The Etiology of Balkan Endemic Nephropathy: Still More Questions than Answers

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Balkan endemic nephropathy (BEN) has attracted increasing attention as a possible environmental disease, and a significant amount of research from complementary scientific fields has been dedicated to its etiology. There are two actual competing theories attempting to explain the cause of this kidney disease: 1) the mycotoxin hypothesis, which considers that BEN is produced by ochratoxin A ingested intermittently in small amounts by the individuals in the endemic regions, and 2) the Pliocene lignite hypothesis, which proposes that the disease is caused by long-term exposure to polycyclic aromatic hydrocarbons and other toxic organic compounds leaching into the well drinking water from low rank coals underlying or proximal to the endemic settlements. We outline the current developments and future prospects in the study of BEN and differentiate possible factors and cofactors in disease etiology. *Key words*: aromatic compounds, Balkan endemic nephropathy, chronic interstitial nephropathy, environmental disease, ochratoxin, Pliocene lignite, urinary tract tumors. *Environ Health Perspect* 106:689–700 (1998). [Online 9 October 1998]
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A matter of debate for more than four decades (*1*), Balkan endemic nephropathy (BEN) is a chronic tubulointerstitial nephropathy (CIN) described so far in several rural regions of Bulgaria, Romania, and the former Yugoslavia (Serbia, Croatia, and Bosnia). Most of the endemic villages are located on alluvial valleys of streams that are tributaries of the Danube River, and the area where the disease occurs does not exceed 20,000 km^2 (*2*). The topography of the disease sites varies from the flood plains in the former Yugoslavia to the undulating hills around Drobeta Turnu Severin in Romania and the low montane region of the Vratza District in Bulgaria (*3*) (Fig. 1). The geographical distribution of the disease has not changed significantly since it was first described in the 1950s. Villages afflicted in the past continue to be afflicted today, while nonendemic villages and towns, sometimes located in close proximity to afflicted villages, have remained free of BEN. At least 25,000 individuals may suffer from BEN or are suspected of having the disease, while the total number of people at risk in the three countries may exceed 100,000 (*4,5*). Some of the intriguing features of BEN are 1) focal occurrence of the clinical cases; 2) familial aggregation without an obvious pattern of Mendelian genetic inheritance; 3) long "incubation" period before the clinical onset of the disease (people coming from outside must reside in the endemic region for more than 10–15 years to acquire the disease); 4) occurrence only in adults (ages 30–50 years are most heavily affected, while almost no children or individuals over 70 years of age develop the disease); 5) a slight sex distribution, with a female:male sex ratio of about 1.5:1; 6) a similar incidence in different ethnic and religious groups; and 7) restriction to a rural farming population. Of particular interest are the upper urinary tract tumors (UTTs) seen in BEN patients at frequencies much higher than in individuals not suffering from BEN from endemic regions or in the general population in the countries with BEN. The distribution of these tumors generally follows the geographical distribution of BEN, with a steep decrease in incidence in nonendemic villages located even 1–2 miles away from the endemic ones. UTTs are frequently multiple and bilateral and rarely occur in the general population. Their high incidence in BEN patients led to speculation that the two diseases could be causally related (*1*) or that the urinary tract tumors should be seen as a symptom, although inconstant, of BEN.

There have been several attempts to trace the history of endemic nephropathy to the beginnings of this century or to the period between the two world wars. Because BEN becomes manifest at older ages and the average life expectancy in the endemic settlements was less than 45–50 years before World War II (some of the major causes of earlier death were parasitic or infectious diseases such as malaria and tuberculosis), no rigorous description of the disease or clue it existed is available from that period (*6*). This, however, does not preclude the possibility that the etiological factor(s) responsible for BEN has been present in endemic areas for centuries or millennia. The increase in the average life expectancy after World War II may have allowed BEN to become manifest and thereafter to be described as a distinct nosological entity. BEN was almost simultaneously recognized for the first time in the three countries involved as well as in each major affected region of these countries by Tanchev et al. in Bulgaria in 1956 (*7*), by Danilovic and others in 1957 in Yugoslavia (*8*), and by Fortza and Negoescu in Romania in 1961 (*9*).

In spite of considerable research performed in the three afflicted countries, the etiology of BEN and of its accompanying uroepithelial tumors still remains a medical enigma. Multidisciplinary studies involving environmental and occupational medicine, microbiology, oncology, toxicology, epidemiology, cytogenetics, and more recently, geology, hydrogeology, and biogeochemistry have added some pieces to the puzzle, but have also raised new questions (*10*). Among these, the most central remains: What is the true cause of the disease?

Clinical Presentation and Laboratory Findings in BEN

BEN is a progressive kidney disease inexorably leading to end stage renal failure. Although some symptoms seem to be more specific, there is no clear-cut clinical or pathological definition that is solely sufficient for disease diagnosis. The onset is without any acute episode, and the anecdotal cases of acute BEN described in the literature seem to be related

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to kidney diseases other than BEN (or going along with BEN) that may occur in the endemic regions. The disease progresses slowly with the occurrence of nonspecific signs (lassitude, fatigue, headache, weight loss and reduced appetite, xanthochromic or pale skin) and specific signs of kidney damage (reduced tubular transport, intermittent proteinuria with low molecular weight proteins such as β-2 microglobulin, and a gradual rise in blood nitrogen) (*11*). Usually, no increased blood pressure is found, even in the more advanced phases of the disease; this was explained by Dammin (*12*) as the outcome of the early impairment of the juxtaglomerular apparatus. Anemia (normocytic, normochromic, or in rare cases, hypochromic or even aplastic) is one of the major symptoms, and it seems to develop well before other signs of BEN become manifest. Although in the advanced stages of the disease anemia is

