Limitations and plausibility of the Pliocene lignite hypothesis in explaining the etiology of Balkan endemic nephropathy

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Background: Balkan endemic nephropathy (BEN) is a chronic, tubulointerstitial renal disease often accompanied by urothelial cancer that has a lethality of nearly 100%.

Introduction: One of the many factors that have been proposed to play an etiological role in BEN is exposure to organic compounds from Pliocene lignite coal deposits via the drinking water in endemic areas.

Objectives: The objective of this study was to systematically evaluate the role of the tenets of the Pliocene lignite hypothesis in the etiology of BEN in order to provide an improved understanding of the hypothesis for colleagues and patients alike.

Methods: A comprehensive compilation of the possible limitations of the hypothesis, with each limitation addressed in turn is presented.

Results: The Pliocene lignite hypothesis can best account for, is consistent with, or has the potential to explain the evidence associated with the myriad of factors related to BEN.

Conclusions: Residents of endemic areas are exposed to complex mixtures containing hundreds of organic compounds at varying doses and their potentially more toxic (including nephrotoxic) and/or carcinogenic metabolites; however, a multifactorial etiology of BEN appears most likely.

Keywords: BEN, Coal, Drinking water, Environmental medicine, Geochemistry, Human health, Medical geology, Organic compounds

Introduction

Over 100 000 individuals may be at risk for developing Balkan endemic nephropathy (BEN),¹ a chronic, tubulointerstitial renal disease often accompanied by urothelial cancer,^{2,3} that has a lethality of nearly 100%.⁴ Prevalence in affected Balkan countries is 2–10%,⁴ but incidence may be over 20%, e.g., in an area in northwestern Bosnia-Herzegovina, where >50% of patients receiving chronic dialysis have the disease.⁵ Transplantation is usually not an option,⁶ and typically 50% of patients have died within 2 years of diagnosis.⁷

Of the many hypotheses that have been proposed for the etiology of BEN,^{8–18} evidence from many studies favors the involvement of an environmental factor(s) in the etiology of the disease,^{1,19–26} for example, exposure to organic compounds from Pliocene lignite coal deposits. The Pliocene lignite hypothesis posits that BEN is caused by long-term exposure to low concentrations of toxic organic compounds leaching into drinking water, that is used

almost exclusively by the population, via groundwater from Pliocene lignites found in the vicinity of endemic settlements.^{16,24,27,28} Many studies have been conducted to test the role of the Pliocene lignite hypothesis in the etiology of BEN,^{27–39} and many characteristics of the disease have been shown to be consistent with the hypothesis. For example, of immigrants who settled into endemic areas, only after a minimum of ~20 years were they likely to develop the disease, suggesting low dose exposure to, e.g., a fixed environmental factor. Also, greater numbers and higher concentrations of organic compounds were shown to be present in endemic vs nonendemic area water samples,^{30,31} and in endemic vs nonendemic area water³⁹ and methanol^{30,34} extracts of lignite samples, showing the potential for differential exposure of the populations to the possible causative agents.

However, many limitations of the hypothesis have been noted in the literature.^{16,24,26,28,35,36,40–47} In addition, criticisms of a number of previous studies that were conducted to test the hypothesis have been raised, casting doubt on its validity. For example, evaluations of the research that addressed the

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Figure 1 Map showing the distribution of Balkan endemic nephropathy (BEN) regions (adapted after Maharaj *et al.*,³⁹ and Stefanovic and Radovanovic⁸⁷).

Pliocene lignite hypothesis concluded that the primary evidence in support of it is merely the spatial association between lignite deposits and BEN endemic villages,⁴¹ but that the spatial correlation is nothing “more than fortuitous.”³⁶ Furthermore, the criticisms have been used as a basis to dismiss the hypothesis altogether. For instance, in another assessment of the hypothesis the authors justified their conclusion that there was “no basis for connecting polycyclic aromatic hydrocarbons (PAHs) or other dissolved organic compounds to the development of BEN”⁴⁶ by citing a report.³⁵ Moreover, it has been suggested that no effort and resources should be spent toward conducting any further research on this subject.³⁶

This study

To date there has been no comprehensive list of the possible limitations of the Pliocene lignite hypothesis. In addition, though some limitations have been addressed in the literature, information exists only in a piecemeal manner, e.g., in studies from 1991¹⁶ to 2013.³⁹ Therefore, herein presented for the first time

is a comprehensive compilation of the possible limitations of the hypothesis, with each limitation addressed in turn. The objective of this study was to systematically evaluate the role of the tenets of the Pliocene lignite hypothesis in the etiology of BEN in order to provide an improved understanding of the hypothesis for colleagues, patients, and those affected by the disease. Possible limitations are presented in the form of statements or questions, and are addressed, within the context of the hypothesis, in the form of responses or answers. Though “answers” are given, the author proposes only potential solutions in an effort to, e.g., reconcile what appears as conflicting information in the literature, and the statement-and-response styled format was chosen for clarity and effectiveness.

