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Endemic (Balkan) nephropathy (EN) is a chronic tubulointerstitial nephropathy frequently associated with upper urothelial cancer exclusively affecting farming villagers [1, 2, 3]. Based on our results, EN is considered to be an environmental form of aristolochic acid nephropathy (AAN) [1,2.3]. AAN was first reported in 1993 in Belgium and subsequently more AAN cases were reported worldwide as AA has been an integral part of traditional herbal medicines [4]. The extent of this problem was recently documented in Taiwan where precise data on prescriptions of herbal products containing AA is available [5]. *Aristolochia* spp. has been used for more than 2000 years in the practice of traditional medicine and European physicians were familiar with the use of this plant as well. After its intrinsic toxicity became known, importing *Aristolochia* herbs was banned in many countries, including Croatia. Nevertheless, products containing AA remain a part of traditional medicine and are sold in many countries that do not have strict control protocols. Recently we reported that AA DNA adducts were present in 95% of patients with EN who underwent surgery for upper urothelial cancers [2] and affirmed the idea that bread contaminated with AA might be the cause of EN [6,7]. However, the causative relationship between AA and EN again raised the question whether bread intake is the only route of ingestion or whether AA was ingested also in EN as a part of folkloric medicine. Gluhovschi et al. reported that although therapeutic remedies based on AA products are used in the EN affected area, no relationship between these remedies and the development of EN or of tumors was observed [8]. However, they used HPLC for detection of AA in plasma, which is less sensitive than the mass spectrometry we recently used [1,2]. In addition, when re-analyzing their data, it does appear that AA was used more frequently in the endemic area. In our opinion, this leaves the question whether herbal tea may play a role in EN still unanswered. In our preliminary study we failed to find any evidence in the group of 1041 Croatian farmers that herbal tea or traditional medicine use is related to EN [9]. The observed differences between Romania and Croatia might reflect cultural and historical differences in traditional medicine. Aiming to resolve this disagreement more conclusively we analyzed whether herbal teas, including those prepared from *Aristolochia clematitis*, were used more frequently in Croatian and Bosnian residents of an endemic area than in farmers from non-endemic villages. A total of 3168 adults from nine endemic and three non-endemic villages were enrolled (the participation rate was 76.73%). The epidemiological survey was designed to collect demographic, medical, and family

history information, as well as dietary and environmental exposures, with an emphasis on the exposure to AA through drinking teas prepared from *A. clematitis*. Farmers were asked questions: 1): Did you ever use any herbal teas when you were sick?; 2): Did you ever buy, prepare or drink tea made of *A. clematitis*?; 3): Did you personally pick up or choose the herbs?; 4): Did you ever buy herbs/herbal teas from an herbalist or in a store? Participants were shown photographs of several common weeds growing in the region, including *A. clematitis* and asked if they recalled seeing the particular weeds in meadows, gardens and wheat fields. Of the 3168 subjects enrolled, 485 subjects were missing some key data elements, 75 subjects had an unreliable urine specimen (specific gravidity <1.002 or >1.030), leaving the remaining 2608 subjects eligible for further analysis. These were classified using the modified WHO criteria: (a) a positive family/household history of EN; (b) tubular proteinuria (α1-microglobulinuria >14 mg/g); (c) a serum creatinine >132.6 μmol/l; (d) anemia (hemoglobin <120 g/l in men and <113 g/l in women); and (e) exclusion of other renal diseases . Population of endemic villages was classified into groups: 1) diagnosed with EN, (a,b,c,d,e) or (b,c,d,e) or (a,b,d,e) (N=52); 2) suspected of having EN: (a,b) or (b,d) (N=139); 3) at risk of having EN (a) (N=455); 4) farmers from EN villages who were not related to EN in any way (N=1425). There were 537 subjects from non-endemic Croatian villages. No EN cases were detected outside of endemic EN villages. This study included 246 Bosnian immigrants who had settled in endemic Croatian villages 15–30 years ago. Aiming to increase statistical power when analyzing differences in herbal tea consumption between affected and non-affected villagers we constructed two larger groups: Group A) Cases = diseased + suspected of having EN (N=191); and Group B) Controls = at risk for EN + not affected villagers from EN villages + villagers from non-EN villages (N=2417). All statistical analyses were performed using SPSS v. 21 (IBM Corp. USA). Testing of exposure among two groups was done using χ^2-test and logistic regression was used to evaluate association between EN status and exposure. All protocols and procedures were approved by the relevant IRBs in Croatia and in the U.S.

As shown in Table 1 observed differences are related to the classification and definition of the groups. There was no difference in farming status. However, in the past Group A observed *A. clematitis* more frequently in their fields and among wheat seeds than those from non-endemic villages. This finding is in concordance with our previous results [2, 7]. We failed to observe differences in their dietary habits,

including baking their own bread from their own wheat (data not shown). The risk of exposure to AA via ingestion of herbal teas was evaluated by analyzing differences in specific questions. For the Question 1, there were no differences in the answers between EN cases and controls (χ^2=2.33, p=0.13; OR 1.11, 95% CI [0.83, 1.48]; p=0.49). On the Question 2, none of the subjects answered positively, except one farmer who had normal renal function, i.e., normal serum creatinine (89 μmol/L) and normal urinary albumin excretion (2.65 mg/l) (Yates' χ^2 = 2.20,Yates' p = 0.14). We failed to find difference in answers to the Question 3 (χ2 = 0.99, p = 0.32; OR 0.92, 95% CI [0.67, 1.28]; p=0.62) and to the Question 4 (χ^2 = 3.80; p = 0.051; OR 1.22, 95% CI [0.90, 1.65]; p=0.20). Evaluating the EN status and exposure to AA through herbal teas using multiple logistic regression analysis we failed to determine any significant association (Table 2). We failed to find that habit of herbal tea consumption is associated either with eGFR (OR 1.31, 95% CI [0.97-1.78]; p=0.08), albuminuria (OR 1.05, 95% CI [0.77-1.45];p=0.74), or with alpha1microglobulinuria (OR 0.99, 95% CI [0.65-1.52];p=0,98). Consumption of non-steroidal-anti-inflammatory drugs was also neither related to the EN status (OR 0.99, 95% CI [0.76-1.32]; p=0.99).