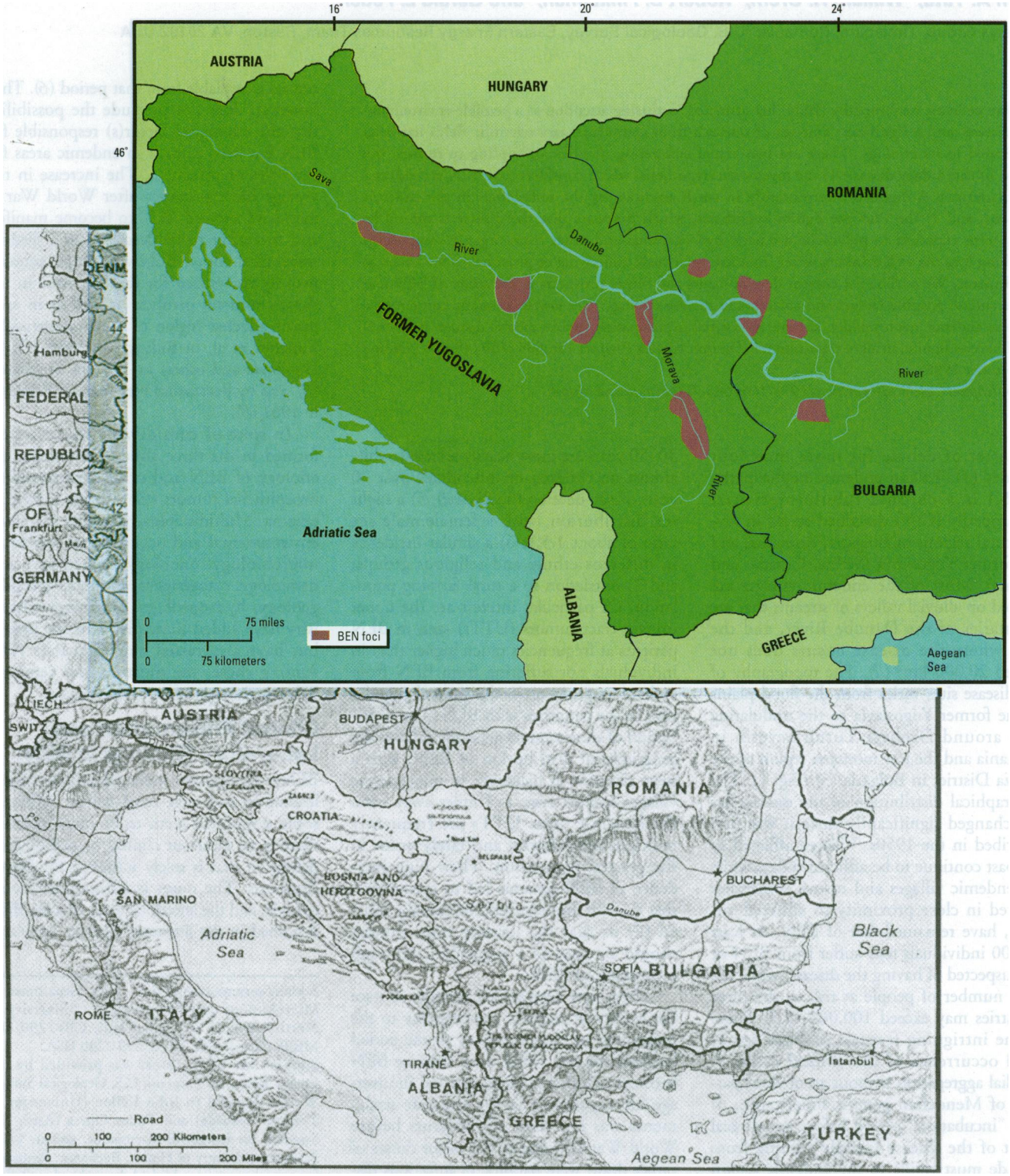

Figure 1. Map showing the distribution of the Balkan endemic nephropathy (BEN) areas in the Balkan Peninsula. Adapted from Feder et al. (*29*) and the CIA World Factbook 1984 (63).

proportional to the degree of kidney insufficiency and, thus, could be attributed to the increased blood urea level and to the reduction of erythropoietin synthesis (as a result of the destruction of the erythropoietin-secreting peritubular endothelium of the kidney by the etiological agent), it usually appears before significant kidney damage takes place. In this regard, it was presumed that the nephrotoxic factor(s) responsible for BEN could also be hematotoxic, inducing anemia and other possible hematological disturbances (*4,13*). Flow cytometric investigation of BEN patients and area control individuals (people born and living in the endemic area and clinically asymptomatic, but having BEN and a BEN death-related family history) revealed in many cases a decreased B-cell count, eosinophilia, and an increased proportion of $CD3^+CD8^+CD16^+CD56^+$ cytotoxic lymphocytes (*14,15*). Because similar modifications can be induced by certain toxic compounds [halogenated aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs)] in experimental animals, the authors linked these alterations to the same presumed toxic agent that caused the kidney alterations. The immunoglobulin (IgM, IgG) levels are normal and no signs of humoral or cellular immunodeficiency were seen, while a decreased phagocytic activity was observed in certain cases (S. Gotia, personal communication). The serum levels of inflammatory cytokines [interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-γ (IFN-γ) are normal in most of the cases [Drugarin et al., Tatu et al., unpublished observations); (*16*)], stressing again the fact that BEN is not an inflammatory kidney disease. The serum levels of albumin, C reactive protein, ceruloplasmin, fibrinogen, and α-1-antitrypsin are normal except for the advanced phases of the disease, when their lower levels could be attributed to uremia and to the reduced liver synthesis.

Morphopathologically, BEN is a tubulointerstitial nephropathy with a relatively nonspecific aspect. In the advanced stages, kidneys are reduced in size symmetrically and can weigh as little as 50 g or less. Histological examination reveals pronounced fibrotic changes of the interstitium without cellular infiltrate or signs of inflammation. The initial lesion seems to be confined to the proximal convoluted tubules, leading to tubular atrophy and pronounced basilar membranes. In the incipient stages of the disease, glomeruli do not seem to be affected, although later periglomerular fibrosis and cysts in the cortical areas can be seen. Atherosclerosis may also occur in kidney vessels (*17*). A peculiar feature of BEN is its frequent association with malignant tumors of the upper urinary tract (renal pelvis and ureters). These tumors develop from the urothelium and usually have an aggressive behavior.

In Search of an Etiology

There are many unknown aspects concerning BEN as a disease, but its etiology is the central topic of dispute. Perhaps no other human disease has generated so many different hypotheses and ideas in an attempt to explain its causal factors. For BEN, which was classified as a disease only in the second half of the century, there have even been rumors among the villagers that the illness is caused by cosmic and astrological influences beyond human control and comprehension. To protect themselves against these dreadful forces, people in certain endemic regions (e.g., from former Yugoslavia, Romania, and probably Bulgaria) still wear amulets or pendants and perform ritual ceremonies to be spared from becoming sick.

The profusion of factors considered to cause BEN reflects, at least in part, the limited knowledge of the etiology of CIN in general. In comparison with other forms of CIN, the main peculiarity of BEN is its restricted geographical distribution, which became more conspicuous after descriptions of the nephropathy from the involved countries became available. Considering only the geography of the disease, an environmental etiological agent seems plausible, presumably acting on a predisposing genetic background of the exposed population.

Other factors that make BEN etiology research difficult include poor epidemiological data gathered from the endemic region; confusing and often contradictory results due to the lack of communication and cooperation of BEN researchers from the afflicted countries; the lack of standardized investigation methods; the communist mentality (e.g., the former communist government from Bulgaria relocated whole endemic villages and never recognized the disease; thus, important medical data was lost) (*5*); a shortage of funds for specialized teams to thoroughly investigate the endemic regions (*18*); and social events (such as the war in former Yugoslavia, which in 1993 cut a fruitful line of research related to the role that low-rank coals may play in BEN). Despite these difficulties, major research efforts have been made over several decades in an attempt to understand the causes of this still confusing disease. BEN is a complex medical condition, and its apparent multifactorial etiology makes it a challenging research topic.

Factors, Cofactors, and Risk Factors

Because the symptoms and pathology of BEN in all the endemic zones are the same regardless of the country where it occurs, there is probably a common etiological factor(s) for the disease. One of the drawbacks of many of the investigations of BEN is that studies performed in one of the three countries were not reproduced by similar studies made in the other two countries, posing questions about their reliability. For instance, the proposal of a viral etiology of the disease was first advanced by Georgescu in Romania (*19*) and Apostolov in Yugoslavia (*20*), but was not confirmed by Bulgarian studies. Similarly, in cytogenetic studies, Bulgarian groups reported the presence of a chromosomal marker (3q25) in BEN (*21,22*), but this has not been demonstrated so far in Romania or Yugoslavia. Other factors inconsistently incriminated in the etiopathogeny of BEN include heavy metals claimed to be present at higher (lead, cadmium, chromium) (*23*) or lower (manganese, cobalt) than normal concentrations in water or foodstuffs from endemic areas; selenium deficiency (*24,25*); X ray-emitting compounds; and industrial pollution. Other studies incriminate low molecular weight protein (LMWP) excretion in patients with BEN as a factor in disease pathogeny (*26*). Microproteinuria with β-2 microglobulin has been found in many BEN patients and area controls. Although these proteins are suspected to be involved in renal diseases in humans and experimental animals, they do not explain the geographical restriction of BEN. In this regard, LMWP involvement is probably secondary, after the etiopathogenic agent has triggered the kidney damage. Immunologic and hematologic alterations (thought not to be of primary role in BEN etiopathogenesis) may also influence the evolution and outcome of the ongoing (established) disease. Anemia and the phenotypic changes in lymphocyte subpopulations (e.g., the increase of a cytotoxic $CD8^+$ T-cell subset and a decrease in the B lymphocyte subpopulation) are considered to be consequential to the action of the same agent(s) inducing kidney pathology (*14,15*) rather than to the nitrogen retention and uremia. It is likely that such modifications, although not marked, could result in an impaired antiinfectious and antitumoral defense, mainly in the advanced stages of BEN when the risk to develop UTTs is highest. Another possible cofactor or risk factor in BEN and UTT is selenium deficiency, which is well documented in rocks, soils, water, and foodstuff from Serbia, as well as in serum samples collected from endemic and nonendemic regions (Fig. 2). In certain endemic locations, levels of Se deficiency approach those encountered in the Keshan province in China, where a severe cardiomyopathy linked to Se deficiency is endemic (*25*).