This study addresses the possible etiology of BEN, a disease that has caused, and continues to cause, significant human suffering in endemic areas. If the Pliocene lignite hypothesis proves to be correct, research on organic compounds derived from coal in water for human consumption will add to the increasing body of knowledge in the re-emerging

discipline known as medical geology,^{48,49} and may potentially lead to the identification and use of effective strategies for preventing the disease, e.g., chlorination and/or filtration of the water or changing the water supply, as well as to insights into the pathophysiology of chronic tubulointerstitial renal disease and/or urothelial carcinoma in humans. In addition, research on organic compounds from Pliocene lignites may have implications for the leaching of organic compounds from similarly ranked coal, coal piles adjacent to many power plants, coal slurry impoundments, the disposal of co-produced water from coalbed methane wells, and/or the increasing prevalence of end-stage renal disease with unknown etiology in many parts of the world.

Discussion

Possible limitations

Other environmental agents are more plausible

Other environmental agents may be more plausible as etiological factors than exposure to Pliocene lignites, i.e., exposure to ochratoxin A, a fungal mycotoxin,^{15,40,50,51} and aristolochic acid from *Aristolochia clematitis* seeds,¹¹ for which there has been much renewed interest.^{44,52–54}

However, ochratoxin A contamination has been found in both endemic and nonendemic regions,^{22,55,56} nonaffected households,⁵⁷ and in Romania, no ochratoxin A or its degradation products were detected in the urine of BEN patients,⁵⁸ raising doubts about its role in the etiology of the disease. Likewise, *Aristolochia* plants are ubiquitous,²⁴ and *Aristolochia* contamination has occurred in nonendemic areas of BEN affected countries.⁴⁵ For example, the plants are widespread in many regions of Romania, including those with similar rural populations and ethnic groups as BEN endemic areas, yet the regions have no identified cases of nephropathy.⁵⁹ Moreover, human exposure to *A. clematitis* in BEN areas has not been apparent.^{25,51} For instance, *A. clematitis* seed bulbs would have been separated from wheat grain by the modern mechanized mills in use for many years in the endemic regions of Croatia and Bulgaria,⁶⁰ and in use for decades in the endemic regions of Romania.⁵⁹ In addition, in Romania, wheat has only been grown sporadically,⁵⁹ maize is the dominant cereal, and it cannot be contaminated by aristolochic acid⁵¹ in the same way as wheat. Also, although uptake of an aristolochic acid solution by maize roots has recently been demonstrated,⁵⁹ differential uptakes by crop plants between endemic and nonendemic regions, for example, appears unlikely. Furthermore, though an epidemiological study showed that exposure to *A. clematitis* has occurred in endemic areas via other routes, i.e., from teas and baths prepared for

therapeutic purposes, the majority of BEN patients had not used such remedies.⁶¹ Finally, the exact mechanism by which aristolochic acid may cause tubulointerstitial nephropathy remains uncertain.⁶²

Thus, although many characteristics of BEN are consistent with the three environmental hypotheses for which there is the most compelling evidence (i.e., Pliocene lignites, ochratoxin A, and aristolochic acid), the Pliocene lignite hypothesis best accounts for the geographical distribution of the disease, as the lignites are not distributed uniformly among both endemic and nonendemic areas, but are proximal to endemic areas.¹⁶ Therefore, exposure to Pliocene lignites can best explain the differential exposure needed in order to account for the spatial distribution of the disease on a regional scale, and moreover, without the need for exposure to an environmental cofactor, as appears necessary in both the ochratoxin A and aristolochic acid etiological hypotheses. Nevertheless, a multifactorial environmental etiology of the disease appears to be likely.

