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Endemic Nephropathy: the Case for Chronic Poisoning by Aristolochia

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Aim To explore the hypothesis that chronic dietary poisoning by aristolochic acid could account for the unique geographical distribution, specific pattern of tubulointerstitial fibrosis, occurrence of chronic renal insufficiency, and an increased risk of developing upper urothelial cancer, all of which are associated with endemic nephropathy.

Methods This case-controlled epidemiologic study consisted of three groups of subjects residing in an endemic region of Croatia: (a) patients meeting WHO criteria for endemic nephropathy (n = 28), (b) individuals who have been treated for renal insufficiency secondary to other forms of renal disease (n = 30), and (c) apparently healthy residents of the endemic village (n = 30). A detailed questionnaire, designed to collect information on demographics, exposure to potentially toxic substances, diet, agricultural practices, and other factors potentially impacting endemic nephropathy was administered to the three study groups. The seeds of *Aristolochia clematitis*, obtained from plants growing in the endemic region, were extracted with ethanol and analyzed by high-performance liquid chromatography for their aristolochic acid content.

Results The majority of subjects, including 90% of endemic nephropathy patients, recall that the plant *Aristolochia clematitis* (birthwort, *vučja stopa* in Croatian) was frequently found in local meadows and wheat fields between 20 and 30 years ago. At that time, endemic nephropathy patients encountered *Aristolochia clematitis* significantly more frequently than controls ($P = 0.035$). Since then, all three study groups reported a significant increase in the use of herbicides ($P < 0.001$) and reduction in the prevalence of *Aristolochia clematitis* ($P < 0.001$). Chemical analysis established that the seeds of *A. clematitis* contain 0.65% aristolochic acid. It is likely that the harvesting process used by local farmers permitted the seeds of *A. clematitis* to mingle with the wheat grain.

Conclusion Flour used to bake bread, a dietary staple in the endemic region of Croatia, is derived from wheat grain which, in the past, is likely to have been contaminated with seeds of *A. clematitis* during harvesting. This observation supports the hypothesis that aristolochic acid, a major constituent of the seeds, plays a central role in the development of endemic nephropathy.

Endemic nephropathy was recognized in the late 1950s as a chronic, ultimately fatal disease affecting residents of rural villages situated near tributaries of the Danube river (1-4). It has been suggested, on the basis of church records and anecdotal observations, that endemic nephropathy actually appeared in endemic regions decades earlier (5-9). Remarkably, half a century later, the striking geographical distribution of endemic nephropathy remains unchanged and

100,000 population are estimated to be at risk (10).

Extensive studies conducted in Croatia, Bulgaria, Bosnia, Romania, and Serbia (3,11-14) revealed the unique epidemiologic features of endemic nephropathy: 1) focal occurrence within certain villages and completely unaffected villages located in close proximity; 2) familial but not inherited pattern, often affecting several members of the same household; 3) occurrence in adults, primarily aged 45-60 but not in children younger than 15; 4) restriction to rural farming populations in the endemic areas; and 5) strong association with upper urinary tract tumors occurring in populations in the same geographical areas. Taken together, this epidemiologic evidence strongly suggested that individuals affected by endemic nephropathy had been exposed to a nephrotoxic and carcinogenic substance(s) present in the local environment.

Scientific conferences on the factors possibly involved in the etiology of endemic nephropathy were held in 1965 by the World Health Organization (15) and in 1967 by the Ciba Foundation (5). Since then, the role of environmental toxins, including mycotoxins, heavy metals, genetic factors, viruses, and trace element deficiencies, has been systematically explored (16-21). Much research over the past decade has focused on ochratoxin A as a causative factor of endemic nephropathy (reviewed in 22-24). Cognizant of the nephrotoxic potential of *Aristolochia* (25), Kazantizis (5) speculated that the flour used for baking bread might be contaminated with constituents derived from this plant, a hypothesis that we consider here in more detail.

