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Invited Review

Current Status and Research Beyond Year 2010 in Balkan Endemic Nephropathy

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Introduction

In the first issue of BANTAO J, in 2003, I have presented the current research in Balkan endemic nephropathy (BEN) and associated upper urothelial tumors (UUT). As I stated in that paper, etiology remains the main problem for research in BEN [1].

Research of BEN in the nineties was hampered by the turmoil in the Balkans, especially in the former Yugoslavia. The Program Committee of the Interacademy Council of South-East Europe (IACSEE) at the second Program Committee Meeting, Belgrade, May 17, 2005, in order to accelerate research on BEN, a major problem in south east Europe (Bosnia & Herzegovina, Bulgaria, Croatia, Romania, Serbia), created the collaborative Project: BALKAN ENDEMIC NEPHROPATHY, with Professor Vladisav Stefanovic, as Project Coordinator. Project "BALKAN ENDEMIC NEPHROPATHY" started in 2005 with participation of investigators from 6 SEE countries (Bosnia and Herzegovina, Bulgaria, Croatia, Macedonia, Romania and Serbia) and 10 countries from Europe and North America. Meetings of the Project Network: Nis, May 2005; Belgrade, SASA, December 2005; Zagreb, October 22-26, 2006; Nis, April 2007; Lazarevac, May 2007; Belgrade, SASA, November 9-10, 2007; Brac, April 2008; Belgrade, SASA, April 17-18, 2009. Participants of the Belgrade 2009 Scientific Meeting agreed that the next Meeting will be held in Sarajevo, 5 May 2010, headed by Professor Senaid Trnacevic.

In conclusion of the Belgrade Meeting, Round Table agreed that etiological and other studies must be multicentric with well defined cases and controls. Several questions are enumerated for further collaborative studies, to give definite answers (Radovanovic, Brac, 2008): Does BEN exist elsewhere?; Has BEN been spreading out of already identified foci?; Do maps of BEN and excessive UUT occurrence overlap?; Do we know current topographical distribution of BEN?; Would genetic research solve the problem of BEN etiology?; How to direct collaborative etiological studies?; Is the exposure to the unknown BEN agent currently going on?; How to assess it?; Which tests should be used to pick up possibly affected individuals in a screening of BEN?; Is mass screening of BEN ethically justified?; Should the population be mass screened for UUT?; How to identify cases outside of BEN foci?; How to make BEN research more methodologically sound?;

Frequent sources of errors in the past were poorly selected cases and controls. Individual diagnosis of BEN may well be wrong. BEN cases: inhabitants of endemic settlements and from families with documented BEN cases (ill or dead), with exclusion of known interstitial and glomerular kidney disease.

For any environmental (as well as most other) research, households should be considered as affected only if at least three members developed BEN and/or upper urothelial tumors. Two groups of control households should be selected – one in endemic and another one in a neighboring nonendemic settlement. None of the control household members should have had a kidney disease or urothelial cancer.

BEN is an important health problem

BEN is a familial chronic tubulointerstitial disease with insidious onset and slow progression to terminal renal failure. It affects people living in the alluvial plains along the tributaries of the Danube River in Bosnia, Bulgaria, Croatia, Romania and Serbia [2]. An estimate of more than 10,000 of affected persons or at risk makes this disease an important health problem in the Balkans. A high prevalence of tumors of the renal pelvis and ureter was described in patients with BEN and in affected families [3].

Etiology of BEN and UUT

Genetics. The aetiology of BEN and associated UUT is not yet fully understood but there appears to be a polygenic susceptibility to the disease in interaction with multiple environmental factors [4,5]. In blood samples of Bulgarian BEN patients and in some of their healthy relatives, 3q24 - 3q26 abnormalities have been established [6]. The frequency of acquired chromosomal anomalies is considerably higher than in healthy persons.

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Oncogenic bands are more frequently involved in structural aberrations and spontaneous chromosomal/chromatid breaks in BEN patients than in the healthy population [7].

Lignites. This hypothesis was first proposed by Feder *et al*. based on the apparent spatial association of endemic villages with subsurface lignite deposits in Yugoslavia [8]. Subsequent publications by this group reported a similar association in Romania, and the presence of complex organic structures both in coal and water samples from the region [9]. In Bulgaria, levels of all polycyclic aromatic hydrocarbons (PAHs) were found low, with none exceeding the maximum contaminant level (MCL) in drinking water for benzo-[a]-pyrene, the most toxic PAH and the only one for which a standard has been set [10]. This study finds no basis to connect PAHs to the etiology of BEN in Bulgaria, and suggests that the evidence in support of the Pliocene lignite hypothesis is limited to the spatial association originally proposed.

