ENVIRONMENT, GEOCHEMISTRY AND THE ETIOLOGY OF BALKAN ENDEMIC NEPHROPATHY: LESSONS FROM ROMANIA

William H. Orem1, Calin A. Tatu2*, Gerald L. Feder3, Robert B. Finkelman1, Harry E. Lerch1, Susan V.M. Maharaj4, Diana Szilagyi2, Victor Dumitrascu2, Virgil Paunescu2, Florin Margineanu5

1US Geological Survey, 12201 Sunrise Valley Drive, MS 956, Reston, VA 20192, U.S.A.
2Department of Immunology, Clinical Laboratory No.1, Pta. E. Murgu No.2, RO-1900 Timisoara, Romania
3Florida Community College at Jacksonville, Jacksonville, FL 32256, U.S.A.
4Department of Environmental & Toxicologic Pathology, Armed Forces Institute of Pathology, 14th and Alaska Ave. N.W., Washington, D.C. 20306-6000, U.S.A
5Center of Hemodialysis, County Hospital, 82 Unirii Str., RO-1500, Drobeta Turnu Severin, Romania

* E-mail: cta@med.unc.edu

Summary. Balkan endemic nephropathy (BEN) is a fatal chronic kidney disease of unknown etiology geographically restricted to several countries of the Balkan Peninsula and described for the first time almost 50 years ago. Along time, many factors have been proposed as etiological agents for BEN (including viruses, bacteria, mycotoxins, industrial pollution, radioactive compounds, etc.) but none of these have been confirmed. However, in the recent years, based on field and laboratory investigations, an environmental etiology of the disease has become more widely accepted, with a prime role played by the geological background of the endemic settlements. In this regard, the most incriminated are toxic organic compounds present in the drinking water, supposed to be leached by the groundwater from low rank Pliocene lignite deposits adjacent to the endemic villages and transported into shallow household wells or springs.

We describe in our study several inorganic and organic geochemical features of water samples collected from the endemic villages, in comparison with water samples from nonendemic locations from Romania. Water samples were analyzed on site and in the laboratory using standard inorganic and organic geochemistry methods. Methanol and aqueous extracts of Pliocene lignite collected from the endemic areas were also analyzed by GC/MS.

While no significant difference could be found in the inorganic parameters (i.e., pH, TDS, anions, alkalinity, oxygen, nitrates, etc.) of the endemic vs. nonendemic water samples, the organic content, as revealed by GC/MS, is more complex and much more higher in the endemic vs. the nonendemic water samples. Also, the Pliocene lignite from the endemic areas seems to have a particular geochemical composition, with many potentially nephrotoxic/carcinogenic aromatic and nonaromatic molecules, leachable into organic solvents as well as into water.

Some of the molecules extracted from the endemic area Pliocene lignite are apparently present in the endemic water samples, suggesting their origin in coal and sustaining the Pliocene lignite hypothesis for BEN etiology.

Key words: Balkan endemic nephropathy, medical geology, geomedicine, Pliocene lignite, GC/MS

Introduction

Balkan endemic nephropathy (BEN) is an irreversible, chronic, tubulo-interstitial nephropathy of unknown origin, geographically confined to several rural regions of the Balkan Peninsula. The first "official" observation of a disease resembling BEN was made almost six decades ago in regions comprising the former Yugoslavia (1). Shortly thereafter, similar descriptions came from Bulgaria and Romania (2-4). The distribution of BEN includes regions of central and southeastern Serbia, southwestern Romania, northwestern Bulgaria, southeastern Croatia, and parts of Bosnia and Kosovo (fig. 1). The geological setting of the endemic areas in the affected countries is similar. Most of the BEN villages are located on alluvial plains of tributaries of the Danube River, with older Tertiary layers (clays, sands and Pliocene lignites) covered by Quaternary river sediments. It is estimated that several thousand people in the affected countries are currently suffering from BEN and that thousands more will be diagnosed with BEN in the coming years.