In 1969, M. Ivić formally proposed that seeds of *Aristolochia clematitis* (Fig. 1), birthwort or *vučja stopa* in Croatian, which is found in meadows and cultivated fields throughout the Balkan region, contained a toxic constituent that might be the etiologic agent of endemic nephropathy (26). In support to his hypothesis, Ivić cited reports that horses consuming hay containing *vučja stopa* developed signs of renal failure including proteinuria (27,28). Histopathologic examinations of tissues of these animals showed marked renal tubular damage and chronic interstitial nephritis with few signs of inflammation or glomerular damage.

Figure 1. Drawing of *Aristolochia clematitis*, illustrating the stages by which plant matures. The fruit, a capsule containing seeds, is shown at the lower right. With the permission of K. Stüber. Kurt Stüber's Online Library (*www.biolib.de*).

Ivić also observed that, during harvesting, seeds from *A. clematitis* were sometimes co-mingled with wheat grain and that the resulting contamination of grain was generally ignored when flour was prepared (26). He performed a series of toxicologic studies on rabbits, which demonstrated the nephrotoxic potential of flour prepared from *A. clematitis* seeds and revealed similarities between the pathologic changes he observed in rabbit kidneys and those reported for endemic nephropathy. He also showed that, in rats, injections of aqueous extract of *A. clematitis* seeds produced sarcomas at the site of injection (29). On the basis of these investigations, Ivić concluded that *A. clematitis* contained a carcinogen, a fact that was subsequently confirmed (reviewed in 30), and noted that both the distribution and nephrotoxic properties of this plant resembled those postulated for the etiologic factor responsible for endemic nephropathy (26). Ivić's well-documented

hypothesis has been frequently cited in published reviews (10,12,16); surprisingly, it has not previously been incorporated explicitly into epidemiologic investigations of the disease.

We were prompted to explore the role of aristolochic acid in endemic nephropathy for several reasons. First, aristolochic acid is an established human carcinogen (30,31) and patients with endemic nephropathy are at significantly increased risk of developing urothelial carcinoma (32-37). Second, chronic renal failure was observed in Chinese patients treated with herbal medicines containing *A. manchuriensis* (38) and in a group of healthy Belgian women who accidentally ingested *A. fangchi* as part of a slimming regimen (reviewed in 39). Both herbs, as well as other *Aristolochia sp.*, are widely used in traditional Chinese medicine (38,40). Third, the clinical and histopathologic findings in the cluster of Belgian cases (41, reviewed in 42), bear a striking resemblance to those reported in patients with endemic nephropathy (43, 44). Finally, we believe that the numerous etiologic hypotheses put forward over the past 40 years (1-12,16-21) have failed so far to provide significant clues to the pathogenesis of endemic nephropathy. For these reasons, we determined that Ivić's promising hypothesis should, at long last, be explored.

As the initial step in evaluating the role of aristolochic acid in the pathogenesis of endemic nephropathy, we designed a questionnaire to assess (a) dietary exposure to aristolochic acid among residents of the endemic region of Croatia, including patients with endemic nephropathy; (b) changes in residents' life styles and the microenvironment over the past 30 years; and (c) agricultural practices that influenced the growth of *A. clematitis* in cultivated fields. We also determined the aristolochic acid content of *A. clematitis* seeds collected from plants growing near wheat fields in the endemic village of Kaniža.

Participants and Methods

Study Subjects

Three groups of adults (total, n = 88) living in the endemic region of Croatia (north of the Sava river and west of Slavonski Brod in the region of Zapadna brodska Posavina) participated in the study. Fifty-eight persons were undergoing hemodialysis in the General Hospital in Slavonski Brod at end-stage renal disease. Of the 58 patients on hemodialysis, 28 met the World Health Organization (WHO) criteria (15,45,46) for endemic nephropathy. The remaining 30 patients ("non-endemic controls") were being treated for renal insufficiency secondary to other forms of chronic renal disease, including diabetes mellitus, chronic pyelonephritis, hypertension, and polycystic kidney disease. The third group of 30 subjects ("endemic controls") comprised apparently healthy residents of the endemic village of Kaniža (Table 1).

