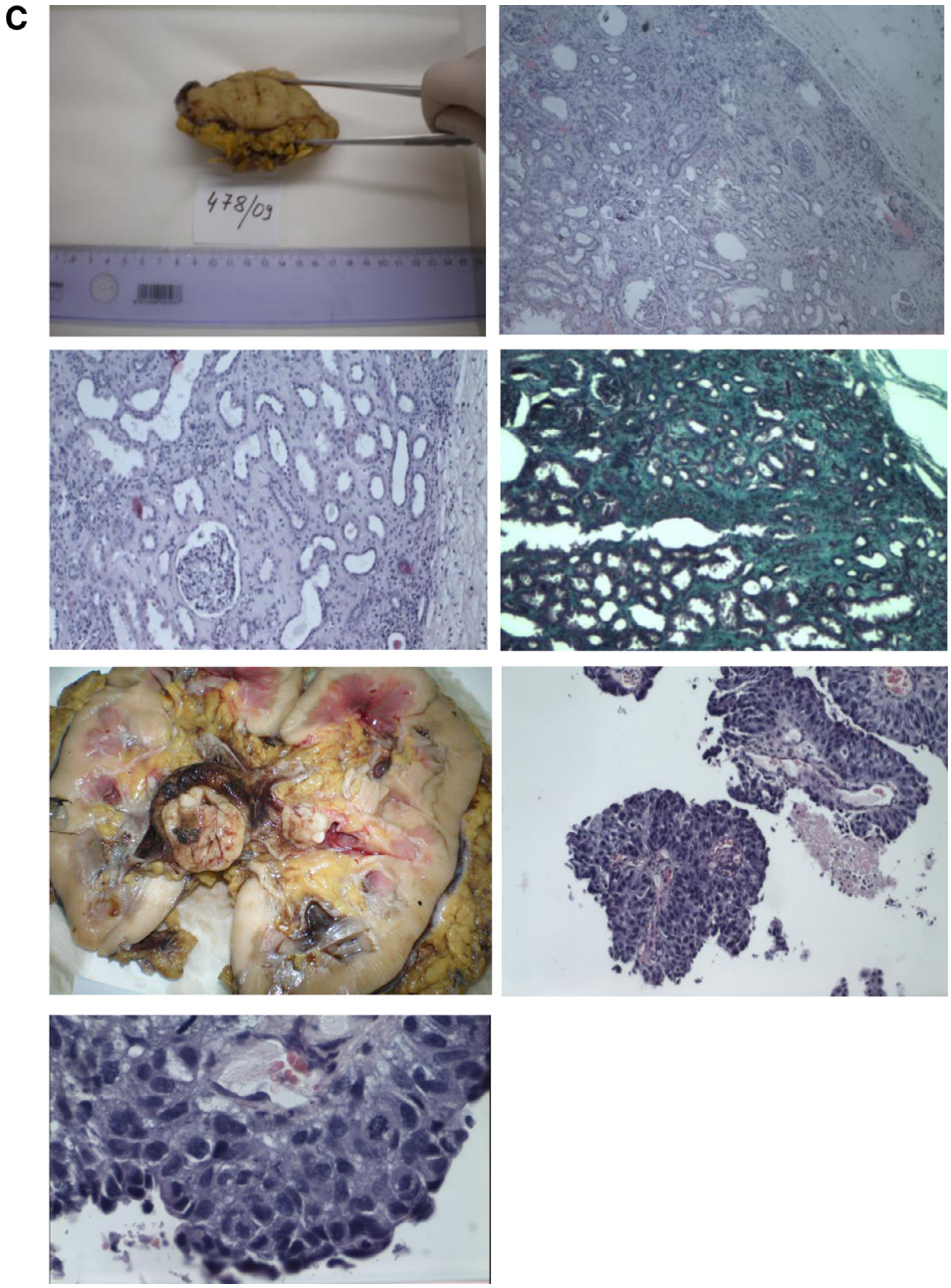

Figure 1. (*Continued*)

were reported in patients with different chronic kidney diseases, thus accumulation of OTA is a consequence rather that the cause of impaired renal function; and (4) the established tolerable weekly intake of 120 ng/kg body weight was significantly higher than the highest values ever reported in BEN patients. In addition to inconclusive epidemiology, histopathology differences and different types of cancer between BEN patients and OTA-induced animal tumors were reported. Finally, there is no evidence of general OTA toxicity in human beings.[16]

