DOI:
10.1016/j.semnephrol.2019.02.007

Document Version
Peer reviewed version

Balkan Endemic Nephropathy and the causative role of aristolochic acid

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Financing: none

Conflicts of interest: none

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Abstract

Balkan endemic nephropathy (BEN) is a chronic tubulo-interstitial 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 Balkan peninsula in rural areas around tributaries of the Danube River. After decades of intensive investigations, the causative factor was identified as the environmental phytotoxin aristolochic acid (AA) contained in *Aristolochia clematitis*, a common plant growing in the wheat fields, which was ingested through home-baked bread. AA was initially 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 tumour 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.

Keywords: Balkan endemic nephropathy, aristolochic acid nephropathy, upper tract urothelial carcinoma, aristolochic acid, *Aristolochia species*

1. Introduction

In 1993, the occurrence of a rapidly progressive form of renal interstitial fibrosis associated with a weight loss diet which 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] Ever since, secondary nephropathies resulting from the toxicity of plants containing AA have been described worldwide.[3] The similarity between the histological aspects of this particular nephropathy and the so-called Balkan endemic nephropathy (BEN) proved instrumental in reviving an old hypothesis on the etiology of BEN.[1,4] In 1969, Ivic had suggested that the latter, occurring in certain villages throughout the Danube Valley, might be caused by the chronic ingestion of the seeds of the *Aristolochia clematitis,* a common plant growing in the wheat fields of these endemic regions.[5] This hypothesis has now 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 tumours 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 herbs nephropathy”), or by the environmental

contaminants in food (BEN).[10]

Although the initial Belgian cohort only included over one hundred 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 themselves 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* sp. 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, *Aristolochia clematis* 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, *Aristolochia 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 *Aristolochia 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] Since the plants were also 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).

Besides 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] Due to 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.

2. Epidemiology and etiology of BEN

BEN is a chronic tubulo-interstitial nephropathy associated with urothelial carcinoma of the upper urinary tract (UTUC).[19,20] It was described in South East Europe at Balkan peninsula in rural areas located in the values of

great tributaries of the river Danube. The first cases were recognized in the middle of 20th century. Disease was observed only in harvesting farmers affecting certain villages. Family or more precisely household aggregation was reported in all BEN countries (Bosnia and Herzegovina, Bulgaria, Croatia, Romania and Serbia) (Figure 1A). Inherited pattern of the disease was ruled out by the fact that BEN often affected several members of the same household not necessarily blood related. At the WHO meeting which was held in Dubrovnik, Croatia, in 1964 the disease was named endemic nephropathy. Average prevalence of diseased subjects in the past years ranges between 2-5%. However, the prevalence of farmers suspected to have BEN is much higher and was reported to be 10-15%. There were no gender differences, although slight insignificant female predominance was found (1:1.2). Importantly, it was never reported in children indicating that long period of exposure to the environmental agent is needed. In last decades, the age when BEN patients start to receive dialysis was shifted to older ages, raising the question whether the etiological agent is still present or active. This is in concordance with the results obtained in Croatian field surveys conducted between 2005 and 2015, where neither new BEN nor new UTUC patients were detected in some previously established BEN villages.[21] Similar trends were observed in Serbia.[22,23]

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 pathological findings – good preservation of glomeruli and a gradient of

tubular atrophy with severe interstitial fibrosis which decreases in severity from outer-to-inner cortex -. Two target tissues were identified in BEN patients: 1) proximal renal tubular cells, leading to the interstitial fibrosis and eventually chronic renal failure, and 2) urothelial (transitional) cells, leading to high prevalence of UTUC. Specific mortality of UTUC was reported to be 55 times higher in Croatian EN county vs. other parts of Croatia and similar findings were found in Serbia and other countries where BEN was observed.[19,20,23,24] Epidemiological findings and striking geographical correlation of two otherwise very rare diseases (chronic interstitial nephropathy and UTUC) pointed to a common etiological agent. Čeović *et al.* (1985) reported first firm evidence on the importance of environment and life style 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.[25] On the contrary, Ukrainians who settled at the same time the Croatian non-endemic villages did not develop BEN, and finally, BEN has never been described in 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 metabolic alterations, heredity and environmental toxins).[19,20] In the critical evaluation of environmental exposure agents suspected in the etiology of BEN, Voice *et al.* (2006) concluded that mycotoxins and AA are the primary targets.[26] 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 concentration were reported in patients with different chronic kidney diseases, thus accumulation of OTA is a consequence rather that the cause of impaired renal function; 4) established tolerable weekly intake (TWI) 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 type of cancer between BEN patients and OTA-induced animal tumours were reported. Finally, there is no evidence of general OTA toxicity in humans.[16]