Disease incidence and prevalence is decreasing

If e.g., aristolochic acid enters the human food chain through contamination of wheat from *A. clematitis* seeds, the number of BEN patients should at least be decreasing, because of, e.g., less exposure to the causative agent from herbicide use, modern milling procedures that remove the seeds, the purchase of non-local sources of flour, and fewer families baking their own bread. However, the envisaged disappearing scenario of BEN^{52,63} has not occurred.⁶⁴

For example, a stable incidence⁶⁵ and prevalence of BEN over a period of decades has been reported for the Kolubara region (the most affected) in Serbia,⁶⁶ and for Romania, where modern harvesting and herbicide application methods have been extensively employed for decades.⁵⁹ Moreover, the disease may be increasing, for instance: tumors of the renal pelvis and ureter have been increasing in endemic regions;²⁰ there has been a gradual involvement of more villages and households affected by BEN and urinary tract tumors in the Vratza District, Bulgaria,⁶⁷ and; in the recent (~1995–2009) years in Serbia incidence of BEN has also been reported as increased.⁶⁴

However, there have also been reports of decreasing incidence⁶⁸ and prevalence⁶⁹ in the Vratza region (and decreasing incidence in Serbia),⁷⁰ but these may be due to flaws in the surveillance system, and/or prophylactic measures, e.g., population redistribution.⁶⁸ Conflicting trends in the literature of BEN incidence in different endemic settlements may also be a result of the different methodological approaches⁶⁴ used in studies, and more research is needed to determine if reported decreases in incidence accurately reflect decreases in the disease.

If in the future, decreases in BEN incidence in the Vratza region are confirmed as real, they may be due to decreased exposure to organic compounds as a result of water being supplied to affected villages since the early 1960s from deep (80 m) aquifers or from the mountains and treated by filtration and chlorination.³¹ Likewise, tap water captured from deep aquifers has also been provided since the 1960s to endemic areas in Serbia,⁷¹ e.g., Lazarevac and Nis, and a slight decrease in incidence has been reported.⁷¹ Additional research is needed (using liquid–liquid extraction and gas chromatography–mass spectrometry) to determine the composition and concentration of organic compounds in the drinking water at the above locations. Furthermore, if decreases in BEN are real, they could also be due to the use of more beverages entering the endemic areas from outside.³⁰ Therefore, even if real, decreases in BEN incidence may not necessarily reflect an elimination of the original causative factor, especially given that the causative agent has been deemed as still prevalent.⁷² Typically there has been an oscillating pattern with lows and highs of disease incidence,³⁴ and not only can another epidemic wave not be ruled out,⁷³ but highs are likely to occur in the future.^{30,34}

Thus, BEN remains localized even where living conditions have changed drastically.¹⁰ No new endemic areas have emerged, and no known endemic locations have become nonendemic.^{57,74} A fixed environmental factor like exposure to Pliocene lignites may best explain the constancy of the disease.

Spatial correlation is nothing more than fortuitous

The spatial correlation between endemic BEN regions and Pliocene lignite deposits has been deemed as nothing “more than fortuitous.”³⁶ However, good agreement between the deposits and BEN endemic areas has been demonstrated for the former Yugoslavia,¹⁶ and a similar spatial correlation has been reported to exist between the deposits (and also for Pliocene lignite coal mines)³³ and endemic sites in Romania and Bulgaria,⁶ (see also the All Endemic Foci are not Linked to Pliocene Lignites section).

In addition, a greater number of organic compounds and in higher concentrations were shown to be present in endemic vs nonendemic area water samples.^{28–31} Well and spring water samples from endemic villages contained more aliphatic and aromatic compounds, [many of which are potentially toxic (including, nitrogen-, oxygen-, and sulfur-containing heterocyclic compounds, aromatic amines, and phenols)], and in higher concentrations than water samples from nonendemic villages.^{30,31} (However, see the Exposure Pathway is Unlikely section for contradictory results.)

Also, greater numbers and higher concentrations of organic compounds were shown to be present in endemic vs nonendemic area water³⁹ and methanol^{30,34} extracts of lignite samples. Furthermore, human mesenchymal stem cells exposed to a water extract of an endemic area Pliocene lignite showed increased cellular proliferation and differentiation compared to the control,³⁷ and human kidney cells exposed to concentrated high-molecular-weight organic compounds from an endemic village water sample showed excess cell death or proliferation compared to controls.³⁸ Results from each above study support the likelihood that the association between Pliocene lignite deposits and endemic BEN areas is not simply due to chance.