Questionnaire

A detailed questionnaire was developed to solicit information on demographics, medical history, exposure to potentially toxic substances, diet, agricultural practices, tobacco, alcohol consumption, and other factors potentially having impact on endemic nephropathy. As the lifestyle of many residents of endemic villages has changed in recent years, we also collected information on ag-

Table 1. Summary of selected demographic information among the three study groups

Patient characteristic	Total (n=88)	Endemic nephropathy (n=28)	Endemic healthy control (n=30)	Non-endemic control (n=30)	*P*†
Age (years±SD)*	56.4±16.6	64.3±5.3	50.0±22.5	55.2±14.7	0.006
Years lived at the address (±SD)	42.8±20.9	42.4±21.9	42.1±22.5	43.8±18.9	0.95
Education (years±SD)	8.0±4.0	8.0±3.8	7.0±4.0	9.1± 3.9	0.13
Gender (No., %):					
female	39 (44.3)	14 (50.0)	16 (53.3)	9 (30.0)	
male	49 (55.7)	14 (50.0)	14 (46.7)	21 (70.0)	0.15
Nationality (No., %):					
Croatian	79 (89.8)	27 (96.4)	25 (83.3)	27 (90.0)	0.19
other	9 (10.2)	1 (3.6)	5 (16.6)	3 (10.0)	
Family member with nephritis (No., %):					
yes	27 (30.7)	22 (78.6)	4 (13.3)	1 (3.3)	<0.0001
no	61 (69.3)	6 (21.4)	26 (86.7)	29 (96.7)	
Smoking status (No., %):					
yes	16 (21.6)	4 (16.0)	8 (27.6)	4 (20.0)	0.58
no	58 (78.4)	21 (84.0)	21 (72.4)	16 (80.0)	

*SD – standard deviation.
†Analysis of variance and Duncan post-hoc test for the first three variables and McNemar test for all other variables.

ricultural practices and dietary habits recalled from 20-30 years ago. This questionnaire was administered by medical students (TH, AK, JK) in the period June-September 2004. The average interview time for each subject was 24 minutes.

Analysis of A. clematitis *Seeds*

The aristolochic acid (aristolochic acid I+aristolochic acid II) content of *A. clematitis* seeds was determined by high-performance liquid chromatography (HPLC) analysis following extraction with 70% EtOH, using a 1/10 ratio of plant material to solvent. Authentic standards were obtained by HPLC purification of a commercial mixture of isomers purchased from Fisher Scientific Co., U.S. Finely chopped seeds were mixed with a 70:30 mixture of ethanol and water and refluxed at 60°C for 2 h. The mixture was allowed to cool and the extract was removed by aspiration. The plant material was washed with 70% ethanol and the resulting supernatant combined with the initial extract. The volume was measured and an aliquot was filtered and subjected to HPLC analysis. Three additional extracts were performed on the residual plant material.

Filtered aliquots (50 μL) of the four extracts were analyzed using a Waters HPLC system (Milford, MA, USA) and a 996 Photodiode Array Detector. Data were analyzed using the Empower® software (Waters Corp.). Efficient separation of aristolochic acid I and aristolochic acid II from other components in the mixture was achieved using an X-Terra MS C_{18} column, 5 μ (250×4.6 mm; Waters Corp.) and a solvent gradient of 20% to 30% acetonitrile eluting over 30 minutes with 0.1 mol/L TEAA buffer pH 7.5. Retention times for aristolochic acid I and aristolochic acid II were 25.1 and 30.9 minutes, respectively.