Although BEN may have existed for centuries in the affected areas, it was acknowledged to the medical community only in the 1950's when the first epidemic outbreaks were reported. Prior to World War II, average life expectancy in the endemic areas was < 45 years, with major causes of short life expectancy being parasitic or infectious diseases like malaria and tuberculosis. As BEN becomes manifest at older ages (40-50 years),
it is possible that the shorter life expectancy in the endemic areas before the war did not allow for a "critical mass" of diseased people to accumulate, and an epidemic kidney disease to be noted. After the war, improvements in sanitation in the affected countries (including anti-malaria and anti-TB measures) coupled with better diagnostic procedures in nephrology made possible the description of BEN, as a distinct nosological entity. BEN was almost simultaneously recognized in all affected countries (in Bulgaria, Tanchev and others in 1956 as reported in (3); in Yugoslavia, Danilovic et al. in 1957 (1); and in Romania, Fortza and Negoescu in 1961 (6)), and the endemic regions were relatively clearly delimited. The geographic distribution of BEN has not changed significantly since the first descriptions, and villages afflicted in the past continue to be afflicted, while nonendemic villages (sometimes located only a few km from endemic villages) have remained free of the disease (7,8). Reports mentioning a kidney disease resembling BEN in the Balkans have been traced back to the early XXth century. For instance, German doctors documented a large number of deaths from uremia in Serbia in 1915, while in the village of Bistretz (Bulgaria) a disease with the clinical features of BEN was reported in 1910. In Romania, a possible link exists between BEN and familial kidney atrophy observed here in 1905 (Susa, 1976, cited in (2)) raising the possibility of BEN having a genetic component.

Some of the medical and epidemiological features of BEN are: focal occurrence of the clinical cases; family aggregation without an obvious pattern of mendelian genetic inheritance; long subclinical "incubation" period with a rapid onset of end-stage renal disease (ESRD); at least 10-20 years residence in one or more of the endemic villages; occurrence only in adults (age 30 to 50 being most heavily affected, while almost no children or individuals above 70 develop the disease); a slight sex distribution (female: male ~1.5:1); a similar incidence in different ethnic and religious groups; restriction to a rural, farming population; presence of high blood pressure in only 20% of the affected people; normochromic and normocytic anemia; a very high frequency of upper urinary tract tumors (UTT) in BEN patients. Most epidemiological studies have revealed no obvious differences in the nutritional habits or other significant differences between the endemic and nonendemic households in the same village.

BEN is a slow-evolving disease, characterized by irreversible progression to renal failure and terminal uremia. Once end-stage renal disease is diagnosed in BEN patients, the therapeutic approaches are only symptomatic. As renal transplantation is not a feasible option for most of the BEN patients, hemodialysis is the only approach left. However, kidney transplantation has been attempted in a few people with BEN and no recurrence of the disease has been noted on the transplanted kidneys. Due to adverse economic conditions of the countries affected by BEN, kidney transplantation is not a viable option for the present and near future. The direct consequence of this situation is a poor quality of life for BEN patients in the ESRD phase.

Due to its medical and economical consequences, BEN has been a major research priority through the years, involving multidisciplinary approaches and varied scientific disciplines (e.g., environmental medicine, microbiology, oncology, radiation biology, epidemiology, cytogenetics, geology, hydrogeology, biogeochemistry) and several international symposia have been dedicated to its medical aspects and origins. Despite these research efforts by regional and international scientific groups, BEN is still a medical enigma in many respects, and its etiology a matter of contradiction and controversy. Many factors have been proposed as etiological agents for BEN, including: bacteria and viruses, heavy metals, radioactive compounds, trace element imbalances in the soil, chromosomal aberrations, mycotoxins (ochratoxin A), plant toxins (aristolochic acid), and industrial pollution (for a review see (9)). Rural villagers in the endemic areas often invoke astrological influences to explain the occurrence of BEN.