Aristolochic acid (AA). That AA produced by *Aristolochia clematitis* is the cause of BEN is an old hypothesis, formulated as early as the 1970s, based on epidemiologic, environmental and agricultural research [11]. Ivic has shown that *Aristolochia clematitis*, a plant native to the endemic region, often grows in cultivated fields where its seeds, containing AA, commingle with wheat grain during the annual harvest. As bread, the dietary staple of the region, is prepared traditionally from flour made from locally-grown wheat, residents of the endemic region consuming home-baked bread may be exposed, over time, to toxic amounts of AA. Ivic has also made an experimental model of BEN in rabbits poisoned with Aristolochia, who developed tubulointerstitial changes and urothelial atypia. However, Grollman *et al* are the first to provide 'direct' instead of 'circumstantial' evidence that AA is indeed the 'culprit' of BEN [12].

It is currently unclear why only 2–5% of the people living in the endemic regions is affected by the disease, although a substantially larger number of individuals is likely to have been exposed to toxic amounts of AA. A large genetic component in the susceptibility to the disease has been suggested, but further research is warranted.

Both, agricultural developments (i.e. introduction of herbicides, new harvesting techniques) and lifestyle changes (i.e. diminished daily intake of bread, fewer families baking their own bread) has lead to a decrease in dietary exposure to AA. Three recent epidemiological studies carried out in Serbia and Bulgaria have demonstrated a decrease of incidence of BEN and associated UUT [13-15]. A high incidence of BEN is still observed in the Kolubara River region in Serbia [16]. Even higher activity of BEN was found in Semberia (Bosnia and Herzegovina). The recent reports favor the hypothesis that different endemic regions have certain local characteristics, including environmental toxicant.

Mycotoxins. Ochratoxin A (OTA) is a mycotoxin probably implicated in development of BEN and associated urothelial cancer [17,18]. OTA was found to be nephrotoxic to all animal species tested, including birds and mammals. The role of OTA has been questioned because of its high toxicity [19]. Some authors have argued that OTA had never been linked to any nephropathy in humans, but endemic nephropathy in Tunisia share clinicopathologic similarities with BEN [20]. DNA adducts related to OTA and CIT are found in human kidney tissues taken from patients in the Balkans, Fran-ce, and Belgium whereas no DNA adducts related to AA could be found in any tumors of BEN patients from Croatia, Bulgaria, or Serbia [21]. Interestingly, specific OTA related DNA adducts have been found in several kidney tumours from Balkan region [21].

Aristolochic acid (AA) is an important risk factor for BEN [11,12]. The geographic correlation and presence of AA-DNA adducts in both BEN and associated urothelial cancer, support the speculation that these diseases share a common etiology. Dietary exposure to AA is a significant risk factor for BEN and its attendant transitional cell cancer. These are cases of well known AA induced urothelial carcinoma, and could be detected worldwide. The presence of more than one risk factor is possible and it is important to test etiological hypotheses in different endemic foci, preferably as a multicentric research.

Since research conducted in one place/region could not hold true elsewhere, it is important to test etiological hypothesis in different endemic foci, i.e. to run studies as a multicentric research, especially as there may be different risk factors in BEN. Studies presenting molecular biology evidence on aristolochic acid as a risk factor were done on a small number of cases from a peculiar endemic region, including immigrants to the Brodska Posavina region. Ochratoxin A was questioned as the etiologic agent, mostly by those favoring AA. The pathway for exposure to ochratoxin A is well defined and there is evidence that humans have ingested ochratoxin A. Factors causing differential exposure to ochratoxin A and how ochratoxin A is implicated in BEN are not defined. Although there is evidence of human exposure to aristolochic acid and that its effects are consistent with BEN, a pathway for exposure to aristolochic acid has been suggested but not demonstrated. Factors causing differential exposure to aristolochic acid are not known. Exposure analysis results suggest that neither ochratoxin A nor aristolochic acid can be firmly linked to BEN [22]. However, this approach suggests future research directions that could provide critical evidence on exposure, which when linked with findings from the health sciences, may be able to demonstrate the cause of this disease and provide a basis for effective public health intervention strategies. One of the key unknowns for both agents is how differential exposure can occur.

