
A Translational Metabonomic Assessment of Aristolochic Acid-Induced Nephropathies

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Abstract

Aristolochic acid nephropathy (AAN) is a global term including any form of toxic interstitial nephropathy that is caused either by the ingestion of plants containing aristolochic acids (AA) as part of traditional phytotherapies or by the environmental contaminants in food. Originally, AAN was reported in Belgium in individuals having ingested slimming pills containing powdered root extracts of a Chinese herb, *Aristolochia fangchi*. However, it is estimated that exposure to AA affects thousands of people all over the world, particularly in the Balkans, Taiwan and China. Despite warnings from the Regulatory Agencies regarding the safety of products containing AA, many AAN cases remain frequently described worldwide. This chapter aims at giving a global picture of AAN through the descriptions of clinical cases and animal models, which were developed to better understand the mode of action of AA when inducing acute/chronic kidney diseases. Major advances in the translational research on biomarkers of AAN are reviewed, with an intended emphasis on the “omics” assessment of this nephrotoxicity.

Keywords: aristolochic acids, nephropathy, biomarkers, metabonomics, animal models

1. Introduction

The kidney plays a major role in the body homeostasis by regulating volume and composition of water fluids and by removing from blood waste products such as metabolites, drugs and xenobiotics. Due to these functions, the kidney is highly susceptible to toxic insults. During lifetime, the body is continuously exposed to numerous potentially toxic agents such as drugs

[1], chemicals and natural nephrotoxins [2]. Among these, herbal remedies and traditional phytotherapies constitute a major challenge. Indeed, traditional herbal remedies are considered harmless by the general population because they are from natural origin. Moreover, most patients using these natural products fail to inform their physicians of their use [2]. The following story demonstrates that the use of natural products, as all drugs, should be submitted to rigorous pharmacological and toxicological studies to determine their efficacy/safety. Some natural products have displayed therapeutic effects [3], whereas some others have been found to be highly toxic for the human body [4]. Among them, the toxicity of aristolochic acids (AA) has been extensively studied during the last decades. The term aristolochic acid nephropathy (AAN) includes 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 (Balkan endemic nephropathy, see below) [5]. AA are compounds found in plants from the genus *Aristolochia*, belonging to the plant family Aristolochiaceae. In addition to its nephrotoxic effects, AA exposure has also been frequently associated with the development of urothelial malignancies [6] and was classified as a human carcinogen class I by the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) in 2002 [7]. Since the identification of AAN in the early 1990s in Belgium [4], increasing cases of AA intoxications have been reported all over the world [8]. AAN incidence is particularly high in Asian countries because traditional medicines are very popular and the complexity of the pharmacopeia represents a high risk of AA intoxication due to some confusion between close species. In the Balkan areas, the chronic exposure to AA has been considered as the causative agent responsible for the so-called Balkan endemic nephropathy (BEN) that occurs following ingestion of food prepared with flour derived from contaminated grains [5, 9–14].

Despite warnings from the Food and Drug Administration (FDA), the European Medicines Agency (EMA) and IARC regarding the safety of products containing AA, AAN cases remain frequently described worldwide [5, 15, 16]. Moreover, given the fact that the nephrotoxic effect of AA is irreversible and that chronic kidney failure as well as carcinogenic effects may develop very slowly after the initial exposure, AAN and associated cancers are likely to become a major public health issue in the next few years [15, 16, 17].

This chapter aims at giving a global picture of AAN with an intended emphasis on the “omics” assessment of this nephrotoxicity, especially on the metabonomic investigation of urine samples. Indeed, it could represent a strategic tool allowing to identify early biomarkers following AA intoxication thereby providing an early detection of the toxicity and a rapid therapeutic intervention [18].