The AA hypothesis was first launched in 1969 by Ivić,[5] who observed that the plant *A clematitis* grew much more abundantly in Serbian-endemic than in non-endemic areas. Ivić[5] realized that farmers unaware of the plant's toxicity brought grain contaminated by *Aristolochia* seeds for grinding into flour and concluded that the bread of those peasants was poisonous. He conducted several in vitro experiments and proved that pathologic findings in rats fed with *Aristolochia* seeds completely corresponded to the changes characteristic of BEN. Ten years previously, Martinčić and Dumić[28] reported horse poisoning with *A clematitis* and found strict similarities in epidemiology, clinic, laboratory data, and renal pathology between horses and BEN. Remarkably, over

the next 35 years, no attempt was made to confirm or follow up these prescient observations until the first description of "Chinese herb nephropathy"[1] in Belgium, which called attention to similarities in the pathologic features of AAN diagnosed in Belgium and BEN.[1,4] In 2003, Croatian and US physicians and scientists started a collaboration that aimed to test the hypothesis of whether chronic, low-dose dietary ingestion of AA, in conjunction with individual genetic susceptibility, accounted for all epidemiologic and clinical features of BEN. First, Hranjec et al[29] in 2005 confirmed that seeds of *A clematitis* co-mingled with the wheat grain used by villagers in Croatian-endemic regions to prepare bread. BEN patients observed *A clematitis* in their meadows and farming fields, as well as *Aristolochia* seeds among harvested wheat 20 years ago, significantly more frequently than other end-stage renal disease (ESRD) patients undergoing dialysis (Fig. 1B). Wheat cultivating, homebread baking, and differences in observations of *A clematitis* in farming fields as a risk factor for UTUC was confirmed in larger groups of residents in Croatian and Serbian villages.[30] Arlt et al[6] raised the question of whether aristolactam-DNA adducts might be present in BEN patients. Prima facie evidence of exposure to AA in BEN was published in 2007 when aristolactam-DNA adducts were detected in the renal cortex and urothelial cancer tissue in several Croatian BEN patients. Furthermore, in a group of Croatian and Bosnian BEN patients, mutation of A:T pairs of the tumor-suppressor *TP53* gene accounted for 89% of all mutations, with the majority (78%) being A:T to T:A transversions (ie, AA mutational signature).[31] Later, the presence of aristolactam-DNA adducts and characteristic *TP53* mutations were confirmed in Serbian and Romanian BEN patients.[7,8] Jelakovic et al[7] found aristolactam-DNA adducts in 95% of cases with A:T to T:A transversion mutations, proving a clear association of biomarkers of exposure and a carcinogenic effect; furthermore, they chemically identified AA in DNA adducts using liquid chromatography-electrospray ionization tandem mass spectrometry. After this final evidence that in genetically susceptible individuals dietary exposure to AA is causally related to BEN and UTUC, De Broe[10] proposed that the terms *Chinese herb nephropathy* and *BEN* should be abandoned and the term *AAN* should be introduced to cover both clinical conditions. Interestingly, it also was reported that exposure to AA in Croatian, Bosnian, and Romanian BEN patients was associated not only with UTUC, but also with renal cell carcinomas.[32,33] In addition, use of a whole-exome sequencing method proved that the AA mutational signature is not restricted to the *TP53* gene, but is present genome-wide in Croatian and Bosnian BEN/UTUC patients.[34] It was questioned whether bread consumption was the only route of AA ingestion in BEN patients in the past. Although reports from Romania suggested that herbal medicine also might be a risk factor for AA exposure in BEN,[35] Ivković et al,[36] in a large cohort of more than 2,500 Croatian farmers, rejected this hypothesis and confirmed that the use of herbs and herbal teas was not associated with BEN. Gruia et al[37] and Pavlović et al[38] speculated whether other plants (maize and cucumber) were capable of absorbing AA and thus being a secondary source of food poisoning. Chan et al,[39] using high-performance liquid chromatography coupled with a fluorescence detection method, identified and quantitated AA in corn, wheat grain, and soil samples collected from the endemic village Kutleš in Serbia. They hypothesized that the AA present in edible parts of crops originating from AA-contaminated soil could be one of the pathways by which AA could enter the human food chain.

The next question was how to explain the decreasing prevalence of BEN and how this could fit the AA hypothesis knowing that *A clematitis* is still growing in wheat fields in BEN countries (Fig. 1B). Croatian physicians and agronomists analyzed harvesting and milling practices and found that important improvements occurred in the 1970s: large common mills were built and used instead of small village mills; and combines became popular with much smaller holes in sieving machines, enabling better separation of much bigger *Aristolochia* from the wheat seeds (Jelaković et al, unpublished data). This could explain why the incidence of BEN is decreasing despite *A clematitis* still being present in farming fields. Jelaković et al[30] proved this by analyzing another natural experiment, determining the prevalence of BEN and proximal tubule damage in the group of Bosnian-nonendemic immigrants who settled Croatian-endemic and nonendemic regions after agricultural improvements were performed. In contrast to Ukrainians 70 years ago, the immigrant status of Bosnians who settled endemic villages was a protective predictor for proximal tubule damage. This observation was confirmed with a similar report from Serbia.[40] Thus, the presence of *Aristolochia* in farming fields is a risk factor, but only if associated with particular agricultural practices and lifestyle. BEN prevalence will decrease and finally disappear, but in the next few years, because of past exposure, new BEN patients still will start dialysis and, even more importantly, new BEN/UTUC patients will be diagnosed.