In recent years, field and laboratory investigations have supported an environmental etiology for the disease, with a prime role played by the geological background of the endemic settlements (9-11). In this regard, there is a growing body of evidence suggesting the involvement of toxic organic compounds present in the drinking water of the endemic areas. These compounds are hypothesized to be leached by groundwater from low rank Pliocene lignite deposits topographically linked to the endemic villages, and transported into shallow household wells or village springs (10,12,13). The population of the villages in the endemic areas uses well/spring water almost exclusively for drinking and cooking, and is therefore potentially exposed to any toxic organic compounds found in the water. The presumably low levels of toxic organic compounds present would likely favor relatively slow development of the disease and over a time interval of 10 to 30 years or
more. The frequent association of BEN with upper urinary tract (urothelial) tumors suggests the action not only of a nephrotoxic, but also carcinogenic, factor.

Several inorganic and organic geochemical parameters of water and coal samples collected from a Romanian BEN endemic area were assessed in our study, in an attempt to find additional links between the environment and BEN etiology.

Methods

Field investigations and water and coal samples collection

Location survey

We performed two field trips in March and August 2000 in the main Romanian BEN endemic area (Drobeta Turnu Severin), located in SW Romania. Ten (10) endemic locations and seven (7) non-endemic sites in the area were visited on these occasions. We also visited three Pliocene lignite mines (Husnicioara, Pietris, and Livezile), located in the BEN endemic territory. Pliocene coal was sampled from each coal mine and water samples were also obtained from springs found at Husnicioara and Pietris mines.

Villages for well water sampling were selected based on medical records showing their endemic character, which we obtained from the Drobeta Turnu Severin County Hospital. Well water and spring sites were classified as endemic (E) and non-endemic (NE). Endemic sites were defined as water sources used on a regular basis (at least 10-20 years) by a family or several families (if the water source is located outside the private household) with described BEN cases and/or family history of BEN. The non-endemic sites were defined as water sources used (for 10-20 years) by a family or families without any described BEN cases or family history of BEN.

Location, elevation above sea level, proximal presence or absence of coal deposits and/or coal mines were noted for each site. Observations of the surroundings were recorded and the general placement of the villages with respect to the adjacent forms of relief was also noted.

Water collection and analysis

Water samples were collected at each site for both inorganic and organics analyses. Standard "clean" procedures for both inorganic and organic sample collection were employed (14,15). For organics, water was collected in solvent-cleaned, amber, glass bottles (2 liters for each sample), after on-site filtration through pre-cleaned and pre-weight glass fiber filters. Methylene chloride (60 ml) was added to each bottle to retard bacterial activity during storage and transport to USGS laboratories in Reston, VA. Water samples were liquid/liquid extracted with 4 sequential volumes of 100 ml of methylene chloride to isolate the dissolved organic compounds of interest. The combined extract (460 ml) was then concentrated to a volume of 50 µl by rotovaporation and evaporation in nitrogen. 1 µl of each sample was subsequently used for GC/MS analysis. We previously attempted using solid phase extraction (SPE) columns to concentrate organic compounds from well water samples, however, this method proved untenable because of time constraints in the field and problems with contaminants bleeding from the C18 SPE cartridges interfering with GC/MS analysis. Despite the inconvenience of shipping large volumes of water between the Balkans and the USA, liquid/liquid extraction provided much more precise and accurate results for this study.

The glass fiber filters used in the filtration of the water samples were saved for analysis of particulate organic compounds. The filters were dried at room temperature and reweighed to estimate the particulate material content of the water samples. The filters were then extracted with sequential 100 ml volumes of GC/MS grade methylene chloride in a clean (baked at 450°C) beaker with sonication. The combined extract was then filtered through a new clean glass fiber filter, and the filtered extract processed in a manner identical to that used for the water extracts for GC and GC/MS analysis.