Although no known human kidney disease has been associated with a low Se intake, the chemoprotective effects make this metal an essential oligoelement in the defense against chemicals inducing oxidative damage and cancer. The immune antitumor response is also boosted by Se (*27*) and, in this regard, Se deficiency cannot be completely ruled out, given the high incidence of UTTs in the BEN endemic territories.

Genetic polymorphisms in environmentally regulated genes (such as those involved in the distribution, metabolism, and disposal of toxic compounds, and DNA repair pathways and their regulators) (*28*) may also account for differential susceptibility to BEN in the endemic region population and particularly to upper urinary tract oncogenesis.

Causal factors

Ochratoxin A and Other Mycotoxins

The present paradigm is that BEN is an environmentally acquired disease. Currently, the two most plausible environmental agents supposedly involved in its etiology are mycotoxins produced by fungi in moldy cereals and various food commodities (grains, meats, beans, etc.) from endemic areas, and aromatic compounds present in the drinking water from the endemic areas (*29*).

The mycotoxin cause for BEN gained support more than 20 years ago (*30–32*) when a striking morphopathological and epidemiological analogy between porcine mycotoxic nephropathy, caused by ochratoxin A (OTA), and BEN was observed. OTA is produced in temperate and subtropical regions mainly by fungi of genus *Penicillium* (i.e., *P. verrucosum, P. cyclopium, P. chrysogenum,* etc.) and *Aspergillus (A. ochraceus, A alliaceus, A. elegans,* etc.) (*33,34*). Subsequent investigations, which revealed a high frequency of OTA contamination of foodstuffs from Yugoslavian and Bulgarian endemic regions, higher levels of OTA in foodstuffs from endemic versus nonendemic villages and in the blood of BEN patients versus individuals from nonendemic regions, and the detection of several characteristic OTA–DNA adducts in urinary tract tumors from Bulgarian individuals living in BEN endemic regions (*35,36*), substantiated this hypothesis. The recent description of a BEN-like nephropathy in Tunisia, Africa (*37,38*), which is allegedly produced by OTA and of a possible causal link between OTA and the karyomegalic interstitial nephropathy (*33*), a form of nephropathy that resembles BEN in certain morphopathological and clinical aspects, also strengthens the idea of an OTA etiology of BEN. OTA, however, is a relatively common contaminant of various foodstuffs in many countries with temperate continental climate and can be sporadically found in the blood of healthy individuals. This hypothesis also leaves unclear the fact whether OTA is the principal etiologic factor in BEN, or only a cofactor that, although required, is not enough to produce BEN itself. A major caveat in studies of OTA related to BEN is that the higher levels of OTA found in the serum of BEN patients compared to controls may be the consequence of secondary accumulation of OTA, following the impairment of kidney transport and excretion functions by the original etiological agent rather than reflecting the natural exposure to OTA. Also, if OTA were the only etiological factor for BEN, one would expect to find cases of BEN or BEN-like disease in many other places around the world where food contamination with OTA has been reported (e.g., in the United Kingdom or Italy where the highest levels of OTA contamination of certain food commodities, moldy wheat flour and moldy bread, respectively, have been observed) or in countries where a high prevalence of human sera contamination with OTA was detected (for instance in France, Denmark, and Germany, where OTA was found in 22%, 54.2%, and 56.5–68.1% of serum samples, respectively) [reviewed by Godin et al. (*33*)]. BEN, however, seems to be unique in its geographical distribution, evolution, manifestation, and pathology. Although several studies claim that BEN could be a disease "beyond the Balkans" (*39*), more research will be needed to clarify this.

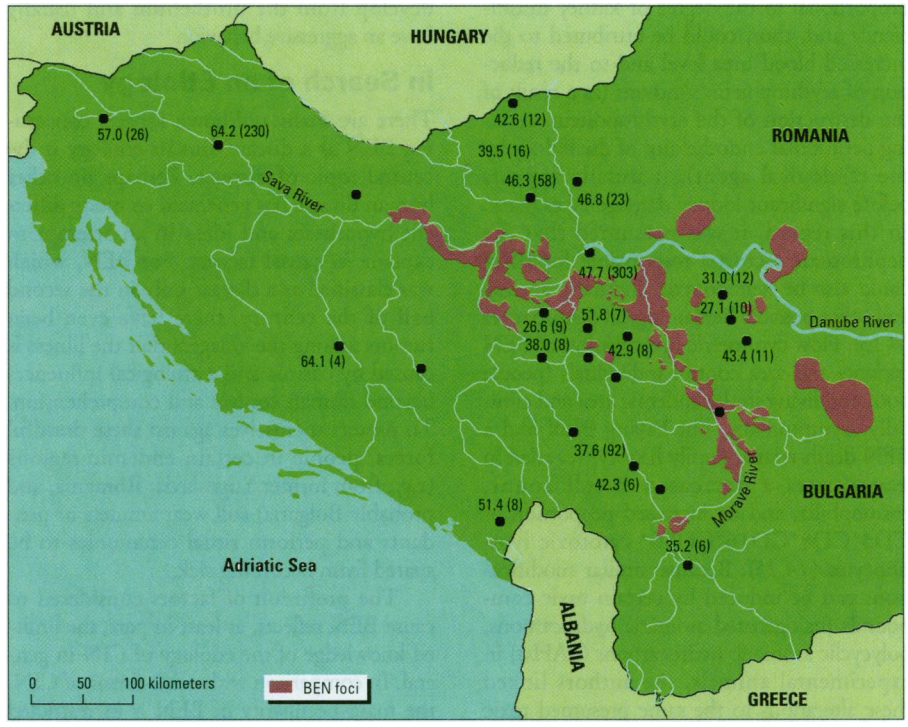

Figure 2. Map presenting the mean serum selenium levels in individuals from former Yugoslavia in relation to the BEN foci. Most values are intermediate between those found in other countries (e.g., 131.0 µg/l in the United States, 144.0 µg/l in Canada, 106.0 µg/l in England) and in the Sichuan Province in China (13.0 µg/l) where Keshan disease is endemic. Values shown are mean serum Se levels in µg/l (no. of samples). Adapted from Maksimovic et al. (*24*).

Pliocene Lignites and BEN

An alternative hypothesis was formulated in 1991 based on the geographical correspondence between the location of Pliocene lignite deposits in the Balkans and the location of endemic areas, as well as preliminary geochemical analyses of well water from endemic villages in Yugoslavia, which showed the presence of organic compounds not observed in well water from nonendemic villages (*29,40*). All endemic regions except one are in close proximity to known deposits of low rank coals of Tertiary age (Pliocene lignites) (Fig. 3). The reason for the one exception is unclear. This endemic area is in central Serbia and does have some nearby Pliocene lignite deposits, although not in as direct proximity as found in other endemic areas. This area of exception may have uncharacterized lignite deposits or be hydrologically connected to nearby lignite beds. Further work will be needed to delineate this.

The hypothesis emerging from these observations is that groundwater leaches toxic organic compounds from these low rank coals, with subsequent transportation of these

organics to wells in the alluvial valleys below the lignite deposits (*29,41*) (Fig. 4). The inhabitants of the rural villages who rely on well water for drinking, cooking, and other purposes are thus exposed to these toxic organics for long periods of time. Although the concentrations of these organic molecules in the well water may be low, long exposure and/or accumulation in body tissues over time may lead to kidney lesions, the development of urothelial carcinomas in some individuals, and ultimately to full-blown BEN.