The next intriguing question was why BEN should be restricted only to small well-defined areas in several South-East European countries, while *A clematitis* is more or less a ubiquitous plant.[41] Nikolić[42] analyzed the prevalence and space distribution of UTUC in Serbian-endemic and nonendemic villages, and was the first who proposed that BEN cases also could be found in villages that were not acknowledged as endemic villages, so-called *sporadic BEN cases*. Recently, his hypothesis was confirmed in a molecular-epidemiologic study in which aristolactam-DNA adducts and signature mutations were found in 10 Croatian and Bosnian

farmers living in nonendemic villages (Jelaković et al, unpublished data).

CLINICAL-PATHOLOGIC FEATURES OF BEN

There are no diagnostic features that are pathognomonic of BEN.[4,43,44] The most dominant morphologic characteristic is extensive hypocellular interstitial fibrosis associated with tubular atrophy involving medullary rays that decrease in intensity from the outer medulla and the cortical labyrinth to the inner cortex (Fig. 1C). Chronic interstitial inflammatory cells, mainly in medullary rays and/or outer medulla, usually less than what might be expected in other renal diseases, were found in less than one third of cases.[43] As the disease progresses, glomerular and vascular lesions are associated with periglomerular fibrosis, ending with obsolescent (collapsing type) glomeruli, occasional thrombotic microangiopathy-like lesions, and focal segmental sclerosis-like lesions. Vascular lesions include arteriolar hyalinosis, intimal fibrous hyperplasia, occasional mucoid arterial intimal fibrosis, and multifocal thickening and splitting up of peritubular capillary basement membranes. At end-stage, the kidneys are extremely small, symmetrically contracted, weighing only 20 to 30 g each, with smooth outlines. This type of interstitial fibrosis shares remarkable similarities with the type of renal fibrosis initially described in the Belgian cohort of AAN patients, which also was associated with a similar prevalence of UTUC.[45] Because similar interstitial fibrosis has been reported after exposure to cadmium, lead, cyclosporine A, ifosfamide, pamidronate, lithium, nitrosoureas, and some herbal tea, exposure to these agents, as well as consumption of nonsteroidal anti-inflammatory drugs, should be ruled out. The other important feature is frequent occurrence of UTUC (40%-46% cases).

Major characteristics of BEN and AAN are shown in Table 1. BEN has an insidious onset and slowly progress to ESRD. There is no leading typical symptom (fatigue, loss of appetite, nocturia, polyuria). In the early phases, aseptic leukocyturia and very seldom urine cylinders can be detected and urine-specific gravidity is low. Low-molecular-weight (tubular) proteinuria and enzymuria could be found. It was reported that anemia was more severe for the stage of chronic kidney disease (CKD), probably owing to destruction of peritubular cells that secrete erythropoietin. Another important feature is initially normal blood pressure and development of arterial hypertension in advanced phases of CKD, which is related mostly to tubular damage and salt-wasting. Recently, very probably because of later onset and milder forms of hypertension, lower arterial stiffness and slower vascular aging was reported in Croatian and Bosnian BEN patients undergoing dialysis compared with other ESRD patients.[46] However, a recent report from Croatia showed that the prevalence of hypertension in BEN villages does not differ from other rural parts of Croatia, very probably reflecting changes in lifestyle (high salt intake), obesity, and more stress.[47]

Different clinical courses were reported and BEN patients could have the following: (1) only chronic tubulointerstitial nephropathy leading to ESRD; (2) simultaneously present UTUC (either unilateral or bilateral) with renal impairment and typical BEN histopathology; or (3) initial deterioration of kidney function followed by UTUC (either unilateral or bilateral) (Fig. 1C). According to analyzed case reports, different clinical courses do not seem to be related to differences in exposure, but more likely to differences in metabolic activation or detoxification of AA and/or DNA repair resulting from different genetic polymorphisms.[48]

DNA ADDUCTS FORMED BY AA AS MARKERS OF EXPOSURE AND EARLY PHASE OF UTUC

Over the past decade, AA has emerged as a causative factor for BEN, and based on molecular epidemiology studies AA has been shown to be responsible for the development of BEN-associated UTUC.[49] DNA adducts can be used as biomarkers of exposure and as markers of cancer risk.[50] The detection of aristolactam-DNA adducts in renal tissue unequivocally showed AA exposure in BEN patients.[6-8] DNA damage produced by aristolactam-DNA adducts is one rare example directly linking environmental exposure to cancer development (UTUC) in human beings.[9,51] Previous studies have shown the role of aristolactam-DNA adducts in AAN-associated tumorigenesis.[50,51] Subsequently, AA was classified as carcinogenic to human beings (group 1) by the International Agency for Research on Cancer by a genotoxic mechanism.