Data Analysis

The questionnaire data were translated into English and entered into an Excel spreadsheet. Statistical software package SAS, version 8.2 (SAS; 1999. SAS Institute Inc., Cary, NC, USA), was used for statistical analysis. Values that were out of range or missing, and ambiguous answers, were checked against the original questionnaire and, in several instances, by re-interviewing the subject. For continuous variables, including age, education level, bread intake, and others, means, standard deviations, and ranges were calculated; for categorical variables, such as gender and herbicide use, frequencies and percentages were summarized. Parametric and nonparametric one-way analysis of variance and Duncan's post-hoc group comparisons were used to compare the three groups. For categorical variables, χ^2-tests and Fisher's exact tests were used to evaluate differences. For certain informational items, current practices were compared with those 20-30 years ago. We used matched-pairs analysis to evaluate changes in items, such as agricultural practices, among the three study groups in the earlier period as compared to the present. For continuous variables, McNemar's test for categorical variables (combining categories if needed) and paired t-tests for parametric and non-parametric analyses were used.

Results

Demographic information obtained from the study participants is summarized in Table 1. The endemic nephropathy-affected group was significantly older than the other two groups, ($P=0.006$). Most (89.8%) of the study subjects were Croatian, 44.3% were women, and 21.6% were smokers.

There was no significant difference among the three study groups with respect to ethnicity, proportion of women, smoking status, or education level. The average formal education for all subjects was 8.0 years. The average length of residence at the person's current address was 42.8±20.9 years. There were no differences in length of residence among the three study groups or between genders. Significantly more endemic nephropathy cases reported having family members diagnosed with "nephritis" (78.6%) compared with the two control groups (13.3% and 3.3%, for endemic village controls and nonendemic village controls, respectively; $P<0.0001$). Clustering of endemic nephropathy cases within households is well-documented (47).

Sources of Water

It has been postulated that drinking water is the route of exposure to the environmental agent responsible for endemic nephropathy (critically evaluated in 16). However, we detected no difference among the three groups with respect to the drinking-water source they currently use. A public water system was brought into the endemic regions in the 1970s. Today, 55% of study subjects

use this public water system and 12.5% use their own well water. In contrast, 20 to 30 years ago, only 6.2% of these persons used the public water system and 85.2% used their own wells ($P<0.001$). Both sources of water were carefully monitored by the regional Department for Public Health and showed no significant levels of toxic agents (48). Thus, we have no evidence to support a hypothesis implicating drinking water as a factor in the etiology of endemic nephropathy.

Prevalence of A. clematitis

Significant social and environmental changes have taken place in the endemic region of Croatia over the past 20-30 years. These include changes in agricultural processes (increased use of fertilizer, herbicides, and combines); lifestyle (residents now rarely bake their own bread and some no longer farm); and changes in the microenvironment (installation of pumps and drainage ditches near the wheat fields). Any or all of these factors could have affected the prevalence and/or abundance of *A. clematitis* in the endemic region. In Table 2, we compared the three study groups according to current information and, separately, with information recalled from 20-30 years ago. We then evaluated the three groups as a whole in terms of changes from 20-30 years ago.

When they were shown a photograph of *A. clematitis*, many subjects recalled clearly seeing this plant in their fields and meadows at one time. At present, only 9.7% of all subjects reported seeing the plant in meadows, which is significantly less than the 66.7% who reported seeing the plant 20 to 30 years ago. Specifically, 20 to 30 years ago, 78.2% of endemic nephropathy patients "always" or at least "sometimes" observed *A. clematitis* in their farm fields compared with 33.3% nonendemic controls and 38.0% endemic controls ($P=0.035$) (Fig. 2). The frequency of these observations has now decreased so that, today,

Table 2. Comparison among the three study groups of current information and information recalled from 20-30 years ago