The AA hypothesis was first launched in 1969 by Ivić, who observed that the plant *Aristolochia clematitis* grew much more abundantly in Serbian endemic than in non-endemic areas.[5] Ivić realized that farmers unaware of plants 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 pathological findings in rats fed with *Aristolochia* seeds completely corresponds to the changes characteristic of BEN. Already ten years before, Martinčić and Dumić had reported horse poisoning with *Aristolochia clematitis* and found strict

similarities in epidemiology, clinic, laboratory data and renal pathology between horses and BEN.[28] 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" in Belgium[1], 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 collaboration aiming to test the hypothesis whether chronic, low-dose dietary ingestion of AA, in conjunction with individual genetic susceptibility, accounts for all epidemiological and clinical features of BEN. Firstly, Hranjec *et al.* (2005) confirmed that seeds of *Aristolochia clematitis* co-mingled with the wheat grain used by villagers in Croatian endemic regions to prepare bread.[29] BEN patients observed *Aristolochia 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 (Figure 1B). Wheat cultivating, home-bread baking and difference in observation of *Aristolochia clematitis* in farming fields as a risk factor for UTUC was confirmed in larger groups of residents of Croatian and Serbian villages.[30] Arlt *et al.* (2002a) raised the question whether aristolactam-DNA adducts might be present in BEN patients and *prima facie* evidence of exposure to AA in BEN was published in 2007 when aristolactam-DNA adducts were detected in renal cortex and urothelial cancer tissue in several Croatian BEN patients.[6,31] 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, i.e. 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] Jelaković *et al.* (2012) 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 carcinogenic effect; furthermore, they chemically identified AA in DNA adducts using LC-ESI/MS/MS^3.[7] After this final evidence that in genetically susceptible individuals dietary exposure to AA is causally related to BEN and UTUC, de Broe proposed that the terms “Chinese Herb Nephropathy“ and BEN should be abandoned and the term “AAN“ had to be introduced to cover both clinical conditions.[10] Interestingly, it was also 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, using whole-exome sequencing method proved that AA mutational signature is not restricted to the *TP53* gene but is genome-wide present in Croatian and Bosnian BEN/UTUC patients.[34] It was questioned whether in the past bread consumption was the only route of AA ingestion in BEN patients. Although reports from Romania suggested that herbal medicine also might be a risk factor for AA exposure in BEN, Ivković *et al.* (2014) in a large cohort of more than 2,500 Croatian farmers rejected this hypothesis that the use of herbs and herbal teas was not associated with BEN.[35,36] Gruia and Pavlović speculated whether other plants (maize and cucumber) were capable of absorbing AA and

thus being a secondary source of food poisoning.[37,38] Chan *et al.* (2016), by the use of high-performance liquid chromatography (HPLC) coupled with fluorescence detection method, identified and quantitated AA in corn, wheat grain, and soil samples collected from the endemic village Kutleš in Serbia.[39] They hypothesized that 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 decreasing prevalence of BEN and how could this fit the AA hypothesis knowing that *Aristolochia clematitis* is still growing in wheat fields in BEN countries (Figure 1B). Croatian physicians and agronomists analyzed harvesting and milling practices and found that important improvements occurred in the 1970s: 1) large common mills were built and used instead of small village mills; 2) 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 *Aristolochia clematitis* still being present in farming fields. Jelaković *et al.* (2015) proved this by analyzing another "natural experiment" determining the prevalence of BEN and proximal tubule damage in the group of Bosnian non-endemic immigrants who settled Croatian endemic and non-endemic regions after agricultural improvements were done.[30] In contrast to Ukrainians 70 years ago, the immigrant status of Bosnian 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 life style. BEN prevalence will decrease and finally disappear, but in next few years, due to past exposure, new BEN patients will still 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 *Aristolochia clematitis* is more or less ubiquitous plant.[41] Nikolić *et al.* (2006) analyzed the prevalence and space distribution of UTUC in Serbian endemic and non-endemic villages, and was the first who proposed that BEN cases also could be found in villages which were not acknowledged as endemic villages, so-called "sporadic BEN cases".[42] Recently, his hypothesis was confirmed in a molecular-epidemiological study, where aristolactam-DNA adducts and signature mutation were found in 10 Croatian and Bosnian farmers living in non-endemic villages (Jelaković *et al.*, unpublished data).