The plant extract AA is a mixture of structurally related nitrophenanthrene carboxylic acids, with aristolochic acid I (8-methoxy-6-nitro-phenanthro-[3,4-*d*]-1,3-dioxolo-5-carboxylic acid; AAI) and aristolochic acid II (6-nitro-phenanthro-[3,4-*d*]-1,3-dioxolo-5-carboxylic acid; AAII) being the major components.[16] Both compounds are mutagenic and genotoxic,[52] but AAI is considered to be responsible for AA-mediated nephropathy. Although AAI might directly cause interstitial nephropathy, enzymatic activation of AAI is required to exert its genotoxic (ie, DNA damaging) properties. Reduction of the nitro group is considered the major activation pathway of AA. This reaction is catalyzed primarily by cytosolic nitroreductases, such as nicotinamide adenine dinucleotide (phosphate) hydrate (NAD(P)H):quinone oxidoreductase, and microsomal enzymes such as nicotinamide adenine dinucleotide phosphate hydrate (NADPH):cytochrome P450 (CYP) oxidoreductase and CYPs, predominantly CYP1A1 and CYP1A2.[53] Another AA-activating enzyme is cyclooxygenase, which is highly expressed in urothelial tissue. Besides activation, CYP

Table 1. Characteristics of BEN and Iatrogenic AAN

	BEN	Iatrogenic AAN
Prevalence of affected subjects in exposed population	2%-5%	3%-5%
Sex*	No difference	More women
Familial/household aggregation	Yes	No
Awareness of plant toxicity	Unaware	Inadvertent
Route of ingestion	Home-baked bread	Herbal remedies
Pathology	Identical	Identical
Incidence of UTUC	30%-50%	44%
Clinical course†	Insidious onset, slow progression	Rapidly progressive to ESRD, Fanconi syndrome

*More women than men in AAN owing to the high number of Belgian women who underwent a slimming regimen.
†Clinical course is dose-dependent (ie, in Belgium and in most other AAN cases worldwide a high dose of aristolochic acid was ingested in a shorter period).

enzymes also can be involved in the oxidative detoxification of AAI through *O*-demethylation (ie, the formation of 8-hydroxyaristolochic acid I, also known as aristolochic acid Ia), resulting in a decrease in the actual AAI concentrations that can lead to the attenuation of nephropathy and/or (geno)toxicity. Differences in AA metabolism (activation versus detoxification) might contribute not only to an individual's susceptibility, but also could be an important determinant of cancer risk. Because not all individuals exposed to AA suffer from BEN, in addition to differences in the cumulative dose of AA and the duration of AA intake, differences in the activities of AA metabolizing enzymes may predispose certain residents in areas endemic for BEN.[54] However, studies evaluating genetic polymorphisms of AA-metabolizing enzymes have resulted in only controversial results (reviewed by Stiborova et al[49]), and thus this phenomenon remains to be investigated further.

The most abundant aristolactam-DNA adduct found in renal tissue of BEN patients living in endemic regions in Croatia, Serbia, Bosnia, and Romania is 7-(deoxyadenosine-N^6-yl)-aristolactam I.[6-8] This adduct also shows a long persistence in renal tissues of AAN patients[9] and still is detectable decades after AA exposure.[50] Based on the structure of the aristolactam-DNA adducts, it has been suggested that a cyclic *N*-acylnitrenium ion with a delocalized positive charge (aristolactam-nitrenium ion) is the ultimate electrophilic species that binds preferentially to the exocyclic amino groups of purine nucleotides in DNA through the C7 position of the phenanthrene ring.[51] For several decades, aristolactam-DNA adducts have been analyzed by the ^{32}P-postlabeling assay; however, recent advances in analytic chemistry have allowed the use of mass spectrometry in both fresh and formalin-fixed paraffin-embedded tissue for the identification of these adducts, thus providing an alternative to the ^{32}P-postlabeling technique.[55]