Water samples for inorganic geochemical studies were collected at each site in 60 ml plastic bottles. Sampling and analysis followed standard procedures for inorganics in water (16). Water samples for inorganics were filtered at the sampling site using 0.45 µm Nuclepore filters, and a plastic Millipore filtering apparatus. All plasticware used for the inorganic analyses was pre-cleaned with acid using: (1) a 10% HCl soak and distilled/deionized water rinse for anions, and (2) a 10% HNO3 soak, 1% ultrapure HNO3 soak, distilled/deionized water rinse for metals. Filtered water samples for metals analysis were stabilized with one drop of concentrated, ultrapure HNO3 for storage. Metals in the filtered, acidified water samples were quantitatively determined by inductively coupled plasma mass spectrometry (ICP-MS) (16). Filtered water samples for anion analysis were stored in clean, 60 ml plastic bottles at room temperature; anions were analyzed by ion chromatography (17), and by colorimetry for phosphate and ammonium (18) at USGS labs in Reston, VA. Dissolved oxygen, pH, conductivity, salinity, and total dissolved solids were determined on site in unfiltered well/spring water using portable meters and electrodes (18,19). Small water samples were collected in pre-cleaned bottles for the determination of sulfide and alkalinity at the end of each day using standard electrochemical methods (18).

Appropriate field and laboratory blanks were run to account for any operational or reagent contamination.

Coal analysis

Pliocene lignite samples were collected from the three coal mines mentioned earlier. Methanol extracts of the lignite were made, using approximately 5 g of coal and 5 ml of chromatographic purity methanol. The extraction was performed in clean glass tubes for two days. The extract was analyzed by GC/MS at the Clinical Laboratory No.1, Timisoara, Romania, using the
Gas Chromatography-Mass Spectrometry (GC/MS)

The concentrated extracts from both the dissolved and particulate organics in the well/spring water samples were analyzed first by GC with flame ionization detection (GC-FID), and then by GC/MS. The initial GC-FID analysis was used to screen the samples for complexity, and to determine if further separation by liquid chromatography (LC) was needed prior to GC/MS analysis. The GC-FID also provided initial quantification of peak area prior to identification of the organic component in the chromatogram. A Perkin Elmer (Boston, MA) model 8500 GC-FID with a 30 m × 250 µm × 0.25 µm J&W DB-5 column (95% dimethyl 5% diphenyl polysiloxane) was used for the analysis.

GC-FID conditions were as follows: 1.0 µl splitless injection, injector temperature of 150°C, detector temperature of 325°C, temperature program 70-325°C at 4°C/min and a final hold at 325°C for 20 min, and a column pressure of 13 psig. GC/MS was used for structural identification of the organic compounds and for additional quantification. A Hewlett Packard (Agilent Technologies, Palo Alto, CA, USA) 6890 series GC and a 5973 electron ionization (EI) mass selective detector (MSD) was used for GC/MS analysis. The GC machine was fitted with a 30 m × 250 µm × 0.25 µm HP-5MS column (95% dimethyl 5% diphenyl polysiloxane). The following conditions were used for the GC/MS run: 1.0 µl splitless injection, constant flow of 1.0 ml/min, solvent delay of 6.0 min, injector temperature of 150°C, interface at 300°C, temperature program of 70-300°C at 4°C/min and a hold at 300°C for 15 min, and mass scan from 50-550 Da. Individual organic compounds in the sample chromatograms were identified by comparison of mass spectral features to libraries of mass spectral data included in the GC/MS system (NIST98 and Wiley 7), by mass spectral identifications, and/or by comparison to standard compounds.

Previous experience showed that GC/MS is the best method for determining low molecular weight (up to 500-600 Da) organic compounds in the water. Only few of the compounds present in the chromatograms, however, are positively identified (>95% probability) using the available mass spectral libraries. This is not unusual in organic geochemical studies of environmental samples. Our attention was primarily focused on aromatic compounds and their functional derivatives: PAHs (anthracene, pyrene, benz[a]pyrene), aliphatics (alkanes, alkenes, and cyclics), and heterocyclic (especially O-, N- and S-containing) compounds.