Plus, an important part of scientific hypothesis validation is the ability to make predictions based on the hypothesis, and the Pliocene lignite hypothesis has been used in a predictive mode.²⁴ For example, an area in southeastern Serbia-Kosovo was recognized as endemic after it was found to be near a Pliocene lignite field.^{16,24} Also, as more studies are conducted, additional possible links support the validation of the hypothesis as a predictive model, e.g., between coal deposits (including lignites) and kidney disease and renal pelvic cancer in Louisiana⁵⁸ and Wyoming,⁷⁵ USA, and preliminarily between lignites and renal disease in Texas, USA⁷⁶ and northern Portugal.⁷⁷

All endemic foci are not linked to Pliocene lignites

Few geological studies have been carried out that address the tectonic and stratigraphic evolution of the basins that formed endemic area Pliocene lignite deposits or their geochemical features compared to, e.g., Pliocene lignite formation in Greece.^{78–82} Therefore, limited information is available on the distribution of the Pliocene lignite coal deposits and the hydrology in the endemic areas.^{16,27,32,83}

All endemic regions in the former Yugoslavia except one located in central Serbia are in close proximity to Pliocene lignites.¹⁶ The one endemic exception in central Serbia has some nearby Pliocene lignites, only they are not in as close proximity as found in the other endemic areas.^{6,24} However, the proximity may be sufficient to be hydrogeologically connected to the known lignite beds,^{6,24} or the area may have unmapped Pliocene lignites,²⁴ or deposits too small to have even been documented, much less mapped. For example, uncharted and of low economic importance Pliocene coal deposits occur in the Resita endemic region in Romania,³⁰ and in 2001 a colleague (Dr. Nikola M. Pavlovic) in Serbia discovered a previously unaccounted Pliocene lignite coal mine at the border between Serbia and Bulgaria.⁸⁴ In addition, as the bedrock adjacent to

all endemic areas in the former Yugoslavia contain coal,¹⁶ the one endemic exception may be influenced by organic compounds being leached from adjacent bedrock coal (and other organic-rich sedimentary rocks), which may also contribute organic compounds to the well and spring water of endemic regions that are linked to Pliocene lignites.

Colleagues who have traveled extensively throughout the Vratza and Montana endemic regions in northwestern Bulgaria report no evidence of lignite deposits.³⁶ However, both endemic regions encountered similar paleoenvironmental conditions to the Romanian Drobeta Turnu Severin endemic region,^{27,33} occur at the Tertiary Dacian southern basin margins like most other endemic sites,²⁷ is geologically defined by Pliocene sediments,^{27,33} and have extensive coal fields north and south of the regions.^{27,33} Also, as in the above case for the Serbian endemic exception, the Bulgarian endemic areas may be hydrogeologically linked to known Pliocene lignite deposits, or there may be undocumented lignites in the endemic areas.³³ For example, the presence of at least two types of coal in the Bulgarian endemic areas, one being a low-rank lignite, very similar to Romanian and Serbian endemic Pliocene lignites was discovered in 2009.³⁹

Furthermore, weathering of coal can impact the quality of groundwater, including affecting the types of organic compounds present.¹⁶ Atmospheric oxygen is a principal chemical agent responsible for the weathering of coal, and low-rank coals react more readily with oxygen than high-rank coals, e.g., oxidation experiments on lignite samples showed that the formation of dihydroxybenzenes occurred during weathering.⁸⁵ Percolating surface water will also contribute to natural weathering processes and leach organic compounds,⁸⁵ and carcinogenic soluble organic compounds⁸⁶ are derived from the weathering of coals.¹⁶ Therefore, weathered particles of lignite and shale in the unconsolidated alluvium from adjacent hills may contribute soluble toxic compounds to the groundwater in endemic villages.^{27,33}

Thus, although not all endemic areas have been geographically linked to Pliocene lignites, their proximities may be sufficient to be hydrogeologically connected to known lignite beds, or the areas may have unmapped Pliocene lignites, or deposits too small to have been documented. In addition, evidence for previously undocumented lignites, including for Pliocene lignites, and in and around the endemic Bulgarian regions, has grown as more fieldwork has been conducted, and this trend is likely to continue in the future. Furthermore, the endemic exceptions (and other endemic areas) may be at least influenced by other proximal organic compound-rich leachable

rocks and sediment that includes, e.g., shales, and products of coal weathering.