Aristolactam-DNA adducts (ie, 7-[deoxyadenosine-N^6-yl]-aristolactam I) are poorly removed by DNA repair processes (ie, nucleotide excision repair) and thus can induce mutations, predominantly characteristic A:T to T:A transversions, in cancer-related genes including *TP53*. The same hotspot mutations were found in BEN and Taiwanese AAN patients located on the nontranscriptable DNA chain, and thus unrepairable.[56] It also is noteworthy that in rodents characteristic A:T to T:A transversion mutations have been observed in codon 61 of H-*ras*, highlighting the underlying mechanism of AA carcinogenesis in experimental animals.[57] Different environmental carcinogens such as AA are known to cause specific *TP53* mutations and this is referred to collectively as the *TP53* mutation signature.[58] In addition to the determination of aristolactam-DNA adducts in urothelial tissue of BEN patients, the *TP53* mutation signature of AA in BEN-associated tumors has been used as biomarker of effect to show AA exposure in BEN patients.[57,59] Indeed, a high prevalence of A:T to T:A transversion mutations in *TP53* has been found in urothelial tumors of BEN patients originating from Croatia, Serbia, Bosnia, and Romania.[59] This mutation type is otherwise rare in urothelial tumors not associated with AA exposure, thereby providing a molecular link between AA exposure and the formation of BEN-associated urothelial carcinoma (UC) across several geographic foci.[49] Similar *TP53* mutation signatures have been observed in AAN-associated urothelial tumors originating in other parts of the world (eg, Taiwan).[56] Furthermore, studying AA-induced *TP53* mutagenesis using human *TP53* knock-in (Hupki) mouse embryo fibroblasts (HUFs) not only confirmed the *TP53* mutation signature of AA in HUFs immortalized after AAI exposure in vitro,[58] but also that AAI-treated HUFs share so-called hotspot *TP53* mutations observed in UTUC from BEN patients.[59] These findings explain the molecular mechanism whereby AA causes urothelial cancer[49] (Fig. 2).

Massive next-generation sequencing has allowed the analyses of thousands of cancer genomes (exomes and whole genomes) across most cancer types and these data are recorded in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (http://cancer.sanger.ac.uk/cosmic). Currently, 30 different base substitution

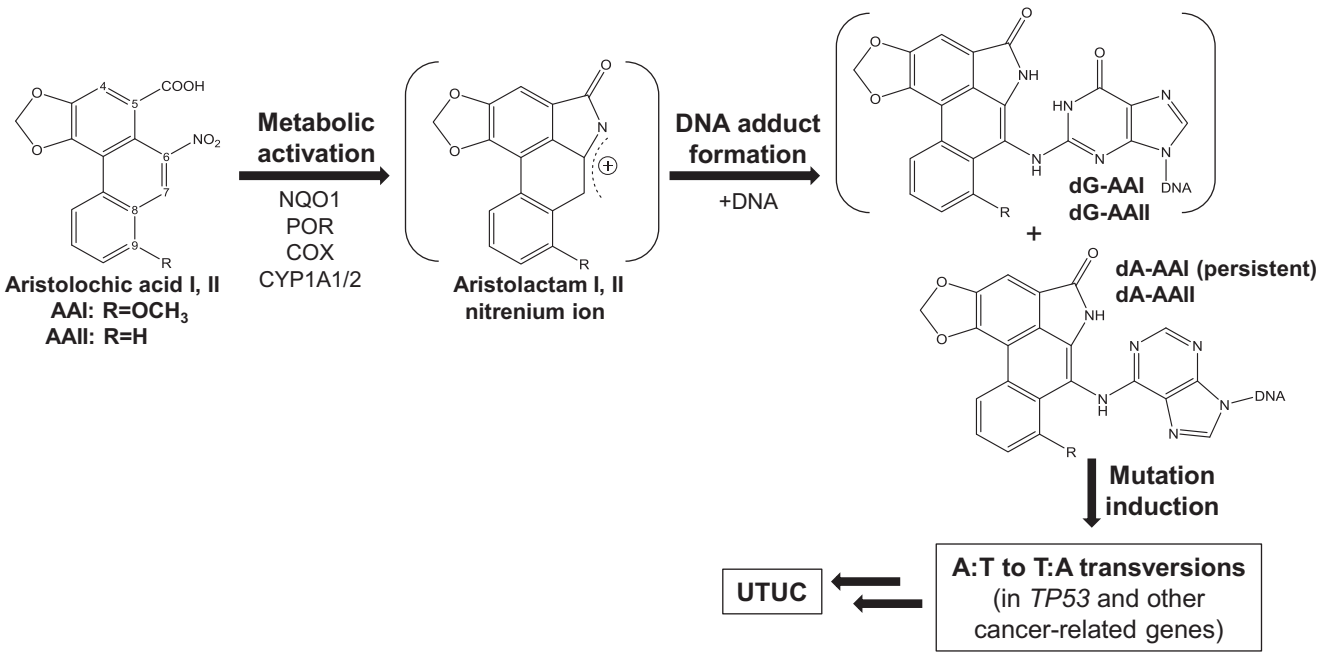

Figure 2. Metabolic activation and DNA adduct formation by AA. COX, cyclooxygenase; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; dG-AAII, 7-(deoxyguanosin-N^2-yl)aristolactam II; dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dA-AAII, 7-(deoxyadenosin-N^6-yl)aristolactam II; NQO1, NAD(P)H:quinone oxidoreductase; POR, NADPH:cytochrome P450 oxidoreductase.