Results

The setting of the endemic villages

All endemic sites visited during the 1999 and 2000 fieldtrips are located at elevations below 250 m, in valley bottoms or on plains, and are surrounded by smooth hills. There is usually a main drainage (river or stream) passing through the village that collects smaller drainages originating from the hills. These main drainages are tributaries of the Danube River. Field observations and discussions with villagers indicated that Pliocene lignite deposits exist in the surrounding hills, and that coal mines are in close proximity (less than 1 km) to several endemic villages (Pietris, Erghevitza, Livezile, Prunisor, Husnicioara) (fig. 2).

Inorganic analysis of water samples

Results from inorganic analysis of water samples collected from endemic and nonendemic sites in Romania in March and August 2000 are shown in Figures 3-5. No consistent or significant differences for anions, nutrients, alkalinity, sulfides, or the general inorganic parameters measured were observed between samples from the endemic and nonendemic sites. Phosphate was somewhat elevated at one of the endemic sites and ammonium was elevated at two of the endemic locations (fig. 4). The relevance of this is unclear, but the higher nutrient concentrations could be attributed to the usage of fertilizers in the area. Nitrate concentrations were elevated in most of the water samples, reaching values as high as 225 mg/l, and exceeding USEPA guidelines for drinking water at all sites (fig. 5). Since no relevant difference was observed between the endemic and nonendemic sites for nitrate, however, it cannot be directly linked to BEN etiology. It is possible though that nitrate is a factor contributing to disease causation in the presence of toxic organic compounds in the water samples. Nitrate can react with various functional groups found in organic molecules (e.g. amino-, hydroxy-, etc.), and could increase the toxicity and carcinogenicity of these compounds. High nitrate concentrations were also found in endemic and nonendemic water samples from Bulgaria (Dr. T. Voice -Michigan State University, personal communication), and likely originate from the inorganic and organic (manure) fertilizers used by the villagers. Concentrations of metals in the water samples were also generally similar at endemic and nonendemic sites, except for zinc, which appeared to be deficient at the endemic sites (data not shown).
Fig. 2. Map showing the distribution of the coal deposits in Romania. 1 and 2 are the two Romanian BEN areas, topographically linked to Pliocene lignite deposits.

Fig. 3. pH and the total dissolved solids (TDS) values for the endemic and the nonendemic water samples.

Fig. 4. The sulfate, phosphate and ammonium values for the water samples. As for the pH and the TDS, no significant difference could be found between the endemic and the nonendemic locations.
The nitrate concentration shows high values for both the endemic and the nonendemic water samples. Nitrate is also much higher in the wells, compared to spring water supplies.

Organic analysis of water and coal

In contrast to the inorganic geochemistry, dissolved organic constituents in the well and spring methanol samples collected from the main Romanian BEN endemic area contain a greater number of different compounds (aliphatic and aromatic), and in much higher abundance (>10x), compared to water samples from the nonendemic sites (fig. 6). Some of the compounds found in the endemic area water samples were also observed in the water extracts of the Romanian Pliocene lignites (see below), suggesting a possible connection between leachable organics from the coal and organics in the well water samples. The identification of the hundreds of individual compounds in the water samples analyzed by GC/MS is continuing. Preliminary assessment shows the presence of both aliphatic and aromatic compounds, rich in oxygen (hydroxy, phenol, methoxy, aldehyde, keto, carboxy, etc.) and nitrogen (amino) containing functional groups. All the samples, without exception, contained various phthalates, which are some of the most common man-made environmental contaminants. Pesticides were absent except for one nonendemic water sample where compounds from the atrazine family were found in µg/l concentration.