Endemic and nonendemic villages are only a few kilometers apart

Most nonendemic villages in alluvial valleys in the former Yugoslavia are not in the vicinity of Pliocene-Miocene lignites.¹⁶ However, where endemic and nonendemic villages are only a few km apart,⁸⁷ the aquifer system supplying the drinking water in the nonendemic village may not be affected by the lignites.¹⁶ Evidence for this is suggested by the greater number of organic compounds and in higher concentrations present in endemic vs nonendemic area water supply samples,^{30,31} the source for which, at least in part, is most likely the endemic area Pliocene lignites.³⁹

Geologic structure differs from one village to another,^{10,16} and the underground water transport pathway may be highly variable, even within the same endemic area, e.g., in Bulgaria, where there is karst terrain, which is often highly fractured.⁸⁸ In addition, the shallow wells and springs in endemic regions are largely supplied by aquifers with a relatively fast flow rate, e.g., from rain. Therefore, the location of endemic and nonendemic villages only a few km apart may be due to, for instance, the proximity to deposits containing the organic compounds, differences in number and/or concentration of the organic compounds in the rocks and sediment, differences in flux of groundwater through the rocks and sediments, and/or differences in dilution of the compounds due to rainfall and permeability.^{27,33}

In the same village, all households are not endemic

There have been no obvious differences in, e.g., living and hygienic standards, and nutritional habits between endemic and nonendemic households within the same endemic village¹ (nor between endemic and nonendemic villages).⁷³ Similar local hydrogeological variations as outlined above, but on a smaller scale, may also explain why in the same endemic village, all households are not endemic.

Organic compounds in the groundwater in endemic villages would not be expected to equally contaminate all wells. Just as higher exposure to organic compounds occurs via drinking water in endemic vs nonendemic villages,^{30,31} higher exposures may also occur in endemic vs nonendemic households within the same endemic village. For example, differences in fluorescence excitation/emission matrix spectra between drinking water samples from BEN and non-BEN households within the endemic village of Brestovac in the former Yugoslavia were due to higher organic compound concentrations in samples from BEN households.²⁹ However, more research is needed, including well-by-well evaluations to

determine if greater numbers and/or higher concentrations of organic compounds consistently occur in endemic vs nonendemic households within the same endemic villages.

In the same household, all individuals do not develop the disease

Balkan endemic nephropathy not only affects only certain households in endemic villages, but also only certain individuals in an endemic household.¹⁰ Provided a spouse has lived with an affected family for 15 or more years, the risk of contracting the disease is as high as it is for the indigenous occupants of a house,⁷ suggesting the cause is not due primarily to genetic factors,⁴ but to an environmental one. However, if BEN is caused by exposure to organic compounds, why would not all individuals in an endemic household develop the disease, as all individuals would use the same water for drinking, cooking, bathing, irrigation, and other purposes?

Exposure is likely to differ at least by gender (see The Hypothesis cannot Explain the Gender Difference section), and the greater total amount of water containing the organic compounds one is exposed to may contribute to the development of the disease in one individual in a household, but not another. Other contributory factors may include: a greater length of exposure, poorer health status of individuals, and the presence of pre-existing conditions such as diabetes and other kidney diseases.

Additional research needed in endemic households include person-by-person evaluations of total household water exposure linked to the number and concentration of organic compounds in their well or spring sources to determine if patients have been more exposed, as well as prospective studies where individuals are followed over time to determine whether a positive correlation exists between total endemic water source exposure (from household, workplace, neighbor/friend's household, restaurants, etc.) and the likelihood of developing BEN. Nevertheless, exposure to organic compounds via endemic household (and other endemic) water would likely not account for all evidence regarding the seemingly sporadic occurrence of BEN within a household, and additional contributory factors, e.g., genetic susceptibilities involving polymorphisms in xenobiotic metabolism enzymes⁸⁹ are needed.