Pliocene lignites are some of the youngest coals in the Balkans and are relatively unmetamorphosed. As such, they retain many of the complex organic compounds contained in their decaying plant precursors, and many kinds of potentially toxic organic compounds may be leached from them (*29*). Semiquantitative analysis of shallow well water from endemic villages revealed the presence of PAHs (pyrene, anthracene) and aromatic amines (aniline, naphtylamine). Some PAHs (e.g., benzopyrene, benzanthracene, benzofluoranthene) are known or suspected carcinogens, and aromatic amines have been linked to urinary tract cancer and tubulointerstitial nephropathies. Thus, long exposure to these and/or similar compounds in well water in a population dependent on this source of water could be linked to BEN etiology and the high incidence of urothelial cancer in individuals with BEN. This hypothesis is appealing in that it accounts for the geographical restriction as well as for the epidemiological and clinical features of BEN. Based on the present status of knowledge in nephrotoxicology, however, it should be noted that the currently observed PAHs and aromatic amines in the well water from endemic villages are not sufficient to explain the tissue (kidney)-confined toxicity in BEN. More specific (kidney-oriented) toxic molecules present in well water and derived from Pliocene lignites also will need to be identified to further validate this hypothesis. Many such compounds may indeed be present in Pliocene lignites, given the complex organic structure of coal. On the other hand, BEN could be a unique experiment of nature, the concentration, the route of entry, the association with other nephrotoxic compounds (such as mycotoxins), and the intermittent intake pattern of the toxicant(s) characterized by seasonal variations, causing some simple molecules such as PAHs to be specific in their target tissue action, with chronic nephrotoxicity and slow carcinogenic activity as their most salient biological effects.

Besides the Balkans, Pliocene lignite deposits are also found in Italy, Turkey, Greece, and Myanmar. Also, low rank, highly unaltered coals such as the Pliocene lignites from the Balkans are distributed worldwide (e.g., Australian brown coals, North Dakota and Gulf Coast lignites, Chinese lignites, etc.). However, in the Pliocene lignite hypothesis of BEN etiology, other factors besides the presence of low rank coals must also be in play. The hypothesis also implies many or all of the following circumstances: the right hydrologic conditions for leaching and transport of the toxic organic compounds from the coal to the wells; a rural population largely dependent on untreated well water; a population with a relatively long lifespan (BEN commonly becomes manifest in people in their 40s and 50s); a relatively settled population for long exposure to the source of nephrotoxic/carcinogenic substances; and a

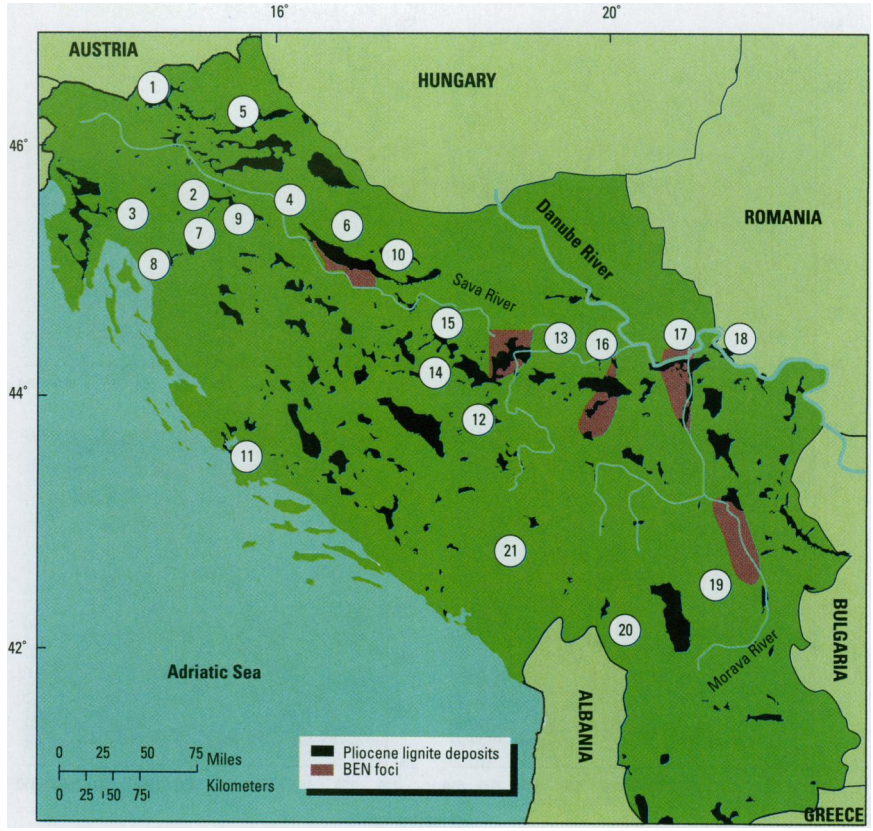

Figure 3. Map showing the distribution of coal deposits and the major BEN foci in former Yugoslavia. The encircled numbers indicate Pliocene-age coal fields. Most of the BEN foci are associated with such coal deposits. One BEN region apparently is not in the proximity of an identified coal field. Large coal deposits in Bosnia have no endemic areas in their vicinity, one possible explanation being that they have a higher degree of alteration (higher rank) than those associated with BEN foci. Adapted from Feder et al. (*29*).

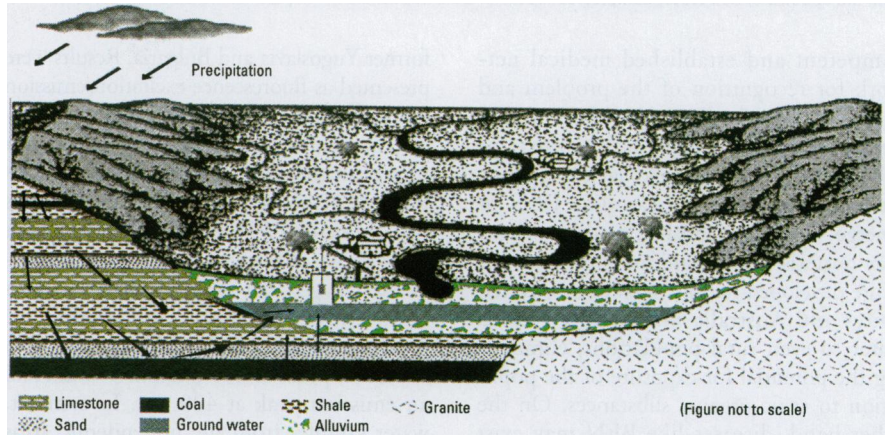

Figure 4. Schematic representation of the hypothesized weathering of Pliocene lignites proximal to or underlying the BEN foci located in alluvial valleys, and of the transportation process (indicated by arrows) of the leached organic compounds into the ground and well water. Adapted from Feder et al. (*29*).

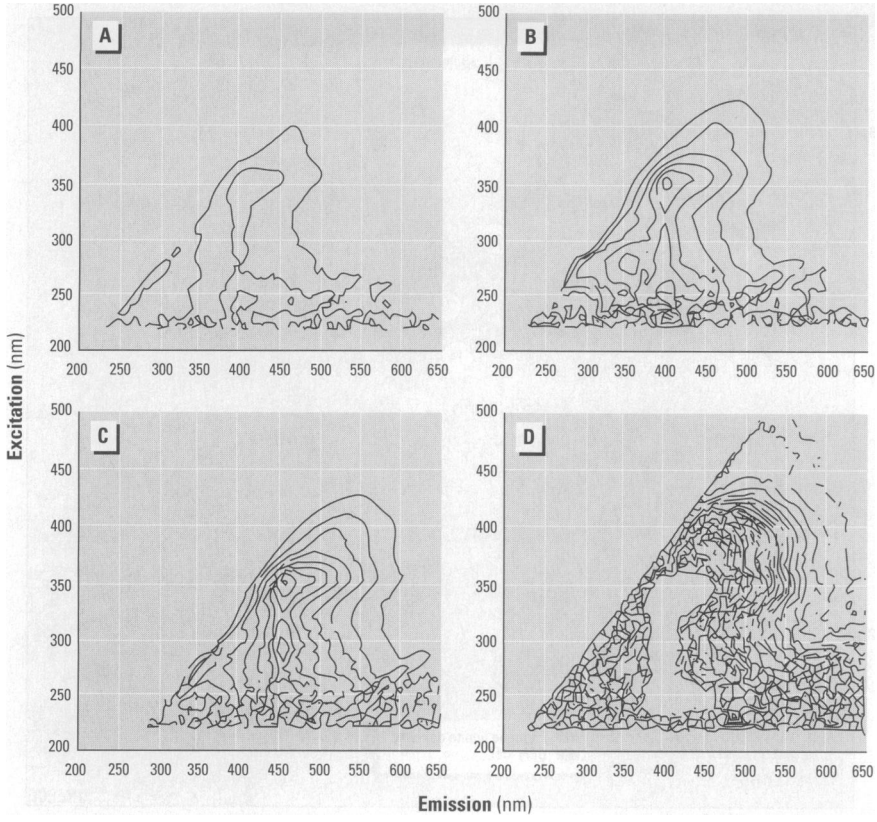

Figure 5. Excitation-emission matrix (EEM) spectra of well water samples collected from Bulgaria and the former Yugoslavia. BEN, Balkan endemic nephropathy. (A) Water sample from a nonendemic village in the former Yugoslavia. (B) Water sample from a nonendemic household in a Bulgarian BEN endemic village. (C) Water sample from a BEN household in an endemic village in the former Yugoslavia. (D) Composite EEM spectra resulting from subtraction of the spectra of two water samples collected in nonendemic locations from the spectra of two water samples collected in BEN households. Adapted from Goldberg et al. (*40*).