3. Clinical-pathological features of BEN

There are no diagnostic features which 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 outer medulla and the cortical labyrinth to the inner cortex (Figure 1C). Chronic interstitial inflammatory

cells mainly in medullary rays and/or outer medulla, usually less than that might be expected in other renal diseases, were found in less than one-third of cases.[43] As 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 20–30 grams 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 was also associated with a similar prevalence of UTUC.[45] As similar interstitial fibrosis has been reported following exposure to cadmium, lead, cyclosporine A, ifosfamide, pamidronate, lithium, nitrosoureas and some herbal tea, exposure to these agents, as well as consumption of non-steroidal antiinflammatory 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 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 due 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 mostly related 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 to 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 1) only chronic tubulo-interstitial nephropathy leading to ESRD; 2) simultaneously present UTUC (either unilateral or bilateral) with renal impairment and typical BEN histopathology; 3) initial deterioration of kidney function followed by UTUC (either unilateral or bilateral) (Figure 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]

4. 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 demonstrated 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 humans.[9,51] Previous studies have demonstrated the role of aristolactam-DNA adducts in AAN-associated tumourigenesis.[50,51] Subsequently, AA has been classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) acting 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 (i.e. DNA damaging) properties. Reduction of the nitro group is considered the major activation pathway of AA. This reaction is primarily catalyzed by cytosolic nitroreductases, such as

NAD(P)H:quinone oxidoreductase (NQO1), and microsomal enzymes such as NADPH:cytochrome P450 (CYP) oxidoreductase (POR) and CYPs, predominantly CYP1A1 and CYP1A2.[53] Another AA-activating enzyme is cyclooxygenase (COX), which is highly expressed in urothelial tissue. Besides activation, CYP enzymes can also be involved in the oxidative detoxification of AAI through *O*-demethylation (i.e. the formation of 8-hydroxyaristolochic acid I, also known as aristolochic acid Ia [AAIa]) 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 not only contribute to an individual's susceptibility but could also be an important determinant of cancer risk. Since not all individuals exposed to AA suffer from BEN, besides differences in the cumulated dose of AA and the duration of AA intake, differences in the activities of AA metabolising enzymes may predispose certain residents in areas endemic for BEN. However, studies evaluating genetic polymorphisms of AA-metabolising enzymes have only resulted in controversial results [reviewed in [49]] and thus this phenomenon remains to be further investigated.

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 (dA-AAI).[6-8] This adduct also shows a long persistence in renal tissue of AAN patients [9] and is still 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 delocalised 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 analysed by the ^{32}P-postlabeling assay; however, recent advances in analytical chemistry have allowed 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 (i.e. dA-AAI) are poorly removed by DNA repair process (i.e. 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 being located on the non-transcriptable DNA chain, thus unrepairable.[56] It is also 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 like AA are known to cause specific *TP53* mutations and this is collectively referred to as *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 in order to demonstrate 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 UC across several geographical foci.[49] Similar *TP53* mutation signatures have been observed in AAN-associated urothelial tumors originating in other parts of the world (e.g. Taiwan).[56] Further, 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] (Figure 2).

Massive next generation sequencing has allowed the analyses of thousands of cancer genomes (exomes and whole genomes) across most cancer types and this data is recorded in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (http://cancer.sanger.ac.uk/cosmic). Currently 30 different base substitution mutational signatures have been published in the COSMIC database in order 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 tumours.[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]

Using whole-genome sequencing exposure to AA in Romania was recently 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 demonstrating 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 Croatian study.[32]

5. 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 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, 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 comparable obtained results of conducted studies in different BEN countries, providing better insight in understanding still unanswered questions of the disease and enabling to provide better medical care for the population at risk.

Criteria for diagnosing BEN and classification of BEN village population are summarized in Table 2.

Screening The entire adult population of BEN villages should be screened by mass screening every five years.[43] This screening should include determination of tubular proteinuria (α1-Microglobulin), estimated Glomerular Filtration Rate (according to CKD-EPI equation), red blood cell count, dipstick urinalysis and urine cytology. Those detected as "Diseased" should be referred to local nephrologists. "BEN suspected" 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 non-endemic villages and members of their households should be screened for sporadic BEN/UTUC.[43] Patients at high risk for developing UTUC (patients with histopathological 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 yearly using urine cytology, ultrasound, and other available imaging techniques if needed.[43] Patients with previous UTUC, bladder cancer or having hematuria should be examined every 3 months, and those with hematuria should be evaluated by cystoscopy. If UTUC is highly suspected, ureteropyeloscopy and CT scan should be performed as well. In all UTUC patients from farming villages, 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 mutation on tumor tissue.[43]