2. Clinical cases

Originally, AAN was reported in Belgium with more than 100 individuals having ingested slimming pills containing powdered root extracts of a Chinese herb, *Aristolochia fangchi* [4, 19]. A total of 75 patients have been treated in our Nephrology Department at Erasme Hospital. Among them, 50 out of 57 (F/M ratio 56/1) received a kidney transplant because of end-stage

renal disease (ESRD); 21 presented with urothelial carcinoma of the upper tract (invasive in two cases) or the bladder (three cystectomies required), leading to five deaths. Four additional kidney recipients developed cancer of the digestive tract, one developed a brain lymphoma and 8 lethal cardiovascular or infectious complications. Among the seven patients still followed in 2018 for chronic kidney disease (CKD), a left nephroureterectomy had to be performed for pelvic carcinoma. Only one case of metastatic urothelial carcinoma was diagnosed without concomitant CKD [6]. The causal link with the intake of pills containing AA was demonstrated by the detection of DNA adducts specific to AA metabolites in renal tissue samples (see Section 3).

Although, initially, the Belgian cohort only included over 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), 8,000,000 people in Taiwan and more than 100,000,000 in mainland China [20, 21]. In Asia, *Aristolochia* species is considered as an integral part of the herbology used in traditional Chinese medicine, Japanese Kampo and Ayurvedic medicine [8]. It is found within the same therapeutic family such as the *Akebia*, *Asarum*, *Cocculus* and *Stephania* plants. Referred by common names such as *Mu Tong*, *Mokutsu* and *Fang Ji*, they are used in a multitude of herbal mixtures for therapeutic use [22]. In the initial cohorts concerning iatrogenic nephropathy due to AA, most of the patients were described, exhibiting a rapid and progressive evolution toward CKD or ESRD [16]. A large series of patients described in China fixed the median rate of the degradation in glomerular filtration rate at ~3.5 ml/min/year [20]. The progression rate in environmental nephropathy due to AA is much slower, with the end-stage of renal failure occurring only after an evolution of 15–20 years [23].

3. Correlation between AA-DNA adducts, Aristolochic acid nephropathy and upper urinary tract carcinoma

Under normal physiological conditions, AA is bioactivated by the reduction of nitro compounds into “aristolactams,” which tend to form covalent bonds with purine bases of DNA. The DNA adducts specific to aristolactams remain part of the body’s cell structure for several years after the patients’ initial exposure to AA. Consequently, their discovery in renal or cancerous tissues constitutes a biomarker, which may be related to a previous exposure to AA, which possibly occurred much earlier [24].

As mentioned earlier, 40–45% of end-stage AAN Belgian patients currently treated by dialysis or renal transplantation displayed multifocal high-grade transitional cell carcinomas, mainly in the upper urinary tract [6, 25]. As mentioned earlier, the detection of DNA adducts specific to AA metabolites (aristolactams) in nephroureterectomy pieces is still possible more than 10 years after exposure to AA [26]. The carcinogenic effect of AA can be explained by the fact that the DNA adducts formed in combination with aristolactams lead to a mutation of A: T → T: A in the tumor suppressor gene TP 53 [27]. This mutation has frequently been detected in the urothelial tumors of cases described in Taiwan and in the Balkans, whereas this mutation rarely occurs in tumors which are not related to the exposure to AA [28, 29]. This mutation is therefore considered as a complementary signature mutation for AA intoxication.

Besides the high prevalence of upper tract urothelial carcinoma, it must be underlined that cancers of the bladder appeared in female Belgian patients who had undergone transplants more than 15 years after exposure to AA had been stopped [30]. Frequent iatrogenic exposure to AA in Taiwan explains why this region has the world's highest level of urothelial cancers [31].

4. Experimental models of AA intoxication

In the 1980s, Mengs et al. had already pointed out AA toxicity and carcinogenicity in several experimental studies [32–34]. They developed diverse animal models using rats and mice of both sexes intoxicated with different doses of AA, during variable exposure times and using different routes of administration. They observed that oral or intravenous administration of high doses of AA was followed by death from acute renal failure within the next 15 days. The lethal dose 50 (LD_{50}) ranged from 56 to 203 mg/kg orally and 38 to 83 mg/kg intravenously, depending on species and gender [32]. Mengs reported that the kidneys were the most affected organs after intoxication confirming that they constitute AA main target [32].