The hypothesis cannot explain the genetic link

Studies suggest a genetic component to the development of BEN.²² For example, in Vreoci in the Kolubara region, Serbia, new BEN patients were found only in families in which the disease had already been recorded.⁶⁶ Plus, along with BEN patients, hyper-beta₂-microglobulinuria^{90,91} and increased urinary albumin excretion⁹⁰⁻⁹³ have been observed in

family members of BEN patients. Maternal history of BEN has also been strongly associated with reduced kidney function and size in their adult offspring before the onset of the disease,⁹¹ and was shown to be a risk factor for increased C-reactive protein in the offspring.⁹⁴ In addition, parental history of BEN had direct and indirect effects on offspring, which also predicted the onset of BEN.⁹⁵ Finally, decreased lecithin cholesterol acyltransferase activity,¹⁸ and moreover, as a result of exposure to coal-derived organic compounds⁷¹ has also been proposed to play an etiological role.

However, as clinical investigations have not shown any single etiologic factor as an independent cause of BEN,⁹⁶ and no single nephrotoxic factor is known to produce the specific combination of lesions seen histopathologically,⁵ most authors have agreed on a multifactorial origin for the disease.^{23,40} For example, BEN may be due to an unusual combination of environmental, toxic, and genetic factors¹⁹ where disease is generated by the action of environmental triggers in genetically predisposed individuals,^{23,45,97} and genetic predisposition may also interact with environmental determinants to produce the associated tumors.²⁰

Thus, there is likely an underlying genetic condition that predisposes individuals to susceptibility by another agent to cause BEN. For instance, the high CYP3A5*1 expressor allele frequency among BEN patients may contribute to a metabolic pattern that more efficiently activates⁸⁹ a toxin or toxins, e.g., from organic compound-rich leachable rocks and sediment, that cause BEN. The greater efficiency of oxidative metabolism in BEN patients⁹⁷ may also predispose individuals to the formation of nephrotoxic and carcinogenic metabolites from metabolic oxidation of the organic compounds in drinking water. In addition, it is possible that parental conditions, e.g., exposure to organic compounds via drinking water, may adversely affect kidney function in offspring via an epigenetic effect.

The hypothesis cannot explain the gender difference

Women have higher incidences than men of both BEN^{1,98} and the associated urinary tract tumors^{98,99} with the male-to-female ratio of BEN being: 1 : 1.5;⁷⁴ 1 : ~1.5–1.65;¹⁰⁰ 1 : 1.65;⁴ 1 : 2;⁷ 1 : 3.¹⁰¹ The Pliocene lignite hypothesis is consistent with the gender incidence and prevalence difference in BEN, as women are more exposed than men to household factors,¹⁰ and would be more likely to not work outside the home, thereby being exposed nearly exclusively to the endemic water supply. In contrast, husbands are often employed outside the home,¹⁰ and therefore would be expected to be exposed to more heterogeneous water supplies.

It may also be important to quantify the doses received from exposure routes other than from ingestion (for both men and women), otherwise there may be an underestimation of the total health risk. For example, total internal dose from exposure to certain water contaminants via inhalation or dermal absorption was shown to be greater than exposure through ingestion.¹⁰² Also, in the USA, baths were taken much more frequently by women than men, and both baths and showers were taken for longer durations by women than men;¹⁰³ similar gender differences in exposure histories may exist for individuals in endemic BEN areas. In addition, organic compounds are stored in fat tissue, and as women have much more fat tissue than men¹⁰⁴ given the same amount of organic compound exposure, women would likely receive, retain, and/or accumulate (a) greater total internal dose(s).

Thus, there is a need for epidemiological studies to develop exposure assessment methods that take into account interindividual sources of water consumption and exposure variations in order to evaluate the gender differences in exposure to organic compounds in the water supplies of endemic areas and BEN. However, the sex difference may also be due to, e.g., differences in diagnostic criteria,⁷³ gender specific susceptibility of the kidneys,¹⁰⁵ or decreased detoxification and elimination capabilities in women against xenobiotics.¹⁰⁴

Therefore, the Pliocene lignite hypothesis has the potential to account for the gender incidence and prevalence difference in BEN and associated urinary tract tumors (due to possible differential exposure to the organic compounds in the water from lifestyle factor and fat tissue differences between men and women), or the incidence and prevalence difference may be due to differences in diagnostic criteria, gender susceptibility of the kidneys, gender differences in detoxification and elimination capabilities, or a combination of these or other factors.

Why is BEN not found in towns and cities?

There is an absence of BEN in towns and cities and within the context of the Pliocene lignite hypothesis this may be explained by the use of alternative water supplies from that of endemic villages, e.g., from deep aquifers, and/or the use of water treatment, for instance, filtration and/or chlorination that are commonly used in more populated areas.