Treatment Patients with established BEN should be treated like other CKD patients, with peritoneal dialysis, hemodialysis or renal transplantation in 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 prior to transplantation. In case of living donor transplantation, it is important to perform kidney biopsy of the donor(s) who lived in the BEN region for more

than 15-20 years to exclude BEN and/or 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 UUC or bladder cancer has already been diagnosed or they have family history of UTUC.[43] After transplantation, BEN patients who refused bilateral nephroureterectomy should be monitor closely for urothelial cancer. Immunosuppression with mTOR inhibitors should be considered for BEN-transplanted patients. Regarding treatment of UTUC, total nephroureterectomy with excision of a bladder cuff around ureteral ostium and regional lymphadenectomy is standard therapy. A conservative surgical approach should be reserved only for the highly selected patients with bilateral tumors.[43] These patients have 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 was also performed and bladder monitoring continued by means of cystoscopies with randomized bladder biopsies at least every year. Indeed, a significant amount of patients developed bladder cancer several years after surgical removal of their native kidneys and ureters.[63] In non-invasive bladder cancer, treatment included endoscopic resections supplemented by the endovesical instillation of mitomycin C. Endovesical therapy based on the Bacillus Calmette-Guerin (BCG) was also successfully

performed, even in patients with a renal graft, provided the therapy was combined with modulation of immunosuppression and prophylactic anti-tuberculosis chemotherapy.[64] Radical cystectomy with pyelostomy of the graft remains the ultimate measure in kidney transplant recipients with invasive bladder cancer.

6. Nephrotoxicity assessment of AA by the "omics" approach

Innovative approaches used in System Biology, that encompasses genomics, transcriptomics, proteomics and metabolomics, have gained great interest in the last decades. Such techniques not only facilitate the development of biomarkers and predictors, but they help gaining basic biological insights into the disease etiology. Due to their multifactorial character and the associated co-morbidities, an integrated approach can also 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 datasets to building a comprehensive and dynamic model of the molecular changes in CKD.[65] Several studies have already underlined the advantage of using urine samples for non-invasive data collection throughout disease progression. Moreover, it can also be adapted for patient clustering, identification of diseased and at-risk populations in epidemiological 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, System Biology (also referred in this case to 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 details. 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 section 4): i) aristolactam-DNA adducts, a direct evidence of AA exposure, were identified in various animal and human studies [55] and ii) a specific AA-related mutational fingerprint mostly in oncogenes and tumor suppressor genes was revealed 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 was also 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 (GC-MS), and liquid chromatography-mass spectrometry (LC-MS). Using ^{1}H-NMR analysis of urine samples, Duquesne et al compared the severity of nephrotoxicity of AAI and AAII given alone or in combination to rats.[73] Main metabolic alterations, including increased urine levels of glucose, amino acids and organic acids together with decreased concentrations in hippurate were all together indicative of an acute proximal tubule injury. These dose-dependent damages were confirmed by histology at later time points and for longer period of exposure. Renal damage was more pronounced with the mixture or AAII alone than AAI alone.

The metabolomic approach was also used for clinical and epidemiological purposes. For instance, an NMR study conducted on Romanian and Bulgarian BEN-diagnosed people treated by hemodialysis highlighted the predictive advantage of metabolomics as compared to more conventional criteria.[74] NMR spectra of urine samples collected from Belgian AAN women were actually compared to 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 has thus identified DNA adducts in genes directly involved in the onset, promotion and progression of urothelial cancer. Proteomics has undoubtedly 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 non-invasively accessible biological matrix, namely urine, this metabolic approach opens new perspectives in patient stratification and follow-up, whether in terms of disease progression or effectiveness of the therapeutic strategies provided.

7. Conclusion and perspectives

The combination of chronic interstitial nephropathy with 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 till the middle of last century, and an occupational history of farming should suggest the diagnostic of environmental AAN, i.e. BEN.

Besides the consensus for diagnosing BEN described above, a general consensus exists regarding the definition of 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 displaying interstitial fibrosis with a cortico-medullary gradient, a history of ingesting vegetal or herbal products whose phytochemical analysis has demonstrated 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 demonstrated, 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.