competent and established medical network for recognition of the problem and proper systematic diagnosis. Many underdeveloped nations such as Myanmar or rural regions in China may lack the population lifespan for a disease like BEN to emerge, and an established medical system may not exist for recognition of the disease. More developed nations such as the United States and Australia may supply treated water to even rural populations, eliminating the potential for exposure of the population to toxic organic substances. On the other hand, diseases like BEN may exist unrecognized. In the United States, for example, some of the highest incidences and deaths from urothelial cancer occur in North and South Dakota, rural states with extensive low rank lignite deposits. Additional geologic, geochemical, and epidemiological work is needed to explore this potential environmental–medical problem.

Using an ingenious technique (excitation-emission matrix fluorescence spectroscopy; EEM), Goldberg et al. (*40*) investigated the fluorophoric activity of organic matter in water samples collected from wells in endemic and nonendemic regions in the former Yugoslavia and Bulgaria. Results were presented as fluorescence excitation/emission matrix spectral plots, providing gross chemical characterization of the organic matter present in the water samples. Water samples from BEN households in endemic villages had distinctive EEM matrix spectra, different from those of water samples from nonendemic villages. For example, water samples from endemic villages had distinctive teardrop-shaped EEM matrix spectra, with excitation peaks at 250 nm and 350 nm and an emission peak at 400 nm. In contrast, water samples from the nonendemic areas had EEM matrix spectra that lacked the teardrop shape and the 250-nm excitation maximum characteristic of endemic villages, and had little fluorophoric activity in general. The EEM matrix spectra of well water samples from endemic villages but from households not affected by BEN had characteristics similar to those of BEN-afflicted households, but fluorophoric activity in the non-BEN households was considerably lower (Fig. 5). These results showed significant differences between the dissolved organic constituents in well water from endemic and nonendemic areas of the Balkans, with endemic areas exhibiting far greater fluorophoric activity. Also, well water from BEN households appeared to have higher concentrations of these fluorophoric compounds compared to non-BEN households from endemic villages. Based on these data, one could hypothesize that toxic dissolved organic compounds present in the well water contribute to the onset and progression of BEN. Well water from households from nonendemic villages appeared to lack the specific types of organic fluorophoric compounds observed in endemic villages, and water samples from non-BEN households from endemic villages had these fluorophoric compounds at much lower concentrations compared to BEN households. The specific nature of the fluorophoric dissolved organic compounds present in the well water samples from endemic villages is beyond the scope of this study, but aromatic compounds of less than 600 daltons molecular weight are consistent with the data.

Additional work was performed on the Pliocene lignite hypothesis in 1993 (Orem et al., unpublished data) to examine 1) the chemical structure of Pliocene lignites from the endemic areas in Yugoslavia; 2) to conduct laboratory leaching experiments on these coals; and 3) to determine the concentrations of aromatic compounds in well water from endemic villages and a nonendemic (control) village. Analysis of the Pliocene lignites from endemic areas in the former Yugoslavia using ^{13}C nuclear magnetic resonance (NMR) spectroscopy showed that these coals are of very low rank (highly unaltered) and retain a very high degree of organic functionality (e.g., methoxyl, phenolic, and O-bonded aliphatic hydrocarbons), as shown in Figure 6. The high degree of organic oxygen functionality probably imparts a high degree of aqueous solubility to these coals compared to more altered coal. This high degree of water solubility was also observed in leaching experiments carried out on the coal samples. Pliocene lignites from the endemic areas were leached with distilled water in the laboratory to simulate groundwater leaching. The resulting aqueous extract had a distinctive yellow tint, indicating the presence of dissolved organic matter. The yield of dissolved organic matter from the leaching experiments was 100–500 mg from 30 g of starting coal. The ^{13}C NMR spectra of the dissolved organic matter from the leaching experiments are shown in Figure 7. The spectra show a large peak in the 110–150 ppm region, suggesting a high degree of condensed aromatic character in the dissolved organic matter leachate. This is consistent with the presence of polycyclic aromatic structures. The leachates also contain aliphatic hydrocarbon structures (peak

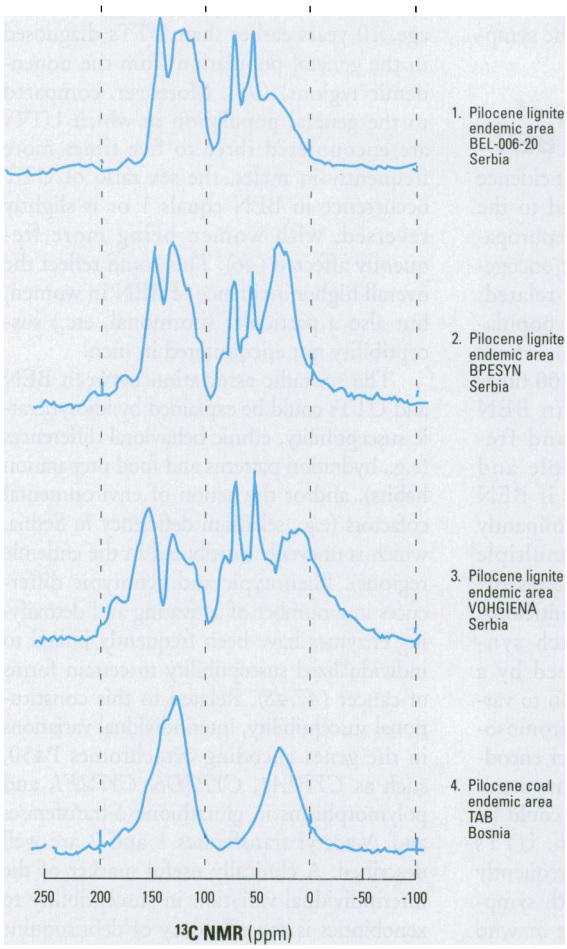

Figure 6. Solid state ^{13}C nuclear magnetic resonance (NMR) spectra of Pliocene coal samples from endemic areas of the former Yugoslavia; 1, 2, and 3 are lignites, while 4 is a higher rank coal. The lignite samples have a high degree of organic functionality and aromatic structure, as indicated by large peaks for methoxyl (56 ppm), O-bonded aliphatic moieties (72 ppm), and phenolic (150–160 ppm) and aromatic hydrocarbons (110–150 ppm).

at 0–50 ppm in the spectra) and a high degree of carboxyl (organic acid) functionality (peak at 175 ppm).

Well water samples from two endemic villages and one nonendemic (control) village in Yugoslavia were analyzed for the presence of dissolved aromatic organic matter using gas chromatography/mass spectrometry (GC/MS). A total of 14 compounds were identified in the samples (Table 1), with concentrations in the low microgram per liter range. Compounds 4, 7, 10, 13, and 14 in Table 1 are phthalates and probably do not arise from a natural source. Phthalates are often derived from plastics and may be an artifact of the sample processing steps. Alternatively, the phthalates could be from plastics accidentally dropped into the wells. All other compounds in Table 1 (except phenol) are PAHs. Many PAHs are known or suspected carcinogens. PAHs are produced during coalification as aromatic substances in woody tissue from plants (notably lignin) are defunctionalized and condensed by heating and pressure after deep burial in sediments. The presence of PAHs in well water suggests a possible source from coal (e.g., the local Pliocene lignite deposits); however, other sources, including anthropogenic ones, are also possible. Concentrations of all of the compounds in Table 1 were higher in well water from the endemic villages. Comparing the concentrations of PAHs in well water from the hyperendemic Petka village to the control village, the Petka village had 10 times higher fluoranthene, 6 times higher phenanthrene and pyrene, 5 times higher anthracene, and 2–3 times higher concentrations of all other PAHs. The higher concentrations of PAHs in the endemic Petka village may be linked to the high incidence of urothelial cancer associated with BEN. Potential nephrotoxic agents such as aminophenols or aromatic amines were not analyzed in this study, although previous work has indicated the presence of aromatic amines in well water from endemic villages (*29*). More additional work including analyses of more well water samples and screening for more potentially carcinogenic and nephrotoxic organic substances is needed to validate the Pliocene lignite role in BEN etiology.