Compared to the water samples, the filter extracts were much "cleaner" in organic compounds, proving that most of the organic contaminants are found in the dissolved phase rather than in the particulate matter. No significant differences were found between the endemic and the nonendemic filter extracts.

Based on the hypothesis that water is the carrier of the BEN causing agent, we conducted laboratory water extraction of different coals as an experimental model for their natural bioavailability. We demonstrated in earlier work (20,21) that methanol extracts of Pliocene lignites from the endemic area have a geochemical signature distinct from that of higher rank coals, and are characterized by a much higher number and abundance of organic compounds from GC/MS total ion current plots. $^{13}$C-NMR experiments have provided similar results, showing that the endemic area Pliocene lignites are rich in aromatic and aliphatic components and high organic functionality (11). Methanol extracts of the Pliocene lignites from the endemic areas also appear to be distinct from methanol extracts of other lignites collected from areas in Romania free of BEN. Total ion currents chromatograms of three endemic zone lignites and one non-endemic area lignite of similar age, are shown in Figure 7. The complexity of the dissolved organics in methanol extracts of the endemic area Pliocene lignites is obvious, and it is not matched by the

![Fig. 5. The nitrate concentration shows high values for both the endemic and the nonendemic water samples. Nitrate is also much higher in the wells, compared to spring water supplies.](image1)

![Fig. 6. Total ion current chromatograms of three water extracts show a much higher organic contents in the endemic area samples (A,B) vs. a nonendemic sample (C).](image2)

![Fig. 7. Total ion current chromatograms of Pliocene lignite methanol extracts show a higher organic content in the lignites collected from the endemic areas (Husnicioara, Kostolac and Bpesyn) compared to a lignite from a Romanian nonendemic area (Jilt Sud).](image3)
any of the nonendemic area lignites analyzed. The total ion current of water extracts of the endemic area lignites show peaks corresponding to aliphatic (mainly cycloalkanes/alkenes and steranic structures) and aromatic (mono- and polyaromatic terpanes, polycyclic aromatic hydrocarbons) compounds. Many of these compounds have attached oxygen-based functional groups (hydroxy-, phenol-, keto-, methoxy-) and some of them contain heterocyclic nitrogen or amino groups, structural features that could make them nephrotoxic and carcinogetic. Very few of these components are encountered in the methanol extracts of lignite from the nonendemic areas. This difference could be due to distinctive paleogeographic coal-forming conditions during the last period of the Tertiary in the corresponding BEN endemic and nonendemic areas (21). A lower extractability in polar solvents of the organic compounds from the nonendemic lignites can also explain the differences in the GC/MS data. We have demonstrated in previous experiments (20) that a three minute methanol extraction of the endemic area Pliocene lignite is sufficient to give a high yield of organic components, the compounds being loosely bound to the coal matrix and readily extractable under the mild experimental conditions used. For higher rank coals and for the nonendemic area lignites, a significantly longer period of time (hours–days) is needed to extract similar quantities of organic compounds.

The endemic area Pliocene lignites also have much more water-soluble organic compounds compared to higher rank coals (fig. 8). The room temperature water extraction process seems also to be time dependent, the 4 months water extract having a double amount of organics compared to the one month extract. All of the individual components have not yet been identified, but they are mainly aromatic and aliphatic and it is likely that many of them overlap with those from the methanol extracts. Similar experiments are underway using nonendemic area Pliocene lignites.

Discussion

The first etiological hypothesis for BEN, advanced by Danilovic in Yugoslavia was that the disease is a chronic intoxication with lead, originating from the milling wheels used to ground wheat and contaminating the flour consumed by the villagers in the endemic area (1). This suggestion was taken seriously by local authorities from Serbia, who banned the use of the watermills in the area. This measure led to serious economic losses for the villagers who had to travel to other localities to grind their grains, but no reduction in BEN incidence was noted in the affected areas (2). Subsequent laboratory investigations carried out in the countries affected by BEN failed to confirm any role for lead in the causation of BEN. In addition, the clinical picture of the BEN patients lacked the elements defining lead intoxication.