mutational signatures have been published in the COSMIC database to shed light on the etiology of human cancer. Each base substitution signature is characterized by a 96-mutation classification that includes the six substitution types together with the bases immediately 5' and 3' to the mutated base. Some mutational signatures have been linked to environmental exposures and this includes COSMIC signature 22, which shows characteristic A:T to T:A transversion mutations and is attributed to AA exposure. One study conducted exome sequencing in 15 BEN patients with UC and identified COSMIC signature 22, confirming the applicability of this approach to investigate the etiology of AA-induced tumors.[60] This study also identified a number of cancer driver genes: *TP53*, *AHNAK*, *ARID1B*, *ATRX*, *BLM*, *CHD2*, *CHD5*, *CHD8*, *CHD9*, *CHEK2*, *CLTC*, *ERBB4*, *FN1*, *HUWE1*, *IARS2*, *KALRN*, *LRRK2*, *MLL2*, *NEB*, *RXRA*, *SMCHD1*, *SPEG*, *STAG2*, *SYNE1*, and *TRIO*. *TP53* was the most frequently mutated gene. Other recurrently mutated genes were related to regulation of transcription, chromatin/histone modification, DNA damage response, and DNA repair.[60]

By using whole-genome sequencing, exposure to AA in Romania recently was implicated in the development of renal cell carcinoma (RCC).[61] Again, COSMIC signature 22 was identified in the Romanian cancer patients. Subsequently, using mass spectrometry, the formation of aristolactam-DNA adducts was confirmed in these patients, unambiguously showing exposure to AA. RCC have not been reported in BEN patients, but studies in Asia (Taiwan) have linked AA exposure to this cancer type.[62] The source of AA exposure remains unclear in the Romanian cohort with RCC; however, it is clear that these patients do not cover the Romanian population of the BEN area.[61] These results are in line with data obtained in a Croatian study.[32]

SCREENING, DIAGNOSING, CLASSIFICATION, AND TREATMENT OF BEN

Until recently, different diagnostic criteria were used in different BEN centers. These criteria involved several combinations of parameters, various cut-off values, and many of them were not in agreement with proposed current international guidelines. Therefore, leading experts developed a consensus on BEN criteria during the International Workshop on Diagnostic Criteria on Endemic Nephropathy held in Brač, Croatia, in 2008. Despite extensive research, no specific diagnostic biomarker for BEN has yet been identified. Thus far, the diagnosis of BEN is based on the combination of several clinical and laboratory criteria.[43] It was the hope of the authors of the Consensus document that the use of these uniform criteria will make results obtained from studies conducted in different BEN countries comparable, providing better insight into understanding still unanswered questions about the disease and enabling physicians to provide better medical care for the population at risk.

Table 2. Criteria for Diagnosis and Classification of BEN

Diseased/affected BEN cases: Biopsy proven/indicative of BEN* or Residency in a BEN household >20 y + tubular proteinuria† + decreased eGFR + anemia‡ or Residency in BEN village >20 y + UTUC + tubular proteinuria†	**Suspected BEN:** Residency in BEN household >20 y + reduced eGFR + anemia‡ or Residency in BEN household >20 y + tubular proteinuria† or Residency in BEN village >20 y + UTUC
High-risk group for BEN: Residency in BEN household >20 y Residency in households with sporadic/suspected BEN cases >20 y	**Sporadic BEN:**§ Biopsy proven/indicative of BEN in patient with UTUC outside of the endemic region or in member of their household

Abbreviation: eGFR, estimated glomerular filtration rate. Reprinted with permission by Jelakovic et al.[43]
*There are no diagnostic features that are pathognomonic of BEN, but the pattern of injury, in the absence of other disease, is highly suggestive of this entity. Detection of aristolactam-DNA adducts and *TP53* fingerprint mutation is diagnostic.
†α1-Microglobulin >31.5 mg/g and α1-microglobulin/urine albumin concentration ratio ≥0.91.
‡Hemoglobin <120 g/L for men and women >50 years, and <110 g/L for women ≥50 years.
§Subjects with chronic interstitial nephropathies where other causes should be excluded (reflux nephropathy, chronic pyelonephritis, recurrent pyelonephritis, hypertensive nephrosclerosis, exposure to lead, cadmium, cyclosporine A, ifosfamide, pamidronate, lithium and nitrosoureas, heavy use of nonsteroidal anti-inflammatory drugs).[43]

The criteria for diagnosing BEN and the classification of the BEN village population are summarized in Table 2.