The link between the low-rank coals and BEN etiology is also reinforced by the detection of phenolic compounds in the drinking water from wells in several Romanian endemic villages and by animal experiments performed in the 1970s in Romania. Bordas et al. (*42*) fed endemic region water extracts to rats and mice for 6 months to 1 year; they observed renal lesions that were not found in the control group. Preliminary investigations of the geological environment of the Romanian BEN locations also revealed the proximity of endemic villages to coal deposits. Hence, endemic villages near the Motru, Jiu, and Cerna Rivers adjoin the massive Pliocene Oltenian lignite deposits, while the villages south of the Carasul River are in proximity with smaller Pliocene lignite deposits from the Carasova and Oravita basins (*43*). Field investigations in several endemic villages in Romania also showed that the presence of only one or a few well water supplies used by all or almost all of the village is apparently associated with a hyperendemic character of BEN. That is, a higher incidence of the disease and more families (living in different households) were affected compared to the normoendemic or hypoendemic regions.

Other studies conducted in the 1960s in Yugoslavia also support a hydrogeological origin of the disease. Field studies performed in a village (Glogovac) located at the border between the endemic and the nonendemic regions in Bosnia revealed a striking association between BEN incidence, mortality due to BEN, and altitude (*44*). Glogovac is made up of two parts: Lower Glogovac (an average 100 m above sea level) and Upper Glogovac (an average 115 m above sea level). While there is no difference between the two parts in population origin, housing conditions, diet, customs, occupations, or economical, social, or ecological conditions, BEN occurs more frequently, and more households are affected in the Lower Glogovac than in the Higher Glogovac. The only notable differences between the two parts are altitude (10–25 m) and the depths of the wells used as a water supply. In Lower Glogovac, the depth of the wells is 5–15 m, whereas wells in Upper Glogovac are 20 m or deeper. No cases of nephropathy have occurred in families using water from wells deeper than 25 m (*44*). Although the hydrology of the region is not very rigorously characterized, Gaon (*44*) hypothesized that the wells of Upper Glogovac derive water mainly from the mountains, whereas those from Lower Glogovac derive water primarily from the River Drina.

The Pliocene lignite theory for BEN etiology was also used in a predictive mode. A previously unknown endemic area in southeastern Serbia (Kosovo region) was described based on the proximity of the area to a previously identified Pliocene coal field (coal deposit #19 in Figure 3) (*5,29*).

A summary of the findings that sustain and disprove the implication of the Pliocene lignites in BEN and the future research needed to reinforce the theory is presented in Table 2.

As long as the BEN etiological agent(s) and its origins remain unproved, it is hard to predict if the exposure to the nephrotoxicant is continuous or discontinuous. However, irrespective of the continuity pattern, there seems to be an aggravation of the disease symptoms and an increased mortality from BEN in certain seasons, usually following a prolonged rainy period. Both the mycotoxin (ochratoxin) and the Pliocene lignite hypotheses can explain this seasonal variation, the former by an increased growth of fungi and OTA production in

foodstuffs in the humid, high water activity periods, and the latter by an accelerated weathering of the coal deposits during the rainy season, increasing the concentration of the weathered compounds into the ground and well water. An acute exposure to higher concentrations of the nephrotoxic molecules will exhaust the functional reserve of the kidney, causing the disease to evolve from the asymptomatic to the symptomatic phase of the renal failure.

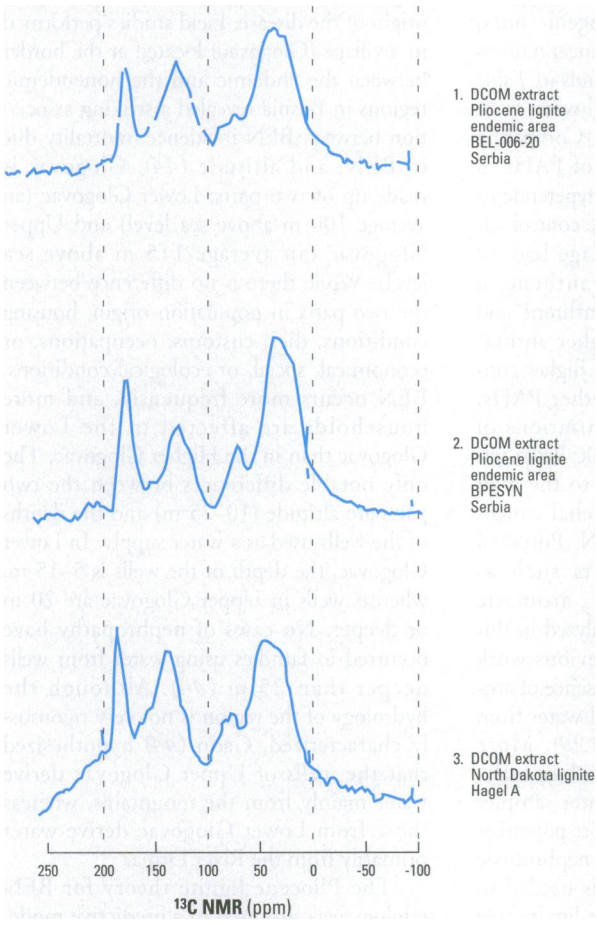

Figure 7. Solid state ^{13}C nuclear magnetic resonance (NMR) spectra of distilled water leachates of two Pliocene lignites from endemic areas of the former Yugoslavia and a North Dakota lignite. The leachates are characterized by a high degree of aliphatic hydrocarbon (0–50 ppm) and aromatic (110–150 ppm) and carboxyl (175 ppm) structures.

The Mechanistic Etiology of the Urothelial Tumors in BEN

The initial description of a high incidence of UTT in BEN patients soon led to the suspicion that the nephropathy and urinary tract oncogenesis were causally related. Rare in the general population (~1/185,000), these tumors have a 100–200 times higher incidence in BEN endemic regions and frequently are multiple and bilateral (*1*). Even if BEN tumors occur predominantly bilaterally and in multiple loci, this does not necessarily mean they have a multiclonal origin. As in Lynch syndrome (characterized by a familial predisposition to various cancers and chromosomal anomalies in loci encoding DNA repair enzymes), the tumors in BEN could be oligoclonal in origin. UTTs are described most frequently in BEN patients with symptomatic renal failure or who were on dialysis, although they can also occur in the asymptomatic stages of the nephropathy.

Not all BEN patients develop UTTs. In Bulgaria and the former Yugoslavia, where the association of BEN with urothelial carcinogenesis was rigorously documented, UTTs were described in only 40% of the BEN cases, and most were diagnosed in patients over 50–60 years of age (on average, 10 years earlier than UTTs diagnosed in the general population from the nonendemic regions) (*45*). Moreover, compared to the general population in which UTTs are encountered three to five times more frequently in males, the sex ratio of UTT occurrence in BEN equals 1 or is slightly reversed, with women being more frequently affected (*46*). This could reflect the overall higher incidence of BEN in women, but also a particular (hormonal, etc.) susceptibility not encountered in men.