Acknowledgements

Work at Charles University was supported by the Grant Agency of the Czech Republic (grant GACR 17–12816S). Work at King's College London is supported by the Wellcome Trust (Grants 101126/Z/13/Z and 101126/B/13/Z) and in part by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Health Impact of Environmental Hazards at King's College London in partnership with Public Health England (PHE) and Imperial College London. The views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR, the Department of Health & Social Care or PHE.

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Figure legends

Figure 1A. Balkan endemic nephropathy (BEN) areas in Croatia, Bosnia and Herzegovina, Serbia, Romania and Bulgaria (in red).

Figure 1B. *Aristolochia clematitis* growing in the wheat fields and having fruits in harvesting time (Croatian endemic area near the endemic village of Kaniža, August 2013- photo by B. Jelaković) and seeds of *Aristolochia clematitis* among wheat seeds (Serbian endemic village of Vreoci, August 2015- photo by J. Nikolić).

Figure 1C. 1C1: Macroscopic finding of a Croatian BEN patient from endemic village of Kaniža (data on positive aristolactam-DNA adducts and p53 signature mutation were published in ref 48: small kidney (length 7 cm, weight 26 gram, smooth surface); Photomicrographs: 1C2: Gradual decrease in fibrosis severity and tubular atrophy from outer-to-inner cortex (HEx40); 1C3: Interstital fibrosis and tubular atrophy with spared glomeruli (Hex 100); 1C4: Extensive interstitial fibrosis (Mallory x 100); 1C5: Macroscopic finding of a Croatian patient with pyelon cancer from endemic village of Slavonski Kobaš (data on positive aristolactam-DNA adducts and p53 signature mutation were published in ref 31: kidney of normal length and weight with pyelon cancer; high grade transitional cell cancer (1C6:HEx100; 1C7 HEx400). Courteously by Karla Tomić, MD, PhD pathologist in General Hospital

Slavonski Brod.

Figure 2. Metabolic activation and DNA adduct formation by AA. NQO1, NAD(P)H:quinone oxidoreductase; POR, NADPH:cytochrome P450 oxidoreductase; COX, cyclooxygenase; CYP, cytochrome P450; 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;

Table 1. Characteristics of BEN and iatrogenic AAN

	BEN	Iatrogenic AAN
Prevalence of affected subjects in exposed population	2-5%	3-5%
Gender [1]	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 [2]	Insidious onset, slow progression	Rapidly progressive to ESRD, Fanconi syndrome

[1]More women than men in AAN due to high number of Belgium women who underwent slimming regime; [2]Clinical course is dose-dependent i.e. in Belgium and most of other AAN cases worldwide high dose of aristolochic acid was ingested in shorter period. BEN = Balkan endemic nephropathy; AAN = aristolochic acid nephropathy; UTUC= urothelial carcinoma of the upper urinary tract.

Table 2. Criteria for diagnosis and classification of BEN

Classification of BEN	
I. Diseased/affected BEN cases	**II. Suspected BEN**
(1) Biopsy proven/indicative of BEN[1] or (2) Residency in a BEN household >20 years + tubular proteinuria[2] + decreased eGFR + anemia[3] or (3) Residency in BEN village >20 years + UTUC + tubular proteinuria[2]	(1) Residency in BEN household >20 years + reduced eGFR + anemia[3] or (2) Residency in BEN household >20 years + tubular proteinuria[2] or (3) Residency in BEN village >20 years + UTUC
III. High risk group for BEN	**IV. Sporadic BEN[4]**
(1) Residency in BEN households >20 years (2) Residency in households with sporadic/suspected BEN cases >20 years	Biopsy proven/indicative of BEN in patient with UTUC outside of the endemic region or in member of their household

[1] There are no diagnostic features which 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; [2] α1-Microglobulin >31.5 mg/g and α1-Microglobulin/ urine albumin concentration ratio ≥0.91; [3] Hemoglobin <120 g/L for men and women >50 years, and <110 g/L for women ≥50 years; [4] 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 non-steroidal antiinflammatory drugs.[43]