	Endemic nephropathy		Endemic healthy control		Non-endemic control		Total		
Characteristic (No., %)	past	now	past	now	past	now	past	now	*P**
Water source:									
own well	23 (85.2)†	1 (3.6)	26 (92.9)†	2 (6.7)	20 (76.9)†	8 (26.7)	69 (85.2)†	11 (12.5)	<0.001
water system	0 (0.0)	14 (50.0)	0 (0.0)	22 (73.3)	5 (19.2)	13 (43.3)	5 (6.2)	49 (55.7)	
other	4 (14.8)	13 (46.4)	2 (7.1)	6 (20.0)	1 (3.9)	9 (30.0)	7 (8.6)	28 (31.8)	
A. clematitis in fields:									
always	5 (21.7)	1 (4.4)	4 (19.0)	1 (3.6)	4 (22.2)	0 (0.0)	13 (20.1)	2 (2.7)	<0.001
sometimes	13 (56.5)	0 (0.0)	4 (19.0)	2 (7.1)	2 (11.1)	1 (4.4)	19 (30.6)	3 (4.1)	
never	5 (21.7)	22 (95.6)	13 (62.0)	25 (89.3)	12 (66.6)	22 (95.6)	30 (48.4)	69 (93.2)	
A. clematitis in the meadow:									
yes	19 (90.5)†	2 (9.1)	9 (50.0)†	3 (15.0)	10 (55.6)†	1 (5.0)	38 (66.7)†	6 (9.7)	<0.001
no	2 (9.5)	20 (90.9)	9 (50.0)	17 (85.0)	8 (44.4)	19 (95.0)	19 (33.3)	56 (90.3)	
A. clematitis in harvested wheat:									
always	3 (13.6)§	0 (0.0)	3 (14.3)‡	0 (0.0)	3 (15.0)‡	0 (0.0)	9 (14.3)‡	0 (0.0)	0.32
sometimes	12 (54.6)	0 (0.0)	1 (4.8)	0 (0.0)	0 (0.0)	0 (0.0)	13 (20.6)	0 (0.0)	
never	7 (31.8)	21 (100.0)	17 (80.9)	28 (100.0)	17 (85.0)	23 (100.0)	41 (65.1)	72 (100.0)	
Flooding of fields:									
yes	10 (41.7)	3 (27.3)	11 (47.8)	6 (28.6)	11 (55.0)	6 (46.2)	32 (47.8)	15 (33.3)	0.14
no	14 (58.3)	8 (72.7)	12 (52.2)	15 (71.4)	9 (45.0)	7 (53.8)	35 (52.2)	30 (66.7)	
Herbicide/pesticide use:									
yes	8 (29.6)	11 (45.8)	3 (11.5)	19 (63.3)	8 (27.6)	12 (52.2)	19 (23.2)	42 (54.6)	<0.001
no	19 (70.4)	13 (54.2)	23 (88.5)	11 (36.7)	21 (72.4)	11 (47.8)	63 (76.8)	35 (45.4)	
Grow wheat every year:									
yes	22 (100.0)	2 (66.7)	14 (87.5)	13 (81.2)	12 (75.0)	4 (66.7)	48 (88.9)	19 (76.0)	0.084
no	0 (0.0)	1 (33.3)	2 (12.5)	3 (18.8)	4 (25.0)	2 (33.3)	6 (11.1)	6 (24.0)	
Making own flour:									
yes	21 (94.4)	5 (100.0)	18 (94.7)	13 (92.9)	15 (93.8)	6 (100.0)	54 (94.7)	24 (96.0)	
no	1 (4.6)	0 (0.0)	1 (5.3)	1 (7.1)	1 (6.2)	0 (0.0)	3 (5.3)	1 (4.0)	
Baking own bread:									
yes	25 (92.6)‡	5 (18.5)	23 (85.2)‡	12 (40.0)	17 (56.7)‡	3 (10.0)	65 (77.4)‡	20 (23.0)	<0.001
no	2 (7.4)	22 (81.5)	4 (14.8)	18 (60.0)	13 (43.3)	27 (90.0)	19 (22.6)	67 (77.0)	
Flour source:									
bought from miller	23 (88.5)‡	2 (25.0)	14 (63.6)‡	11 (73.3)	12 (63.2)‡	5 (55.6)	49 (73.1)‡	18 (56.3)	0.008
bought from store	2 (7.7)	5 (62.5)	0 (0.0)	3 (20.0)	5 (26.3)	4 (44.4)	7 (10.4)	12 (37.5)	
from own wheat	0 (0.0)	1 (12.5)	6 (27.3)	1 (6.67)	2 (10.5)	0 (0.0)	8 (11.9)	2 (6.2)	