The sporadic association between BEN and UTTs could be explained by idiosyncratic susceptibility, ethnic behavioral differences (e.g., hydration patterns and food preparation habits), and/or the action of environmental cofactors (e.g., selenium deficiency in Serbia, which is unevenly distributed in the endemic regions). Phenotypic and genotypic differences in a number of activating and detoxifying enzymes have been frequently linked to individualized susceptibility to certain forms of cancer (*47,48*). Related to this constitutional susceptibility, interindividual variations in the genes encoding cytochromes P450, such as *CYP1A1*, *CYP2D6*, *CYP2E1*, and polymorphisms in glutathione *S*-transferases and *N*-acetyl transferases 1 and 2 are well described. A clinically useful marker of the interindividual variation in susceptibility to xenobiotics is the efficiency of debrisoquine metabolism. Administered in healthy individuals, debrisoquine is converted into a 4-hydroxy derivative by debrisoquine hydroxylase (CYP2D6). Polymorphisms in *CYP2D6* genes resulting in impaired debrisoquine metabolism occur in less than 10% of the Caucasian populations and have been associated with variable susceptibility to chemical carcinogenesis (bladder cancer, leukemias, and possibly melanomas), pesticide-induced Parkinson disease, and iatrogenic reactions to drugs (*49*). The association between the efficiency of oxidative metabolism of debrisoquine and the risk for developing BEN and/or UTT was examined in Bulgaria (*50*). This study included individuals with BEN and/or UTT (both categories from the endemic region), individuals suspected of having BEN, healthy controls from the endemic regions, and healthy controls from nonendemic regions. The extensive metabolizer phenotype was encountered in most of the BEN cases and subjects with BEN and UTT. The mean metabolic ratio (unmodified debrisoquine:hydroxylated debrisoquine) decreased progressively as follows: controls from nonendemic regions > controls from endemic villages > suspected BEN patients > BEN patients. The following conclusion can be drawn from this study: whatever the etiological factor is, it will induce BEN and/or

Table 1. Concentrations of aromatic organic compounds in extracts of well water from two endemic villages and a nonendemic control village in Serbia

Compound	Endemic village-Nis Concentration (µg/l)	Endemic village-Petka Concentration (µg/l)	Control village-near Petka Concentration (µg/l)
1. Phenol	0.43	0.42	0.15
2. Naphthalene	0.40	0.49	0.20
3. Acenaphthylene	0.05	0.06	0.03
4. Dimethylphthalate	0.16	0.14	0.08
5. Acenaphthene	0.08	0.10	0.05
6. Fluorene	0.24	0.28	0.12
7. Diethylphthalate	2.24	1.60	0.82
8. Phenanthrene	0.76	2.53	0.43
9. Anthracene	0.07	0.32	0.06
10. Di-*n*-butylphthalate	39.26	39.76	17.45
11. Fluoranthene	0.13	0.99	0.09
12. Pyrene	0.12	0.68	0.11
13. Butylbenzylphthalate	10.56	8.47	4.21
14. Bis(2-ethylhexyl)phthalate	973.83	1,191.55	672.26

UTT only after metabolical activation in susceptible individuals (i.e., extensive debrisoquine metabolizers). Moreover, the development of UTTs in less than half of the BEN patients may reflect differences in type or degree of enzymatic efficiency in these subjects (*50*). It is not known, however, if the supposed BEN causing toxicant(s) is activated specifically in the kidney, or if it is first metabolized to the active form in the liver and subsequently transported to the kidney where it induces the progressive tissue damage. On the other hand, experiments in animals showed that the nephrotoxicity and carcinogenicity of OTA is not altered significantly by metabolism. One of the major metabolites that preserves almost the full toxicity of OTA is 4(*R*)-hydroxyochratoxin A, generated by the mixed function oxidase system (*51*). This suggests, once again, that it is unlikely that OTA is the (sole) causative factor for BEN and UTT. Ochratoxin A also seems to be metabolized by a different hydroxylase than for debrisoquine, as only the former is inducible by methylcholanthrene or phenobarbital (*51*). Nevertheless, in this case, interspecies metabolization and enzymatic differences make extrapolation from laboratory animals to humans difficult. It cannot be ruled out that the suspected aromatic compounds from the endemic drinking water, besides being nephrotoxic, contribute additionally to the disease etiopathogenicity by inducing enzymes that alter OTA and extend in this way its pattern of toxicity.

The higher incidence of UTTs, with no increase in the incidence of bladder cancer, in non-BEN interstitial nephropathies (e.g., analgesic nephropathy, interstitial nephropathy induced by renal lithiasis) contradicted the carcinogenicity of BEN agent(s), suggesting in the extreme case that BEN causing factor(s) has no direct carcinogenic activity at all (*52*). The occurrence of UTTs only in the advanced stages of BEN (frequently after renal failure is diagnosed and renal dialysis is performed), however, could be the result of a weak oncogenic agent. While no tumorigenic effect is observed in the earlier phases of BEN, when kidney morphology is still preserved, the alteration of the pyelo- and ureterolymphatic drainage system following the kidney destruction leads to stasis and accumulation of the nephrotoxic factor(s) in the urothelium, and a (pro)carcinogenic threshold is reached with subsequent induction/promotion of tumorigenesis.

The etiology of BEN-linked urinary tumors is still speculative, as is the cause of the background disease. However, no other known human disease, with the possible exception of analgesic nephropathy, is so frequently associated with ureteral and renal pelvis carcinomas. The molecular description of BEN-linked tumorigenesis and the influence of a familial or individual susceptibility to UTT could offer an important arena of research for molecular and environmentally induced carcinogenesis.

Other Environmental Factors

Aristolochic Acid

An interesting association has been made between Chinese herb nephropathy (CHN) and BEN (*53*). The incriminated agent in this case is aristolochic acid, a known nephrotoxic and carcinogenic compound synthesized in various portions of *Aristolochia* plants. The nephropathy was described in several hundred young women in Belgium and, more recently, in Japan (*54*) who followed a slimming diet (accidentally containing plant parts of *Aristolochia*, instead of *Stephania tetrandra*) for several years and a cure for atopic dermatitis, respectively. CHN resembles BEN in many aspects: clinical-normal blood pressure; aseptic leukocyturia; low grade, low molecular weight proteinuria; early and severe anemia and morphopathological-extensive hypocellular interstitial sclerosis; tubular atrophy and global sclerosis of

Table 2. Summary of the main findings that support the role of the low-rank coals (Pliocene lignites) and the hydrogeological factor in Balkan endemic nephropathy/upper urinary tract tumor (BEN/UTT) etiology, and disagree with the hypothesis and the additional research needed to confirm the hypothesis

PROs	Earlier research (1970s) showed the presence of phenolic compounds in drinking well water from endemic regions in Romania
	Glogovac was described as a model endemic village composed of two parts with similar ethnical groups, socioeconomic status, and hydric and food preparation habits, but different in the incidence of BEN/UTT and probably in the hydrogeological conditions and well water chemistry (*44*)
	Hyperendemicity of BEN is apparently linked to the use of a single or a few well water supplies by an entire village
	Semiquantitative detection in water samples from endemic regions in Yugoslavia revealed the presence of PAHs and aromatic amines not present in the water samples from the nonendemic regions (*29*)
	Characteristic excitation/emission spectra were identified in water samples from endemic regions in Yugoslavia and Bulgaria (*40*)
	PAHs were detected by GC/MS in higher concentrations in water samples from endemic regions than in water samples from nonendemic regions (Orem et al., unpublished observations)
	Laboratory leaching experiments showed the highly unaltered character of the Pliocene lignites proximal to the endemic regions in Yugoslavia and their potential to release PAHs and aromatic compounds with functional groups that can be nephrotoxic or carcinogenic (Orem et al., unpublished data)
	The Pliocene lignite theory explains peculiar features of the disease, such as the geographic confinement and the seasonal variation in prevalence and death from BEN; it also fits epidemiological data, e.g., the fact that BEN is encountered only in rural areas that depend entirely or almost entirely on wells for drinking water
	The Pliocene lignite theory has predictive power: a previously unknown BEN region was identified based on the proximity to a Pliocene coal field (*5,29*)
CONs	Pliocene lignites also occur in regions outside the Balkans, although their degree of alteration and their potential to release nephrotoxic/carcinogenic compounds in the ground water may be different from the lignites associated with the BEN endemic areas
	The currently identified PAHs and aromatic compounds in the well drinking water from the endemic areas are not solely sufficient to explain the etiology and pathogeny of BEN/UTT
Future prospects	Geochemical analysis of lignites from Romania and Bulgaria topographically linked to the BEN endemic areas may be performed
	Compounds likely to be nephrotoxic/carcinogenic, such as aminophenols, aromatic amines, or other unknown molecules potentially leaching from the Pliocene lignites in the well water from the endemic regions, may be identified
	Biomarkers of PAH exposure may be assessed, e.g., PAH–DNA adducts in blood cell DNA samples and urinary 1-hydroxypyrene levels in patients with BEN/UTT
	An animal model for BEN might be developed by feeding laboratory animals compounds isolated from endemic well water and/or from Pliocene lignite leachates

Abbreviations: PAHs, polycyclic aromatic hydrocarbons; GC/MS, gas chromatography/mass spectrometry.

glomeruli; and mild to moderate atypia or atypical hyperplasia of the urothelium (*53*). In two cases of CHN, UTTs were described, providing another link to BEN.