Screening

The entire adult population of BEN villages should be screened by mass screening every 5 years.[43] This screening should include determination of tubular proteinuria (α1-microglobulin), estimated glomerular filtration rate (according to the CKD-EPI equation), red blood cell count, dipstick urinalysis, and urine cytology. Patients detected as diseased should be referred to local nephrologists. Patients suspected of having BEN and members of BEN households with no signs of either tubular proteinuria or UTUC should be monitored yearly by the aforementioned screening tool. Patients with ESRD of unknown origin from nonendemic villages and members of their households should be screened for sporadic BEN/UTUC.[43] Patients at high risk for developing UTUC (patients with histopathologic findings indicative of BEN, BEN patients in CKD stages ≥3A, BEN transplanted patients or undergoing dialysis) should be monitored every 6 months, while their household members should be monitored yearly using urine cytology, ultrasound, and other available imaging techniques if needed.[43] Patients with previous UTUC, bladder cancer, or with hematuria should be examined every 3 months, and those with hematuria should be evaluated by cystoscopy. If UTUC is highly suspected, ureteropyeloscopy and a computed tomography scan should be performed as well. In all UTUC patients from farming villages, the renal cortex should be excised during surgery (distant from tumor) and analyzed for evidence of BEN, and, if possible, should be frozen at -20°C for subsequent determination of the level of aristolactam-DNA adducts and *TP53* fingerprint mutations on tumor tissue.[43]

Treatment

Patients with established BEN should be treated similar to other CKD patients, with peritoneal dialysis, hemodialysis, or renal transplantation in the ESRD stage.[43] BEN patients are at high risk of developing UTUC and should undergo appropriate examinations to exclude urothelial cancers before being placed on the waiting list for kidney transplantation. Thus, bilateral nephroureterectomy should be performed in BEN patients before transplantation. In case of living donor transplantation, it is important to perform a kidney biopsy of the donor(s) who lived in the BEN region for more than 15 to 20 years to exclude BEN and/or the presence of AA-DNA adducts.[43] Bilateral nephroureterectomy should be performed in all BEN recipients younger than 65 years, and also in those older than 65 years if UTUC or bladder cancer already has been diagnosed or they have a family history of UTUC.[43] After transplantation, BEN patients who refused bilateral nephroureterectomy should be monitored closely for urothelial cancer. Immunosuppression with mechanistic target of rapamycin inhibitors should be considered for BEN-transplanted patients. Regarding treatment of UTUC, a total nephroureterectomy with excision of a bladder cuff around the ureteral ostium and regional lymphadenectomy is standard therapy. A conservative surgical approach should be reserved only for highly selected patients with bilateral tumors.[43] These patients have an increased risk of local

recurrence and should be monitored closely. Systemic chemotherapy is indicated for unresectable and metastatic disease if not otherwise contraindicated.

In the Belgian cohort of AAN patients with ESRD, who were treated with dialysis or kidney transplantation, a prophylactic bilateral nephroureterectomy also was performed and bladder monitoring was continued by means of cystoscopies with randomized bladder biopsies at least every year. Indeed, a significant number of patients developed bladder cancer several years after surgical removal of their native kidneys and ureters.[63] In noninvasive bladder cancer, treatment included endoscopic resections supplemented by the endovesical instillation of mitomycin C. Endovesical therapy based on the Bacillus Calmette-Guerin immunotherapy also was performed successfully, even in patients with a renal graft, provided the therapy was combined with modulation of immunosuppression and prophylactic antituberculosis chemotherapy.[64] Radical cystectomy with pyelostomy of the graft remains the ultimate measure in kidney transplant recipients with invasive bladder cancer.

NEPHROTOXICITY ASSESSMENT OF AA BY THE *OMICS* APPROACH

Innovative approaches used in Systems Biology, which encompasses genomics, transcriptomics, proteomics, and metabolomics, have gained great interest in the past decades. Such techniques not only facilitate the development of biomarkers and predictors, but they help gain basic biological insights into the disease etiology. Because of their multifactorial character and the associated comorbidities, an integrated approach also can be helpful in redefining and stratifying chronic diseases. In the field of nephrology, recent advances in omics technologies has created an opportunity for integrating omics data sets to building a comprehensive and dynamic model of the molecular changes in CKD.[65] Several studies already have underlined the advantage of using urine samples for noninvasive data collection throughout disease progression. Moreover, it also can be adapted for patient clustering, identification of diseased and at-risk populations in epidemiologic studies, and, where appropriate, tailoring treatments to patients via personalized medicine.[66] On the other hand, in the context of risk assessment of chemicals and natural substances, Systems Biology (also referred to in this case as toxicogenomics), enables the study of adverse effects of xenobiotic substances in relation to structure and activity of the genome, proteome, and metabolome.[67] Applied to nephrotoxicity, toxicogenomics allowed for more sensitive and earlier detection of adverse effects in many in vivo and in vitro preclinical toxicity studies, as reviewed by Zhao and Lin.[68] An additional advantage is the possibility of studying the effects of exposure to mixtures in more detail. By using DrugMatrix, a large database including gene expression data from rats exposed to diverse chemicals, a cluster of 30 genes was identified that could assess the nephrotoxic potential of a chemical well before injury actually occurs.[69]