However, in some instances the same water as in an endemic area has been reported to be used in neighboring cities, but the cities do not have BEN (except for cases that originated in an affected village).¹⁰ For example, the absence of BEN in the town of Lazarevac in Serbia that uses the same groundwater as the endemic villages in the municipality, was

proposed as possibly due to the increased flow velocity as a result of a cone of depression in the water table from the large quantity of water pumped for the town, which may reduce exposure of the water to contaminants,⁸³ e.g., the organic compounds (however, see The Hypothesis cannot Account for the Rainfall Correlation section).

The hypothesis cannot account for the rainfall correlation

The intensity of the endemic process has been thought to depend on altitude and the frequency of flooding,⁸⁷ as BEN occurs in floodplain terrain usually only 100–200 m above sea level in, e.g., Serbia.^{7,87} However, the disease also occurs in mountainous districts in Bulgaria¹ that are as high as 600 m in altitude,⁸³ and therefore, is not consistent with frequent flooding, and indeed there is no correlation between altitude and BEN frequency in Bulgaria.⁸⁷

Rainfall, however, may impact BEN progression. For example, a positive correlation between the amount of rainfall and the number of BEN deaths over the following 2 years has been shown as significant ($r=0.80$).⁷ Though this supports a fungal hypothesis for the cause of BEN due to increased growth of fungi and toxin production from damp autumnal conditions,⁷ it is also consistent with the Pliocene lignite hypothesis.

Drinking water in endemic areas is largely influenced by rain, as average depth-to-water of open shallow wells and springs is 0–10 m in Romania³³ and <15 m in Bulgaria.⁸⁸ Therefore, increased rainfall would be expected to increase the leaching of the organic compounds from the source rocks and sediment, and indeed, greater numbers and higher concentrations of organic compounds were present in water samples collected during the wet rainy (spring) season compared to the dry (summer) season.³¹ In addition, greater numbers and higher concentrations of organic compounds were leached in experiments that best modeled rainfall fluxes and groundwater movement using endemic area Pliocene lignites compared to all other experimental conditions and coals examined.³⁹ However, additional fieldwork is needed involving isotopic analysis in order to trace the rainwater as it leaches the lignites and enters the aquifers. Though it normally takes 15–20 years for BEN to manifest, during times of increased rainfall, exposure to the increased amount of organic compounds may cause BEN to evolve from the sub-clinical (undiagnosed) to the clinical (diagnosed) phase,³¹ and/or hasten an acute stage of the disease that culminates in death over the next 2 years in individuals already compromised from BEN.

However, the above scenario is not consistent with the faster drawdown hypothesis of reduced exposure

to water contaminants⁸³ (see the Why is BEN not found in Towns and Cities? section). Instead, the absence of BEN in towns and cities may be due to the use of alternative water supplies and/or water treatment and filtration, e.g., as has been used in the BEN regions of Nis in Serbia and Vratza in Bulgaria.³¹

If, as a result of the above measures, decreases in BEN incidence in the above areas are shown in the future to be real (see the Disease Incidence and Prevalence is Decreasing section), a lower incidence but not eradication of the disease (as has been reported for Serbian endemic villages)⁷¹ would be consistent with the Pliocene lignite hypothesis, and the water treatment being not very effective. For example, though the author has no information regarding the filtration apparatus used in the treatment plants, nor the extent to which their filtering process eliminates organic compounds (if at all), in studies where 1 µm pore size scientific laboratory grade (Whatman GF/C glass microfiber) filters were used to filter water, not only did the filtered water contain abundant organic compounds,^{31,39,75} but most of the organic contaminants were in the dissolved phase,³⁰ i.e., had passed through the filter. Organic compounds in the dissolved phase plus or minus in association with inorganics may persist longer in aquifers and may be more bioavailable than those associated with larger particulate forms.

Balkan endemic nephropathy is a new disease

Pliocene lignites are 1.8–5.3 million years old, yet BEN was first described only 57 years ago in 1956,^{1,106} (though a decade earlier local physicians noted a high prevalence of kidney disease in certain settlements in the Vratza region, Bulgaria).⁵⁷ If BEN is caused by organic compounds from such a stationary source, why is it a relatively new disease?