Following the identification of AA as the agent responsible for AAN and BEN in 1993, various animal models were developed to study underlying mechanisms of AA nephrotoxicity. To do so, Cosyns et al. established a model of female New Zealand White rabbits injected intraperitoneally (i.p.) with 0.1 mg/kg of a mixture of AA (AAI: 44%; AAI: 56%), 5 days a week during 17–21 months [35]. The same authors reported the development of renal fibrosis and urothelial tumors in rabbits as it occurred in patients, therefore supporting the causal role of AA in the pathology.

Our group also developed different models of AAN. In 2002, Debelle et al. showed that daily subcutaneous injection of 10 mg/kg of AA mixture (AAI: 40%; AAI: 60%) in salt-depleted rats induced interstitial fibrosis and renal failure within 35 days [36]. In 2005, Lebeau et al. analyzed the time course of structural and functional impairments of the proximal tubule in a rat model of AAN using the same protocol [37]. A biphasic evolution of renal dysfunction and morphological alterations was described. First, a phase of acute kidney injury (AKI) was characterized with structural abnormalities occurring within the first 3 days as attested by necrosis of proximal tubular epithelial cells (PTEC). Dysfunction of proximal tubule was also highlighted by an increase of biochemical parameters such as tubular proteinuria, N-acetyl- β -D-glucosaminidase (NAG), α -glutathione S-transferase (α -GST), leucine aminopeptidase (LAP) and neutral endopeptidase (NEP) enzymuria. At day 35 of the protocol, a second phase was identified, mainly associated to morphological alterations along with the presence of severe tubular atrophy in an interstitial fibrotic environment. Moreover, tubular proteinuria, NAG, α -GST, LAP and NEP enzymuria were further enhanced at this timepoint characterizing the later phase of chronic kidney injury.

Regarding mice models, it has been shown that mice from different strains displayed variable susceptibility to AA treatment. In this regard, Sato et al. published a study in which they compared three strains of inbred mice, BALB/c, C3H/He and C57BL/6 that received 2.5 mg/kg of AA (AAI: 55%; AAI: 45%) or AA sodium salt daily by i.p. injection or oral administration,

5 days a week for 2 weeks [38]. Characteristic tubulointerstitial lesions as well as parameters of renal dysfunction were observed in both strains intoxicated with AA. However, the susceptibility to AA differed between the three strains tested. The authors postulated that these differences might reflect diversity in AA metabolism and/or in the mechanisms of detoxification. Indeed, genetic polymorphisms have been identified as a major factor affecting the expression level and/or activity of enzymes involved in AA metabolism. The variations observed between the different strains could therefore be linked to such polymorphisms.

More recently, we also confirmed the biphasic evolution in a mouse model of AAN (**Figure 1**). C57Bl/6 J male mice were daily i.p. injected with a solution of AAI (3.5 mg/kg) for 4 days and then sacrificed at 5, 10 or 20 days after the first day of injection [15, 39]. The acute phase was identified at day 5 with necrosis of PTEC while at day 20, cystic tubules and tubulointerstitial fibrosis was observed characterizing the features of a chronic phase. This transition from AKI to CKD has an important significance clinically. Indeed, it has been shown that patients surviving an episode of AKI present a significant risk of progression to CKD [40–42]. However, the mechanisms by which AKI might initiate the onset of CKD have not been fully described. Therefore, a better understanding of the pathological mechanisms of AAN along with the identification of early biomarkers could lead to therapeutic strategies to treat AKI or impede progression to CKD. Along these lines, animal models of AAN could be considered as a useful tool providing important insight on the underlying mechanisms of AKI-to-CKD transition. In this regard, we recently demonstrated that renal nitric oxide (NO) bioavailability was significantly reduced during the AKI-to-CKD transition in our AAN model while L-Arginine supplementation appeared beneficial [39]. Indeed, L-Arg treatment restored renal NO bioavailability and reduced the severity of tubular necrosis, inflammation and fibrosis