Epidemiological characteristics of BEN

Residence in an endemic settlement. Several endemic regions in former Yugoslavia, Bulgaria and Romania, were identified. BEN was described in the Kolubara River valley, but also along the Sava and its tributaries, in flood areas of Podrinje and Mačva, in some areas of Bosnia (near Bijeljina) and in Croatia (around Slavonski

Brod). Later reports described BEN in the valleys of Velika Morava, Juzna Morava, Binacka Morava and Zapadna Morava. In the endemic area not all vilages are affected: those affected are sometimes at a distance of only a few kilometers from unaffected ones [23]. However, we have found sporadic cases of BEN and associated UUT in a wider region.

Family history of kidney disease. In a single household, several members of one or several generations may be affected. Within the same village, affected and spared households live in close proximity. However, studies of BEN in villages around Slavonski Brod, Croatia pointed to environmental factors as key determinants of individual's susceptibility to BEN [24].

History of urothelial tumors. An increased incidence of tumors of the upper urothelium was discovered among the inhabitants of endemic settlements and in families affected by BEN, as well as in up to 40% of BEN patients [3]. Nikolic *et al*. have found that territorial distribution of UUT in Serbia was much wider than distribution of BEN [25]. The same group described 312 sporadic cases of BEN, with concomitant UUT, in 164 nonendemic settlements [26].

Occupational history. BEN was found among farmers of the affected villages. Farming is a common occupation among villages and we were able to prove that farming, at least in some period of life, was characteristic of patients with BEN. Thus, the putative etiologic agent could relate to farming procedures or to dietary habits peculiar to this rural area.

Diagnosis of BEN

Diagnostic criteria for BEN have been described more than 40 years ago. Research groups on BEN use one of at least three described lists of criteria. Comparison of studies using such criteria is difficult, and a recent meeting of investigators (Zagreb, October 2006) has suggested that unified criteria have to be elaborated. An International Panel of BEN Investigators agreed on criteria appropriate to epidemiologic studies and clinical investigations of BEN. A screening procedure of BEN in endemic settlements is proposed [27].

ß2-microglobulin was used as an indicator of tubular proteinuria. However, as alpha-1-microglobulin is more stable in acid urine, its role in diagnostic procedure of BEN requires further investigation. Preliminary collaborative study in the South Morava and Kolubra region, comparing these two markers, favors the use of ß2-microglobulin (unpublished data).

Diagnosis of BEN is made in inhabitants from endemic settlements using epidemiologic criteria, glomerular filtration rate (GFR) decrease, proteinuria, generally below 1 g/24 h, microalbuminuria, tubular markers (renal glucosuria, increased urinary excretion of β2-microglobulin or alpha-1-microglobulin), and decreased kidney size, by ultrasonography. Exclusion of other known kidney diseases (chronic atrophic pyelonephritis, glomerulonephritis, etc.) is mandatory.

Ideally all adults in endemic settlements should be routinely screened for evidence of early BEN and associated risk factors [27].

Diagnostic procedure

Diagnosis of BEN is made in inhabitants from endemic settlements using: 1.Epidemiologic criteria; 2. Demonstration of (a. GFR decrease; b. proteinuria generally bellow 1 g/24 h; c. microalbuminuria; c. scarse urinary sediment; d. tubular markers (increased urinary excretion of β2-microglobulin or alpha-1-microglobulin); e. a typical renal histology showing hypocellular cortical interstitial fibrosis decreasing from the outer to the inner cortex (if renal biopsy feasible); f. decreased kidney size, by ultrasonography); 3. Exclusion of other known kidney disease.

Screening of BEN

In endemic settlements individuals with a family history of kidney disease appear to be at higher risk of developing kidney disease. BEN is still the major problem in several endemic regions in Bosnia, Croatia and Serbia. Many patients are not diagnosed until the late stages of disease, as early kidney disease may be asymptomatic. Ideally all adults in endemic settlements should be routinely screened for evidence of early BEN and associated risk factors.

Urothelial cancer and BEN

Urothelial malignancies of renal pelvis and ureter are significantly more frequent, up to 100 times, in endemic than in non-endemic areas [28-31]. They tend to cluster in families affected with BEN, indicating an association between these diseases and, probably, a common etiologic agent. Tumors develop usually later than the interstitial nephropathy.

Their incidence increases with age at the time of diagnosis and with a longer survival, and is higher in females.

Our analysis of the morphological characteristics of UUT in BEN and nonendemic regions showed that the best characteristic that differentiated them was growth pattern; i.e., solid growth for BEN tumors and papillary for control tumors [32]. In this study, control UUT with solid growth had a higher Ki-67 index than BEN solid tumors. In UUT with a papillary configuration, there was no difference in proliferative Ki-67 activity.