In the current survey, we enrolled autochthonous Croats and Bosnian immigrants. None of the Bosnian farmers enrolled in our survey reported use of herbal teas or other herbal products containing *A. clematitis*. Nikolić used the same questionnaire for EN patients from different Serbian endemic areas and none reported use of herbal products based on *A. clematitis* [2]. Therefore, in three countries (Bosnia, Croatia, Serbia) ingestion of AA through herbal teas was ruled out. Interestingly, Schmeiser et al. recently reported the presence of AA-DNA adducts in Romanian EN patients and found that subjects living in Romanian endemic areas frequently consumed homemade bread, what was a probable route of exposure to AA and did not find a correlation between the use of herbal remedies in the Romanian endemic areas and the development of urothelial cancers.

Our results suggest that farmers from endemic villages do not differ from non-endemic farmers in their herbal tea habits, which might be considered a marker of use of traditional medicine in general. Additionally, we failed to find relation of habit of herbal tea consumption with markers of kidney impairment i.e. reduced glomerular filtration and albuminuria. Even more important is lack of association with alpha1 microglobulinuria since this is an early marker and hallmark of renal damage

in EN. We also failed to find association of usage of analgetics and non-steroidal drugs with EN and kidney function. Results obtained in this survey revealed that farmers from EN villages do not differ from farmers from non-EN villages not only in the usage of traditional herbal remedies but also in the usage of regular and commonly used drugs. According to our results, herbal teas can be excluded as the route of AA ingestion in farming villagers living in endemic areas. This finding is confirmed in Croatia, but can be extrapolated with good probability to other endemic countries.

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Table 1. Basic demographic data and clinical characteristics of the study group

EN group	Endemic villages				Non-endemic villages
	Diagnosed with EN	Suspected of having EN	At risk for EN	Not affected	Not affected
N	52	139	455	1425	537
Male/female (N)	20/32	57/82	177/278	588/837	238/299
Age (years)	71.90 (9.86)	63.61 (14.23)	47.56 (15.57)	50.41 (17.39)	51.54 (18.40)
Height (cm)	162.0 (9.09)	165.72 (10.82)	169.29 (9.44)	167.80 (10.11)	167.94 (9.63)
Weight (kg)	71.02 (14.40)	76.17 (17.40)	79.55 (16.13)	77.20 (16.71)	79.50 (15.97)
Systolic blood pressure (mm Hg)	151.50 (23.44)	150.45 (27.76)	136.96 (23.32)	137.53 (24.73)	141.41 (24.79)
Diastolic blood pressure (mm Hg)	77.78 (11.65)	85.98 (14.75)	82.71 (12.71)	82.61 (12.91)	80.50 (12.67)
Positive family history for EN N (%)	31 (60)	122 (88)	455 (100)	0	4 (1)
Albuminuria (mg/L) #	53.25 (37.59-97.58)	16.30 (11.30-21.90)	5.40 (5.08-5.98)	6.07 (5.74-6.50)	6.00 (5.28-6.61)
α1-Microglobulinuria (mg/L) #	55.40 (46.47-81.18)	15.30 (13.90-16.87)	5.18 (5.05-6.86)	5.18 (5.18-5.53)	6.14 (5.64-6.70)
Serum creatinine (μmol/L)	345.54 (265.49)	108.37 (70.26)	83.04 (14.61)	85.08 (19.93)	84.97 (49.21)
Hemoglobin (g/L)	98.60 (14.82)	132.99 (18.77)	138.02 (13.51)	138.59 (14.39)	138.42 (14.88)

Values are expressed as mean (standard deviation) or as median and IQR (#). EN = endemic (Balkan) nephropathy

Table2. Age- and gender-adjusted multiple logistic regression analysis evaluating the association between EN status and exposure to aristolochic acid through herbal teas.¶

Exposure	B	Standard error	Wald	OR [95% CI]	p-value
Using herbal teas when sick – Yes* **(Question 1)**	0.101	0.147	0.471	1.11 [0.83, 1.48]	0.49
Picking up herbs by themselves – Yes** **(Question 3)**	-0.081	0.166	0.240	0.92 [0.67, 1.28]	0.62
Buying herbs from herbalist or in a store – Yes*** **(Question 4)**	0.199	0.154	1.678	1.22 [0.90, 1.65]	0.20

* Pseudo R^2 = 0.05 (Cox &Snell), 0.10 (Nagelkerkel), 0.08 (McFadden), $\chi^2(3)=121.9$, overall model fit $p<0.001$

** Pseudo R^2 = 0.05 (Cox &Snell), 0.10 (Nagelkerkel), 0.08 (McFadden), $\chi^2(3)=121.7$, overall model fit $p<0.001$

*** Pseudo R^2 = 0.05 (Cox &Snell), 0.11 (Nagelkerkel), 0.08 (McFadden), $\chi^2(3)=123.1$, overall model fit $p<0.001$

¶ Referent values were ”Controls (for dependent variable) and answer “No” (for independent variable)

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