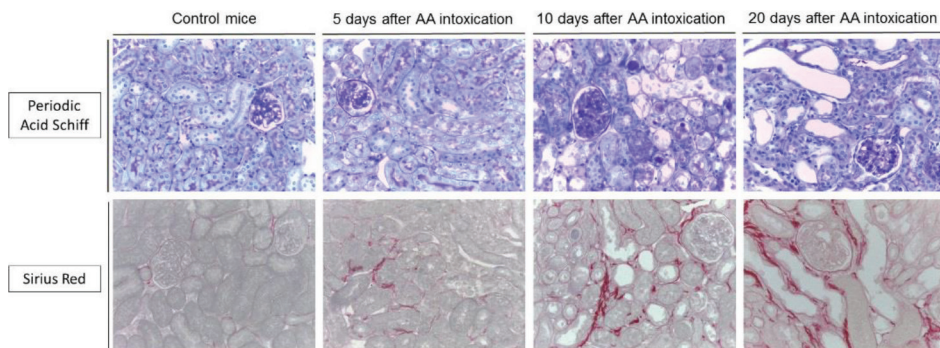


Figure 1. Renal histology of AA-induced tissue injury. A–D. Representative photomicrographs (×400 magnification) illustrating renal tissue injury with periodic acid-Schiff staining in Ctl (A), 5 days after AA intoxication (B), 10 days after AA intoxication (C) and 20 days after AA intoxication (D). Zones of necrotic proximal tubular epithelial cells are observed in AA intoxication at day 5, 10 and 20 after AA (B–D). Moreover, cystic tubules and proteinuria (*) are visible in mice treated with AA at day 10 and 20. E–H. representative photomicrographs (×400 magnification) illustrating collagen deposit with Sirius red staining in Ctl (E), 5 days after AA intoxication (F), 10 days after AA intoxication (G) and 20 days after AA intoxication (H). Deposition of collagen I and III are observed in the interstitium of renal parenchyma in AA-treated mice at day 5 (D). Collagen deposition are even enhanced from day 10 to day 20 (G–H). Abbreviations: G, glomerulus; NT, necrotic tubule; CT, cystic tubules and PT, proximal tubule.

AA-intoxicated mice. We concluded that reduced NO bioavailability contributes to the pathological processes in AAN but also that L-Arg could represent a potential therapeutic tool to decrease the severity of acute-to-chronic transition.

5. Conventional biomarkers

In most AAN cases, renal failure was discovered by routine blood testing [43]. Typically, moderate hypertension developed with increased serum creatinine and severe anemia [8, 44]. Proteinuria from tubular origin was confirmed with elevated urinary excretion of five low molecular weight proteins (β 2-microglobulin, cystatin C, Clara cell protein, retinal-binding protein and α 1-microglobulin) [45]. Levels of urinary neutral endopeptidase (NEP), a 94 kDa ectoenzyme of the proximal tubule brush border, constitute a reliable indicator of the severity of renal disease. Indeed, it has been shown to reflect the proportion of brush borders remaining intact at the apical side of proximal tubules. Urinary NEP was significantly decreased in patient with moderate renal failure and almost undetectable in those with ESRD, indicating the loss of the proximal tubule integrity [46]. A hallmark of the Belgian cohort of AAN cases is the rapid progression to an ESRD in nearly 70% of intoxicated patients [44]. The combination of rapidly progressive interstitial renal fibrosis with urothelial malignancy strongly suggests the diagnostic of AAN. Since 2015, a consensus regarding the definition of diagnostic criteria has been established [16]. AAN will be diagnosed in any individual who suffers from renal failure in combination with at least two of the following three criteria: (1) a renal histology highlighting interstitial fibrosis distributed along a corticomedullary gradient; (2) a history of herbal products consumption with the demonstration of the presence of AA and (3) the identification of AA-DNA adducts or the specific A:T $\rightarrow$ T:A transversion in the tumor suppressor gene TP 53 in a kidney tissue sample or in a urothelial tumor.