Measurements of other metals (Cu, Zn, Ni, Ti, Al, Mb, Co, W, Cd) in water and other environmental samples from the endemic region and in BEN patients have generally produced values in the normal ranges, which in general are in agreement with our recent data obtained from Romania. Some authors (22) have claimed higher concentrations of Mn, Al, Cu and Fe, which could have significance in BEN etiology. According to Bulgarian and Yugoslavian studies (23), silicon was found in high concentrations in drinking water from endemic areas, suggesting a leaching process of the sediment and of the alluvium coming in contact with the ground water. Silicon is known to be nephropathogenic but it has not been found in significant concentrations in the kidneys of BEN patients. Other trace elements like selenium were found to be deficient in the endemic areas, at least in the former Yugoslavia (24,25). The significance of selenium deficiency with respect to the onset of BEN, however, is unclear.

Except for the nitrate concentrations that were found to be high in both the endemic and the nonendemic water samples, all the other inorganic parameters measured by us showed no strikingly high or low values for the endemic and the nonendemic locations. To some extent in contradiction with previous studies made in Yugoslavia, we found zinc to be in lower concentrations in the endemic vs. the nonendemic sites. Zn is a biologically essential element, and it is absolutely required for the development of normal immune response and defense against cancer. Although more extensive geochemical mapping in the affected countries is needed before drawing a final conclusion, it is possible that Zn-deficiency in the endemic region may have a role in BEN causation and in concert with other trace element imbalances (e.g. the selenium deficiency) be responsible, at least in part, for the induction of the UTT’s associated with BEN.
The organic geochemical information obtained from our study in the main Romanian BEN-endemic area during 1999-2000 is consistent with the Pliocene lignite hypothesis of BEN etiology, but it does not provide a final confirmation to this theory. A "smoking-gun", imagined as an organic molecule with known nephrotoxic and/or carcinogenic properties, is still missing, but the presence of much more potentially toxic organic contaminants and in much higher concentrations in the endemic vs. nonendemic water samples is an encouraging finding, which by itself should justify further field work not only in Romania, but in the other affected countries, too. In addition, we have demonstrated that the Pliocene lignites from the BEN areas produce a much greater yield of organic substances in polar extracts compared to higher rank coals and compared to other lignites from nonendemic areas in Romania. These compounds include PAH's, and highly functionalized (O, N, and S) aliphatic and aromatic compounds. Some of these compounds were also identified in the water samples collected from the endemic villages. It may be that only a small number of compounds (perhaps even only one type of structure) out of the hundreds in the water samples are responsible for BEN and the associated UTT's. Moreover, the relatively low concentrations of toxic organic compounds found in the drinking waters (individual compounds generally <1 µg/l) in endemic villages could explain the long period of exposure (decades) leading to BEN.

As the GC/MS method of identifying organic compounds is limited to molecular weights <600 Da, it is likely that some higher molecular weight compounds present in the water samples have remained undetected. Of particular interest from this standpoint are humic substances. There are some studies reporting a potential toxicity of humic substances present in ground water and bioavailable to human populations. Certain humic acids have even been implicated in the etiology of black foot disease and Kashin-Beck disease in China (26,27), and a similar implication cannot be excluded in BEN, the more so as the presence of humic substances in the ground water from the BEN areas is almost a certainty. Such compounds can also be leached from low rank coals like Pliocene lignites and we plan to identify them by HPLC-MS in further experiments.