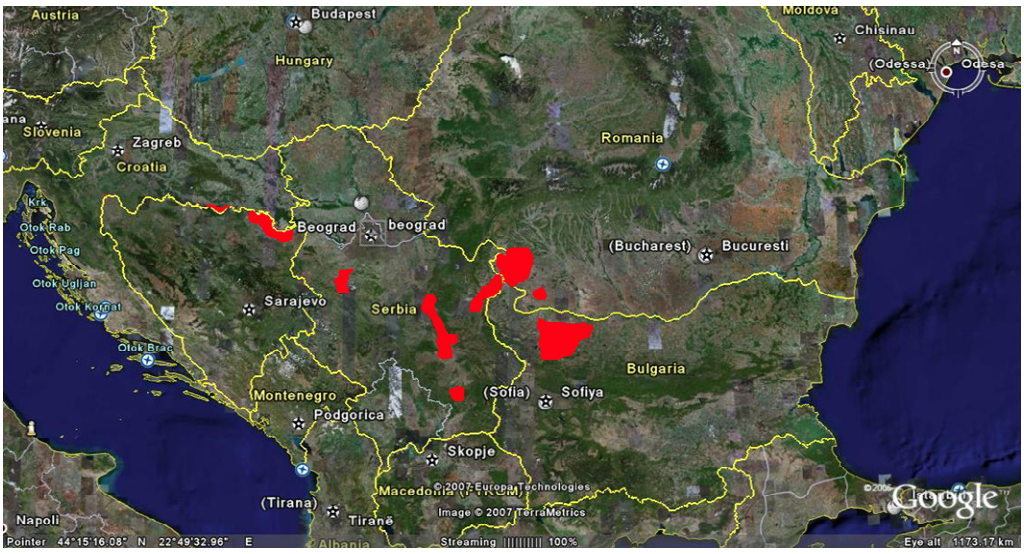
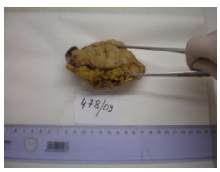
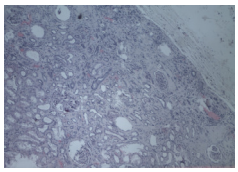


Figure 1A

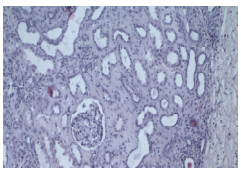
Figure 1B.



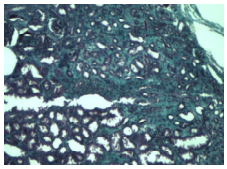
1C1.



1C2.

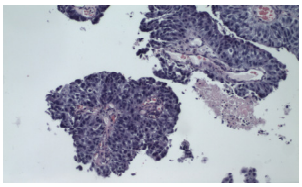


1C3.

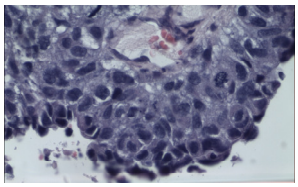


1C4.

1C5.



1C6.



1C7.

Figure 1C.

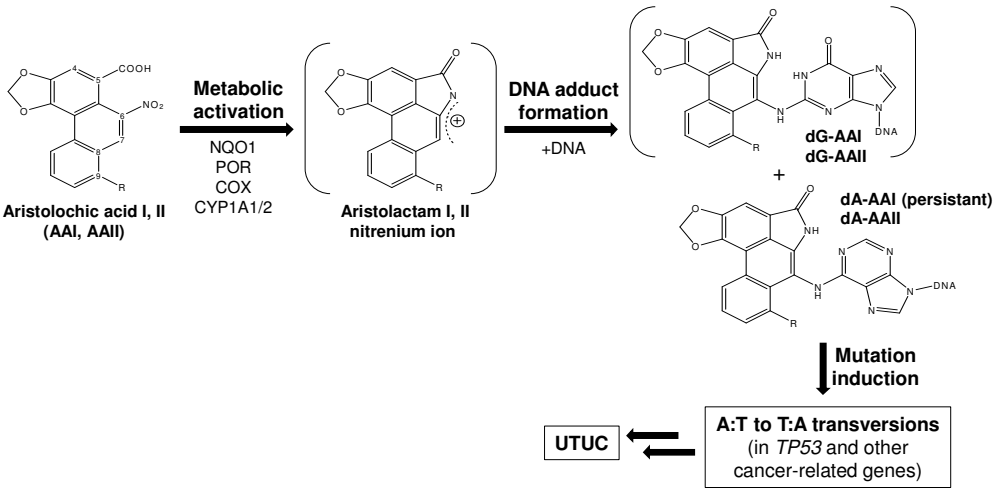

Aristolochic acid I, II
(AAI, AAII)
Metabolic activation
NQO1
POR
COX
CYP1A1/2
Aristolactam I, II
nitrenium ion
DNA adduct formation
+DNA
dG-AAI
dG-AAII
+
dA-AAI (persistant)
dA-AAII
Mutation induction
A:T to T:A transversions
(in TP53 and other
cancer-related genes)
UTUC

Figure 2