Until now, no serum or urinary biomarker has been identified for clinical utility in the diagnosis of AAN or BEN [16]. Although mechanistically informative, the conventional markers described in the previous paragraph display a lack of sensitivity. When abnormal levels of those markers are noticed, irreversible damages were already set up. In this regard, the 'omics' approach could constitute a strategic tool to identify early markers which could allow an early therapeutic intervention [18].

6. Nephrotoxicity assessment by the 'omics' approach

The biomarkers conventionally used in toxicology are particularly useful to detect or to follow the evolution of a very specific effect usually identified in advance. On the other hand, significant variations in the levels of such biomarkers are often too late since they only appear once severe or worse irreversible damages are already developed. Moreover, they are not appropriate to identify unknown or unexpected effects or to appreciate responses that result from several effects occurring simultaneously in various cell types, organs or systems.

Toxicogenomics is a more recent discipline that broadly studies changes in genes (genomics), RNA (transcriptomics), proteins (proteomics) and metabolites (metabol(n)omics) in a whole living organism exposed to a xenobiotic substance causing subsequent adverse health effects [47]. The main goal in toxicogenomics is to obtain a global and integrated view of the molecular and cellular mechanisms underlying a toxic response. It is crucial to distinguish changes in basal expression, for example due to stress, from both adaptive and toxic responses. In addition, according to the principle of molecular homology, it is likely that substances sharing similar chemical structures will display close mode of toxic action by altering the same set of genes, proteins and metabolites. Therefore, reference databases can be built that contain characteristic expression profiles of reference toxicants [48]. The comparison of the expression profile induced by a tested molecule with the database allows a fine classification. From the properties of the identified class, the toxic behavior of the test substance can be predicted. Finally, thanks to the development of 'high-throughput' analytical techniques, the expression of thousands of genes at the level of RNA or proteins can be simultaneously measured in an organ/tissue subjected to the effect of a toxic substance. In parallel, changes in the metabolic composition of biofluids and biopsies can be assessed over time. These global measurements facilitate not only the observation of the effects of a molecule on the targeted tissues (on-target), but also of the harmful effects on non-targeted tissues (off-target) [49].

In a recent review, conventional and toxicogenomics methods were compared to evaluate their potential to predict nephrotoxicity, genotoxicity and teratogenicity of traditional remedies [50]. Toxicogenomics methods present the main advantage to allow a quick and simultaneous acquisition of multiple markers which can be next identified and related to cellular processes targeted by the tested compound. Focusing on the nephrotoxicity assessment, these new approaches could identify a set of proteins and metabolites which might be promising biomarkers indicative of very early cellular changes. Compared to conventional markers such as plasma creatinine and BUN, the "omics" indicators are more sensitive and more specific. On the protein side, Clusterin, Cystatin-C, and N-Gal/lipocalin-2, among others, are now considered as excellent candidates to detect acute or chronic tubulotoxicity [51, 52] to such an extent that they have been accepted by regulatory agencies (FDA and EMA) for the detection of acute kidney injury in preclinical risk assessment. On the metabolite side, since the COMET (Consortium for Metabonomic Toxicology) project that evaluated the benefit of metabonomics in the prediction of potential drug adverse effect [48], numerous nonclinical and clinical studies have reported the usefulness of urine metabolic markers to predict acute and chronic renal injuries. In rats, variations in plasma 3-methylhistidine (3-MH) and 3-indoxyl sulfate (3-IS) are now recognized as early predictors of a decline in glomerular filtration due to acute renal failure, while the combination of simultaneous changes in urine glucose, methylamines, hippurate and certain amino acids is associated with proximal tubule damages [53].