Investigation of multiple molecular markers identifies tumor suppressor p53 as an indicator of the group, where BEN UUT had a higher p53 index than control tumors. In regression analysis, P53 correlated with the grade, growth, and group ($p < 0.05$). This investigation identifies the p53 pathway as the specific cell cycle marker involved in BEN-associated UUT [33].

Patients in a chronic dialysis program frequently develop urothelial cancer, particularly cases from BEN foci [34]. Such cases are also at an increased risk of developing UUT after kidney transplantation [35,36]. There is a striking difference in malignancies in general kidney transplant population (0.69%) and in the BEN population (43.7%). In BEN patients, bilateral nephroureterec-

tomy before transplantation has been suggested as a preventive measure.

Children and BEN

BEN has not been described in children; however, some previous studies in children from families with BEN have revealed abnormalities of the urinary tract. Recently, we demonstrated that children from families with BEN excreted significantly more albumin and total protein than those from nonendemic families living in the same settlements or from children living outside of the endemic region in the villages or the city of Nis [37,38]. In children from endemic and control settlements around the South Morava River, urinary β2-microglobulin, N-acetyl-β-D-glucosaminidase (NAG) and gamma-glutamyl transpeptidase (GGT) excretion was measured nine times during a 3-year period [39]. As BEN is an environmentally induced disease with possible seasonal variation of toxicants, children were studied in different seasons (spring, autumn, and winter). Beta-2-microglobulin excretion in urine, in all three seasons, was highest in children from families with BEN compared with the excretion in children from the city, nonendemic villages, and those from nonendemic families. In multivariate analysis, β2-microglobulin excretion was significant for family status, gender, and the season. NAG emerged as a potentially useful marker for seasonal exposure to an environmental nephrotoxin.

Prevention and treatment of BEN

We just started to unravel the etiology of BEN and associated urothelial cancer. Since BEN was first described, around half a century ago, socioeconomic changes (in housing, farming, living standards, etc.) have been profound and the effect of environmental toxicants has been reduced. Genetically susceptible persons have become ill and dead.

Avoidance of etiological factors is the best prevention. As aristolochic acid is the probable toxicant in the South Morava region and in Bulgaria, the number of BEN and associated UUT has been reduced and will disappear.

Treatment of BEN is similar to that of all chronic interstitial nephropathies. Patients with BEN should pay close attention to cardiovascular risk and stop smoking, eat healthy and balanced diet, and take regular exercise. Hypertension should be treated with ACEIs and ARBs. A low protein diet can be used in CKD stages 3 and 4.

Hemo-and peritoneal dialysis as well as kidney transplantation have been used with success. BEN does not recur after renal transplantation.

With longer survival on renal replacement therapy, patients develop tumors of the renal pelvis, ureter, and urinary bladder. And long term surveillance is required for that. Pretransplant bilateral uretero-nephrectomy is necessary to prevent urothelial cancer on native urinary tract.

BEN worldwide disease

Whatever the causes of BEN and associated UUT, the disease might not be restricted only to southeastern Europe. Rather, the intensity of exposure to risk factors for BEN and, consequently, clustering of cases has more likely determined our knowledge of topographical distribution of an etiological entity that is much more widespread, or that might even be ubiquitous in its sporadic form.

Demonstration of AA as the risk factor of BEN, at least in a part of the BEN cases, has identified BEN as aristolochic acid nephropathy AAN, appearing worldwide [40,41].

As we have predicted in 1991, with the etiologic diagnosis, it will be possible to detect BEN and associated upper urothelial cancer outside of the Balkans, like in AAN, where sporadic cases occur [42]. We still don't have the method sufficiently sensitive to demonstrate AA-DNA adducts in exfoliated urothelial cells from urine, but this could be a possible biomarker.

Further etiologic studies

Although the genetic analysis discloses several genes that might predispose to BEN, etiology of the disease remains unclear. Currently, there is no specific genetic diagnostic marker and predictive genetic testing of BEN. Human genes that influence susceptibility to AA and OTA toxicity may be identified through toxicogenomic studies of BEN.

Analysis of single nucleotide polymorphisms (SNPs) in blood samples, covering the whole genome both in BEN patients and in controls living in the endemic regions could allow determination of polymorphisms that increase the risk of disease development. Association analysis of the polymorphisms in BEN patients with UTT and in BEN patients without UTT could differentiate the polymorphisms that increase the risk for tumor development. The role of environmental toxicants, such as OTA and PAHs, but also AA, should be reconsidered in the coming years.

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