The clinical, scientific and environmental health importance of BEN is not limited by the relatively small number of affected persons, or by the seemingly small extent of the affected regions. As intriguing as its etiology and epidemiology is, BEN could still be considered a local problem, affecting just a discrete part of Europe. However, there are at least four reasons, advanced by Radovanovic et al. (2), why BEN has a universal relevance for the scientific and medical community:

1. The medical geography of BEN is not completely clarified. Although traditionally described in only in a few Balkan countries, kidney diseases similar in etiology to BEN could occur unrecognized on a more or less endemic scale in other regions of the world. This is true not only for regions with low rank coal deposits such as the Pliocene lignites linked to BEN, but also for anywhere where groundwater used for drinking or cooking is in proximity to coal or coal waste stored near power plants or mines. Since coal is a major source of energy worldwide, there is a large potential for contamination of groundwater and surface water by toxic organic compounds from coal (28). BEN-like kidney diseases could remain unrecognized due to factors such as population mobility, short life expectancy in an exposed population, or deficient local medical systems. Also, point sources of contamination, such as coal stored next to a power plant, may affect much smaller areas than those where BEN occurs.

2. Discovering the cause of BEN and revealing its etiopathogenic mechanisms will lead to a better understanding of other kidney diseases, such as analgesic and cadmium nephropathies and of the end stage renal disease. ESRD poses a significant economic burden in many countries, translated into costs for renal dialysis and kidney transplantation. According to a US Renal Data System (USRDS) report, due to the recent growth in the ESRD patient population, total Medicare expenditures for the ESRD program have been increasing steadily, from $5 billion in 1991 to $12 billion in 1998. The total Medicare ESRD program cost is projected to more than double in the next ten years, surpassing $28 billion by the year 2010. As for many ESRD patients the primary cause of the chronic renal failure is unknown, effective preventive measures cannot be considered in this case.

3. The emergence of UTT's in BEN patients has often been attributed to the same etiologic agent(s) causing BEN itself. Thus, identification of this agent(s) may provide insight into the etiology of cancer as well as ESRD.

4. From a humanitarian standpoint, identifying the BEN causal factor, will allow preventive measures to be adopted in the directly afflicted countries, such as by treatment or filtration of the drinking and cooking water or changing the main water supply as has already been done in several endemic regions in Serbia. Such measures could prevent another generation of BEN patients from developing.

A slight decrease in the incidence of BEN has been noticed in the last two years in Serbia (29) however, this seems to follow the usual oscillating pattern of the disease, with low's and high's of incidence, and another epidemic outbreak is likely to occur in these areas in the future. It is interesting that a similar epidemiological pattern was noted in the incidence of diseases related to selenium deficiency in the endemic areas of China. Here, a peak incidence of Se deficiency (Keshan and Kashin-Beck diseases) cases was noted in the late 1950s.
until the 1970s, followed by a reduction in the number of cases (30). An epidemiological parallel can be drawn between BEN and Se-deficiency related diseases, as similar factors (geological, environmental, social, dietary, etc.) could control in a similar way the incidences of these diseases. In both situations, the endemic outbreaks have occurred in stable rural populations, living in similarly complex geological environments (characterized by inorganic as well as organically rich rocks) depending almost entirely on their immediate environment for water and food and having little access to goods outside their areas. As the situation has gradually changed after the end of WWII, with more food and drink products coming into the areas from the outside, this would provide a partial explanation, at least in the case of BEN, for the decreasing incidence of the disease, without eliminating the original causative factor still present in the area.

Although appealing, the Pliocene lignite implication in BEN etiology is still a possibility rather than a certainty. BEN cause probably resides in the complexity of the environment where the disease occurs and a combination of external factors acting together on a susceptible genetic makeup should be considered. Organic compounds found in the groundwater are a potential major culprit, but their source remains elusive; their origin in the Pliocene lignite is likely but the fact that not all the described endemic areas are topographically connected to known Pliocene coal beds (i.e., one endemic area in Serbia and the endemic area in Bulgaria) rises further questions about this possibility. Uncharted and of low economic importance Pliocene coal deposits could be an explanation in this case, as is the situation of the smaller endemic area in Romania (Resita region). However, more extensive geochemical investigations and perhaps some novel methodological and conceptual approaches will be needed in order to answer the main question, that of Balkan nephropathy etiology.

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