Of course, those new approaches in systems biology are far from being validated. Nevertheless, in association with *in vivo* models, they are already useful to mechanistically assess the nephrotoxicity of xenobiotics. Most importantly, many recent studies highlight their unique potential for translational research from benchside to bedside, especially for the diagnosis and follow up of AKI [54].

Both genomic and transcriptomic approaches have already been applied to investigate the cellular mechanisms of AA toxicity and tentatively reveal fingerprints of AA exposure. Human exome sequencing of urothelial carcinoma of the upper urinary tract from individuals chronically exposed to AA revealed characteristic and unusual somatic mutations predominantly located on the non-transcribed strand [55]. This AA-related mutational fingerprint was essentially found in oncogenes and tumor suppressor genes. Using a molecular epidemiologic approach based on genome sequencing, the genome-wide mutational signature for AA was also detected in liver and kidney cancers in targeted Taiwanese and Chinese populations [56, 57]. Exposure to AA in various animal species and in humans is detected by the presence of aristolactam-DNA adducts, with excellent results obtained on renal specimens by ultra-performance liquid chromatography-electrospray ionization/multistage mass spectrometry (UPLC-ESI/MSn) [58]. Such DNA adducts represent the direct evidence of tissue exposure to AA, and according to these authors, they can serve as endpoints for cross-species extrapolation of toxicity data and human risk assessment.

Proteomic analyses of urinary, plasma and renal tissue resulted in differential expression of several cytoskeletal, developmental and inflammatory kidney proteins, in both AA-exposed and control mice [59]. Using fluorogenic derivatization followed by high-performance liquid chromatography analysis and liquid chromatography tandem mass spectrometry with a MASCOT database searching system, two altered proteins, thrombospondin type 1 and a G protein-coupled receptor, were identified as discriminating mice chronically exposed to AA [60]. A proteomic signature of AA-exposure was also identified in rat kidney: upregulated proteins included ornithine aminotransferase, sorbitol dehydrogenase, actin, aspartoacylase, 3-hydroxyisobutyrate dehydrogenase and peroxiredoxin-1, while ATP synthase subunit β , glutamate dehydrogenase 1, regucalcin, glutamate-cysteine ligase regulatory subunit, dihydropteridine reductase, hydroxyacyl-coenzyme A dehydrogenase, voltage-dependent anion-selective channel protein 1, prohibitin, and adenylate kinase isoenzyme 4 were all downregulated [61]. Interestingly, some of those proteins presented obvious biological and medical significance.

From the metabol(n)omics side, several studies were conducted either in experimental animal models or in patients. For example, a GC-MS-based metabolomic study was performed to analyze urinary metabolites in AA-exposed rats over time [62]. Among all metabolic alterations, eight metabolites were selected as potential metabolic biomarkers including methylsuccinic acid, nicotinamide, 3-hydroxyphenylacetic acid, citric acid, creatinine, uric acid, glycolic acid and gluconic acid. Four of them were considered as “early indicator” (methylsuccinic acid, citric acid, creatinine and 3-hydroxyphenylacetic acid), while the others were defined as “late metabolic biomarker.” According to the authors, the metabolomics markers were more sensitive than conventional biomarkers of renal injury. Using a UPLC/QTOF-HDMS analysis, a bench of lipids molecules including cholic and chenodeoxycholic acid were identified as excellent biomarkers to evaluate the progression from early to advanced AAN. Also, indoxyl sulfate, uric acid and creatinine were considered as good markers in severe cases of AAN. Interestingly, they were also reversible under treatments both in AAN rats and in CKD patients [63]. Using a ^1H -NMR-based approach, we compared the urine metabonomic profiles of rats exposed to either AAI, AAI, or the mixture. Metabolic alterations indicating a proximal tubule injury were observed in all treated groups. These dose-dependent effects already noticed at early stages were morphologically confirmed at later