Recent advances in the development of omics-type biomarkers of acute/chronic kidney diseases encouraged researchers to apply toxicogenomics to AAN. From the plethora of genomic studies devoted to AAN, two major findings emerge (detailed in the *DNA Adducts Formed by AA as Markers of Exposure and Early Phase of UTUC* section): aristolactam-DNA adducts, direct evidence of AA exposure, were identified in various animal and human studies,[55] and a specific AA-related mutational fingerprint, mostly in oncogenes and tumor-suppressor genes, was shown in UTUC.[70] From the proteomic side, analyses of urinary, plasma, and renal tissue resulted in differential expression of several cytoskeletal, developmental, and inflammatory kidney proteins in AA-exposed and control mice.[71] A proteomic signature of AA exposure also was identified in rat kidney and, interestingly, some of those proteins presented obvious biological and medical significance.[72] Finally, the metabolomic approach also contributed to a better understanding of AAN both in acute and chronic exposures. In fact, a wide range of metabonomic analytical techniques have been used lately in the modern research of TCM, with a special focus on TCM toxicity issues. These techniques include proton nuclear magnetic resonance (^{1}H-NMR), gas chromatography−mass spectrometry, and liquid chromatography−mass spectrometry. By using ^{1}H-NMR analysis of urine samples, Duquesne et al[73] compared the severity of nephrotoxicity of AAI and AAII given alone or in combination to rats. The main metabolic alterations, including increased urine levels of glucose, amino acids, and organic acids, together with decreased concentrations in hippurate, together were indicative of an acute proximal tubule injury. These dose-dependent damages were confirmed by histology at later time points and for longer periods of exposure. Renal damage was more pronounced with the mixture or AAII alone than with AAI alone.

The metabolomic approach also was used for clinical and epidemiologic purposes. For instance, an NMR study conducted on Romanian and Bulgarian people diagnosed with BEN and treated by hemodialysis highlighted the predictive advantage of metabolomics as compared with more conventional criteria.[74] NMR spectra of urine samples collected from Belgian AAN women were compared with those collected from Croatian BEN patients.[75] Interestingly, both Belgian and Croatian patients presented close urine metabolic profiles, bringing some new evidence that both diseases have a common etiology. In the context of AAN, toxicogenomics thus has identified DNA adducts in genes directly involved in the onset, promotion, and progression of urothelial cancer. Proteomics undoubtedly has advanced the discovery of novel biomarkers of this proximal tubular nephropathy. Many hopes are now

based on the predictive potential of these markers to quickly point the evolution from acute to chronic nephrotoxicity. Finally, metabolomics is certainly not left over. Based on a readily and noninvasively accessible biological matrix, namely urine, this metabolic approach opens new perspectives in patient stratification and follow-up evaluation, whether in terms of disease progression or effectiveness of the therapeutic strategies provided.

CONCLUSIONS AND PERSPECTIVES

The combination of chronic interstitial nephropathy with the UTUC tract should suggest the diagnostic of AAN. In addition, a long-term residence in endemic settlements of Balkan countries, but probably also in some other countries where traditional harvesting was used until the middle of the past century, and an occupational history of farming, should suggest the diagnosis of environmental AAN (ie, BEN).

In addition to the consensus for diagnosing BEN described earlier, a general consensus exists regarding the definition of the diagnostic criteria for AAN.[12] The diagnosis of AAN can be considered as certain in any person who suffers from renal failure, in combination with any two of the following three criteria: a renal histology showing interstitial fibrosis with a corticomedullary gradient, a history of ingesting vegetal or herbal products whose phytochemical analysis has shown the presence of AA, and the presence of aristolactam-DNA adducts (or the specific mutation A:T to T:A of gene *TP53*) in a kidney tissue sample or of a urothelial cancer. Nevertheless, if only one of these three criteria can be shown, the diagnosis of AAN remains highly probable and examinations should be continued in this direction. Whatever the case, the presence of either AA in plant extracts ingested by patients or of aristolactam-DNA adducts in patients' renal tissue samples are central to a diagnosis that provides absolute certainty.

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