*The three study groups were compared both for current information (''Now'') and for information recalled 20-30 years ago (''Past''). The McNemar test was used to compare information for all subjects between the current period and 20-30 years ago (*P*-values for those results are shown in the far right column).
†$P<0.05$ for differences between "past" and "now" data in each of the three subgroups.
‡$P<0.01$ for differences between "past" and "now" data in each of the three subgroups.

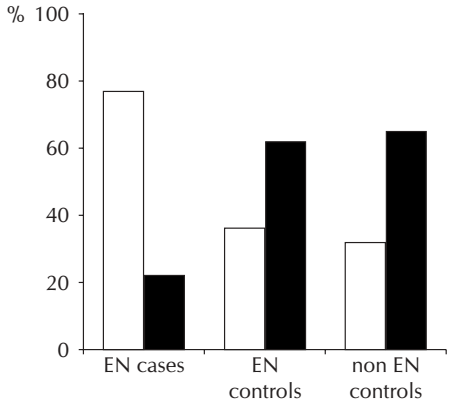

Figure 2. Frequency of sighting *A. clematitis* in fields 20-30 years ago. EN – endemic nephropathy. Open bars – always/sometimes, closed bars – never.

only 4.4%, 10.7 and 4.4%, respectively, reported seeing *A. clematitis* in their fields. This change was most dramatic for endemic nephropathy patients, 90.5% of whom reported seeing *A. clematitis* in meadows 20-30 years ago (Fig. 3) but only 9.1% reported this observation currently ($P=0.05$). Of endemic nephropathy patients, 68.2% stated that they "always" or "sometimes" observed *A. clematitis* plants among harvested wheat 20-30 years ago, compared with only 15.0% of nonendemic and 19.1% of endemic controls (Table 2) ($P<0.001$).

The diminished prevalence of *A. clematitis* could have resulted from changes in the microenvironment and/or the introduction of herbicides. It is known that *Aristolochia* grows abundantly in flooded fields (27,49). Installation of pumps and drainage ditches by the government between 1965 and 1990 reduced dramatically the flooding of local wheat fields, thereby decreasing the overall "wetness" of the habitat. Although the differences were not statistically significant, 33.3% of surveyed subjects reported less frequent flooding of their fields after pumps were installed to remove water, compared with 47.8% reporting flooding of their field 30 years ago. This reduction in flooding was most pronounced in reports from residents of endemic villages (41.7% to 27.3% for endemic nephropathy cases and 47.8% to 28.6% for endemic village controls). In addition, during the same time period, the use of herbicides and pesticides among all subjects more than doubled, from 23.2% 20-30 years ago to 54.6% today. This wider and more systematic use of herbicides may have additionally contributed to the reduced prevalence of *A. clematitis* in wheat fields in the endemic region.

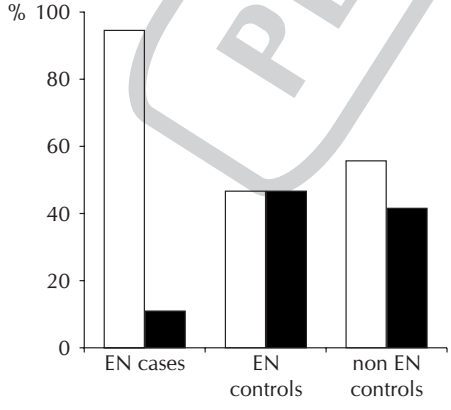

Figure 3. Frequency of sighting *A. clematitis* in meadows 20-30 years ago. EN – endemic nephropathy. Open bars – reported seeing, closed bars – reported not seeing.