The aristolochic acid hypothesis nevertheless fails to explain one of the defining features of BEN, i.e., the geographical confinement. *Aristolochia* plants are widely cultivated and are probably native in many places in Europe, Asia, and North America, places where no cases of BEN or a BEN-like disease have been documented. Also, even though many herbal remedies are used locally in the BEN areas, it is unlikely that *Aristolochia* leaves, seeds, or roots are ingested on such a widespread scale in the endemic locations (*3*). The pungent repulsive odor of the plant makes it equally unlikely to be accidentally ingested, even on an endemic scale. In addition, the pelviureteric sclerosis found in one of the biopsied cases of Chinese herb nephropathy reminds more of analgesic nephropathy than BEN.

Viruses

A viral etiology of BEN was hypothesized based on the endemic character of the disease, the seasonal variation in the incidence and deaths from BEN, and on the discovery of viral particles in the kidneys of some afflicted persons (*55*). The high frequency of urothelial tumors in BEN patients may also be explained by infection with slow oncogenic viruses. Several viruses have been considered as possible agents: the West Nile virus, papilloma viruses (BK and SV40) (*56,57*), coronaviruses (*20*), and arenaviruses (*58*). An extensive search for these viruses in BEN patients has been unsuccessful so far. For example, the West Nile virus (a mosquito-borne flavivirus that caused more than 300 cases of meningoencephalitis in the Danube flood lands, including Bucharest, Romania, in 1996), is probably not involved in BEN because only a few of the investigated BEN patients exhibited antiviral antibodies. The same is true for the BK and SV40 viruses. Although high titers of anti-BK and -SV40 antibodies were found in some of the BEN patients, such antibodies are also frequently encountered in the general healthy population. Coronaviruses transmitted from pigs in the endemic households were suggested as a possible cause for BEN (*20*). However, evidence supporting this hypothesis is lacking. More likely candidates for a viral etiology are rodent-borne viruses such as those responsible for hemorrhagic fever (HF) syndromes. Some of these viruses (Puumala, Hantaan, and Dobrava in the Balkans) can cause more or less severe HF with renal syndrome in rural or urban areas. The classic Hantaan disease in rural China (epidemic HF) and in Korea (Korean HF) has a seasonal pattern of incidence, being most common in spring or fall, possibly in relation to agricultural practices and rodent density (*59*). Infection in humans is acquired through aerosols of rodent urine; the disease is not transmitted from human to human. Some of the features of Hantaan HF are seen in BEN—disease endemicity, incidence in rural settlements, and seasonal variation—but BEN is primarily a chronic kidney disease without any acute episode in its evolution. In contrast, the Hantaan infection usually evolves with acute renal failure. There is an analogy worth mentioning between BEN and the Bolivian hemorrhagic fever, an arenaviral HF disease spread by rodents (*58*). As discussed by Radovanovic (*58*), both diseases are characterized by similarities in the topography of the disease area (flat plains, in river or stream valleys), clustering only in certain settlements and households, lack of evidence of human-to-human transmission, and the first recognition of both diseases after the beginning of malaria control programs using DDT after World War II. Although the evidence is speculative, Radovanovic (*58*) postulates that residual DDT sprayed in the Balkans in the mid-1940s led to the reduction in the number of cats, the most important natural barrier against rodents in the endemic (and nonendemic) households. As a result, a decade later, the rodent reservoir expanded and BEN emerged and was described for the first time as a disease entity.

Although improbable based on the previously mentioned findings, a viral etiology for BEN cannot be completely rejected until more comprehensive studies are available.

Discussion

BEN in the Context of Geomedicine

In a broader framework, BEN and its associated UTTs would not be unique in having a hydrogeological etiology. For instance, the field of geomedicine also records a possible correlation between digestive cancer mortality rates and the consumption of water from coal bearing strata (*60*). From the BEN and UTT perspective, an intriguing observation is that the top five states in the United States in average annual age-adjusted cancer mortality rates from kidney and renal pelvis cancer (1989–1993) are Alaska, Maine, North Dakota, South Dakota, and Minnesota. North and South Dakota have extensive lignite deposits and a largely rural population dependent on wells as a water source. Alaska, Maine, and Minnesota have extensive peat deposits (coal precursors) and also considerable rural populations. Coal is also considered to be an environmental component in the multicausal etiology of other common diseases besides cancer. An increased rate of heart attacks is encountered in Ohio, correlated with the consumption of drinking water from coal-bearing strata (*61*). A significant negative correlation has also been observed between cardiovascular mortality (CVM) rates and the hardness and calcium concentration of potable ground water. Apparently, a harder water and a higher concentration of dissolved calcium salts (from limestone and dolomite) are positively correlated with increased longevity and lower CVM in the northern–midcontinental United States, compared to decreased longevity and higher rates of cardiovascular deaths in the southeastern coastal area where drinking water is softer and Ca^{2+} concentrations are lower (*62*).

The etiology of CIN is variously reported to be secondary to metabolic diseases, drugs (i.e., analgesics or analgesic mixtures), or known environmental factors (e.g., industrial pollution with lead, cadmium, mercury, etc.). About 15–20% of the patients in end stage renal disease (ESRD) treatment centers who have been diagnosed with CIN have no established etiology. Without a known etiology, prevention of disease is an elusive goal. Studies that will yield information on the causes of CIN could have an obvious immediate prophylactic effect and allow a reduction in the costs of kidney transplantation and dialysis, the current final therapeutical solutions for ESRD patients. In this respect, identifying the factor(s) responsible for BEN will lead to a deeper understanding of the etiopathogeny of this disease and to a better knowledge of kidney disease mechanisms and carcinogenesis in general. Unraveling the etiology of BEN will also allow appropriate preventive measures to be taken in the afflicted areas, such as by treatment or filtration of the drinking and cooking water or changing the main water supply.

BEN Etiology as a Conceptual Model for the Genesis of Environmental Diseases

Every human disease has a multifactorial causality. Usually there is a main factor whose *sine qua non* action is sufficient for triggering a pathogenic process that individualizes the disease. Other factors without direct and feasibly quantifiable effects in disease genesis and evolution are frequently neglected, although in their absence the disease pattern and progression should be different.

What is involved and what is not in BEN etiology are questions equally difficult to answer, and some of the reasons for this are stated above. Similar problems are evident when the long-term and sometimes combined effects of twentieth century environmental pollutants, including pesticides

and the largely debated endocrine disruptors, are assessed and straightforward answers are expected. Due to the synergistic (or antagonistic) and not infrequently transgenerational action of a multitude of factors (some even unimagined), a cause–effect relationship is not always obvious. Causative agents interplay with predisposing or constitutional factors (cofactors or risk factors) toward a complex disease phenotype.

Defined initially as a lead nephropathy, subsequently considered a viral disease, a genetic or chromosomal disorder, or a form of chronic pyelonephritis, BEN is presently thought of by most researchers as an environmental disease. Although the environmental etiology is widely accepted, the true factor(s) causing the disease is unclear. Multidisciplinary approaches carried on in various countries have excluded some of the factors claimed to cause BEN. These studies, however, have also outlined some factors that cannot be disregarded when considering BEN etiopathogeny, as they can comfortably explain certain features of the disease (e.g., familial aggregation, geographic confinement, association with uroepithelial tumors, etc.). BEN, from these standpoints, may offer a conceptual and mechanistical model for the complex and integrated topics pertinent to the study of environmental diseases.

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