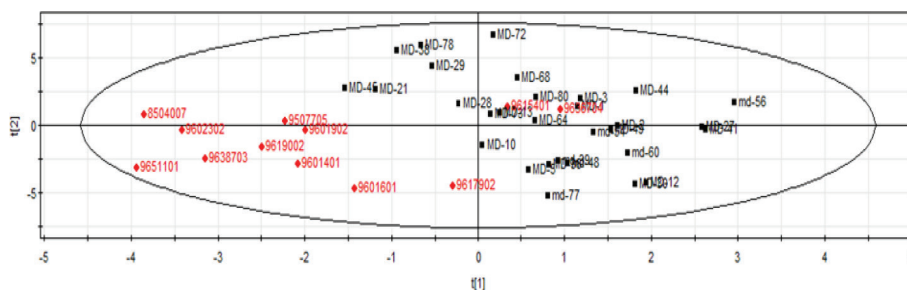


Figure 2. Scores plot comparing the ^1H NMR spectra of urine samples from diseased Balkan women in red with the urine samples collected from healthy Belgian women, in black. PLS-DA model.

time points. Renal damages were more pronounced with the mixture or AAI alone than AAI alone [18].

¹H NMR-based metabolomics was also applied to urine samples collected from BEN-diagnosed people treated by hemodialysis, in Romania and Bulgaria [64]. While samples from healthy volunteers from both countries coincided in the PCA scores plot, Bulgarian and Romanian BEN-suffering patients not only deviated from controls but also from each other. This study also suggested that metabonomic assessment could be predictive of impending morbidity before conventional criteria can diagnose BEN. Similar metabonomic approach was also applied to surplus of urine samples collected from Belgian women (acutely and mistakenly exposed to AA through diet pills) and Croatian patients (chronically exposed to AA through contaminated food) [65] (**Figure 2**). Interestingly, a clear discrimination of the Belgian patients from healthy volunteers was shown, and urine samples collected from individuals living in Croatian endemic regions as compared to Croatian inhabitants from non-endemic villages tended to separate from each other. Finally, both Belgian and Croatian patients displayed close urine metabolic profiles, suggesting a common etiology of both diseases.

In conclusion, the methods in toxicogenomics have already brought water to the mill of researches on nephropathies induced by AA. Thus, aristolactam-DNA adducts specific to the AA exposure were revealed by genomic methods. For their part, proteomic and metabolomic approaches have identified very early and specific biomarkers of tubular renal damage associated with exposure to AA. These same markers have also opened new mechanistic tracks on the toxic modes of action of AA at the molecular and cellular levels.

7. Conclusions

"AAN" is a global designation for any toxic interstitial nephropathy consecutive to the exposure to aristolochic acids. Although the development of accurate experimental models and the validation of robust biomarkers these last decades have allowed a better mechanistic understanding of the mode of action of those natural substances, we are still facing many challenges. In particular, the need for early and more predictive indicators of AAN in both animal models and in clinics is urgent. Also, unveiling the role of altered renal hemodynamics in the pathogenesis as well as the molecular and cellular events leading from an acute to a chronic

kidney disease in patients is essential. To this respect, the omics technologies have already brought new pieces to the puzzle. Specifically, metabonomic applied to urine samples collected from AA-exposed animals or AAN patients stands out as a very promising approach.

More philosophically, the history of AAN reminds us that natural products are not without risk. The easy and uncontrolled access to such substances, for instance from suspicious sources on the Web, increases the risk of renewing this sad experience. A review of the most recent literature in the field of traditional medicines and remedies reinforces our belief that they should be subject to the same safety assessments as drugs, and that omic methods should be included at different steps of the assessment process.

Conflict of interest

Authors declare no conflict of interest.

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