Sources of Grain and Flour

In Croatia, both *A. clematitis* and wheat produce seeds at approximately same time of the year (Fig. 4). Thus, seeds from these plants (Fig. 5) are likely to be mingled in grain brought by farmers to the local mill (Fig. 6) and, as a result, constituents of *A. clematitis* are highly likely to contaminate the flour used to bake bread, a dietary staple of the region.

The source of grain and the preparation of flour differed significantly among the three study groups. For example, a significantly greater fraction of persons living in endemic villages, both endemic nephropathy cases and endemic nephropathy controls, grew their own wheat compared to those from nonendemic villages. This was also true 20-30 years ago, when more persons from endemic villages baked their own bread (85.2% for endemic controls and 92.6% for endemic nephropathy cases) compared with nonendemic controls (53.3%; $P=0.008$). Among the entire study sample, the percentage of subjects "baking own bread" decreased over 20-30 years from 77.4% to 23.0%. During the earlier time period, 73.1% of individuals purchased their flour from the local miller and 10.4% bought flour in stores. Today, 56.3% of all subjects obtained flour from the miller and 37.5% from stores.

Figure 4. *A. clematitis* growing in a wheat field near Kaniža (photographed in June 2004).

Figure 5. Left: seeds of *A. clematitis* weighing ~21 mg each, obtained from the Kaniža region and its fruit, transected to show organization of seeds. **Right:** wheat grains weighing ~49 mg each.

Aristolochic Acid Content of A. clematitis *Seeds*

A. clematitis seeds, obtained in June, 2004, from plants growing near wheat fields in Kaniža, were extracted with 70% ethanol and analyzed by HPLC as described in Methods. The amount of aristolochic acid I and aristolochic acid II in 70% ethanol extracts of these seeds was determined by quantitative HPLC. The two isomers were present at a ratio of ~10:1. We determined the total aristolochic acid content of 1 gram of *A. clematitis* seeds to be 6.5 mg, an amount significantly higher than that reported for ethanol extracts of the roots of *A. fangchi* and *A. manchurensis* (36-38) and confirmed by parallel analyses of these Chinese herbs.

Figure 6. Mill used by residents of the endemic village of Kaniža.

Discussion

The 40-year-long search for the causative agent(s) of endemic nephropathy includes investigations of genetic factors, environmental toxins, heavy metals, viruses, and dietary deficiencies (10-12, 14, 16-18). In this study, we explored a previously unexplored hypothesis, initially offered by Ivić (26), that flour used by residents of endemic villages for preparing home-baked bread is contaminated with aristolochic acid, a nephrotoxic and carcinogenic chemical. This toxin is known to cause both renal interstitial fibrosis and urothelial cancer (31).

Residents of endemic villages located west of Slavonski Brod traditionally farm wheat, providing grain that serves as a major dietary staple of this region. Twenty-thirty years ago, *A. clematitis* was commonly observed in meadows and local wheat fields and the harvesting practices employed at that time led to co-mingling of *A. clematitis* seeds with wheat grain. This contamination, recorded on an anecdotal basis by Ivić (26), was confirmed by farmers participating in our survey who reported little effort during or after harvesting to separate seeds derived from *A. clematitis* and other weeds from the wheat grain. As a result, flour used in baking bread by local residents potentially contained significant amounts of aristolochic acid, the principal chemical constituent of *A. clematitis*. Twenty to thirty years ago, a typical

household in endemic and nonendemic villages consumed at least four loaves of bread weekly, with each loaf weighing approximately 2 kg. Thus, residents of endemic villages may have unknowingly ingested small amounts of highly nephrotoxic aristolochic acid over a period of years.

The essential characteristics of endemic nephropathy – its geographical distribution, familial clustering of cases (47), and occurrence in adults only (1-4,20,21) – are fully consistent with our hypothesis. Household clustering of endemic nephropathy is well-known (47) and may reflect the fact that some families derived their grain from fields where *Aristolochia* grew less abundantly as a result of differences in soil nutrition and/or agricultural practices, especially the use of fertilizers. Importantly, *A. clematitis* grows abundantly in flooded areas, including wheat fields located near tributaries of the Danube river where endemic nephropathy is concentrated.

In addition, the pathologic changes associated with endemic nephropathy (43,44) are identical to those observed in aristolochic acid nephropathy (AAN), a syndrome associated with ingestion of *Aristolochia* species of Chinese herbs (41,42). Hallmarks of both diseases include renal tubulointerstitial fibrosis, a distinctive form of proximal tubule cell damage, and a strong association with upper urothelial carcinomas (33). Such nephrotoxic and carcinogenic effects of aristolochic acid have been reproduced in experimental animals (50,51). Following activation by cellular enzymes, aristolochic acid reacts with DNA to form covalent adducts. In humans, these adducts have been detected in renal tissues up to 89 months following the exposure (39). Renal damage caused by aristolochic acid appears to be cumulative and irreversible (38,39).

The women with end-stage renal disease in Belgium, a cluster which first called attention to AAN, ingested a maximum dose of 560 mg aristolochic acid over a 20-month period (39). In China, patients with chronic AAN ingested an average of 1 mg/day for 1-10 years (38). An equivalent exposure for residents in the endemic region of Croatia is represented by the daily ingestion, over 8-10 years, of one-half a loaf (0.5 kg) of bread prepared from flour contaminated by a single seed of *A. clematitis*.

An important question to consider in the context of our hypothesis is why endemic nephropathy is found only in a subset (2%-5%) of households within an endemic village. We attribute this mosaic distribution, in part, to the more abundant growth of *A. clematitis* in wheat fields with poor soil conditions (26,27,49). Also, polymorphisms among genes involved in cellular metabolism and/or cellular transport of aristolochic acid may have affected individual susceptibility to its toxic effects (52-53).

Changes in farming practices, microenvironment, and lifestyle over the past 25 years are expected to alter significantly the potential dietary exposure to aristolochic acid in endemic villages. For example, drainage ditches constructed between 1965 and 1990 may have contributed to the dramatic reduction in the prevalence of *A. clematitis,* as the plant prefers to grow in swampy soil. In addition, as recorded in our survey, herbicides toxic to this plant were introduced and widely employed over the last 20-30 years. A contributing factor in the 1990s was the war that devastated the economy of the eastern region of Croatia, reducing the profitability of growing wheat. As a result, fewer farm families depend on bread as a dietary staple or bake their own bread.

Decreased dietary exposure to aristolochic acid should translate into a decreased incidence and a delayed age of onset of endemic nephropathy and urothelial cancer. The chronic, low-dose nature of such poisoning, coupled with persistence of aristolactam-DNA adducts in human tissues, suggest that the incidence of endemic nephropathy and urothelial cancer will lessen gradually over a period of years. We predict that such a trend, reported in some but not all, endemic regions (14), will be detected in data gathered during the *perlustracija* scheduled to take place in 2005 in the endemic villages of Kaniža and Bebrina, and in the control village of Klakar.

This epidemiologic study, based on a case-control survey of endemic nephropathy patients and apparently healthy control subjects living in the endemic region of Croatia, suggests that dietary intake of aristolochic acid may be responsible for endemic nephropathy and for its associated urothelial cancer. Additional experimental evidence, including unequivocal identification of aristolactam-DNA adducts in tissues of patients with endemic nephropathy (54), will be required to conclusively establish a link between aristolochic acid nephropathy and this endemic disease. In

the meantime, public health authorities in countries plagued by endemic nephropathy should implement measures to effectively remove the potential source of contaminated grain.

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