Balkan endemic nephropathy is likely not a new disease, but rather a newly recognized and described one. For example, past death records show a number of people suffering from an unidentified kidney disease suggesting that the condition is not new, and it being first recognized in the late 1950s was not a result of its initial appearance, but rather improved rural medical service,¹ or the amplification of an already existing endemic process.⁷³ In addition, BEN typically occurs late in adulthood⁴¹ in the 4th or 5th decade of life,¹⁰⁷ with most cases diagnosed between 40–60 years old.^{24,98} Prior to World War II (WWII) average life expectancy in endemic settlements was <45 years³⁰ due to e.g., malaria and tuberculosis,⁷³ and therefore, not enough afflicted patients may have amassed for the disease to have been recognized.²⁴ The increased average life span after WWII may have allowed the disease to reach the clinical stage,

permitting enough cases of BEN to develop, resulting in BEN being recognized⁵⁷ and later described in the 1950s. Additional evidence also indicates that BEN was likely endemic in the Balkans prior to the early 1950s,¹⁰⁸ had existed since at least the 1920s, and possibly for many decades earlier.⁵⁶

Thus, though Pliocene lignite deposits were present since their formation millions of years ago, and the etiological factors for BEN may have been present for millennia,²⁴ the presence of the combined prerequisites necessary for BEN development and recognition (see also the There is no BEN in Other Areas of the World section) are relatively new.

There is no BEN in other areas of the world

Neighboring Balkan countries, i.e., Croatia and Slovenia, have several large Pliocene lignite deposits that are not associated with endemic villages,¹⁶ and similar Tertiary sediments and low-rank Pliocene coals as found in BEN countries occur in other nearby countries, i.e., Albania, Greece, Hungary, Italy, and Turkey.^{16,27} In addition, low-rank, highly unaltered coals, including Pliocene lignites occur worldwide, e.g., in Australia, Burma, China, Europe, and the United States.^{16,24,45} Yet, all above locations have no reported endemic nephropathy.

The development of BEN is not simply a matter of the presence of a particular rank (i.e., lignite) and age (i.e., Pliocene) of coal. For example, methanol extracts of organic compounds from identically aged and ranked coals showed endemic Pliocene lignites possessed a complexity that was not matched by the nonendemic Pliocene lignite.^{30,34} Complex geological and hydrological factors, e.g., the environment of deposition within the geological setting, the activity of groundwater, the weathering of the coal, the composition of the country rocks and mineralization processes, and/or their weathering and transportation conditions, would be expected to influence whether organic compounds (and which ones, and at what concentrations) are leached from the Pliocene lignites and other organic-rich leachable rocks and sediment, and transported to wells and springs.

In addition, e.g., for disease development, a rural population largely dependent on the untreated water, and a settled population for long enough exposure²⁴ to the low concentrations of organic compounds is required. In the more developed countries mentioned above, treated water may be used.⁶ Provided that the necessary hydrogeologic conditions for leaching and transportation of the organic compounds from the deposits to wells and springs have been met, a BEN-like kidney disease may still exist unrecognized, e.g., in developed countries due to population mobility, and/or too small an area affected (such as coal stored next to a power plant³⁰), and in undeveloped areas,

i.e., Burma and rural China, due to inadequacies in the medical infrastructure for correct diagnoses.⁶ Furthermore, as more data becomes available, more possible links may be supported, e.g., an interstitial nephropathy similar to BEN has been described in Greece;²² in Louisiana and Wyoming, USA, kidney disease and renal pelvic cancer may be associated with coal deposits, including lignites;^{58,75} and in Texas, USA⁷⁶ and northern Portugal,⁷⁷ renal disease may also be associated with lignites.

Thus, the restricted disease distribution of BEN to the Balkans may be due to the distinct organic compound composition of the regional organic-rich leachable rocks and sediment (including, of course, Pliocene lignites), which are unique with respect to petrological and organic geochemistry reflecting differences in the for instance, peat forming vegetation and paleoenvironment as a result of the limiting paleogeographic coal-forming conditions in the Tertiary swamps during the Pliocene,²⁷ coupled with the right hydrological, demographical, and medical infrastructure conditions. Alternatively, the lack of a BEN-like kidney disease in (hypothetical) populations equally exposed to the same organic compounds as BEN patients and that satisfy the necessary additional factors above for disease development and recognition, may be mitigated by genetics, e.g., the Balkan population may have a propensity for BEN development, possibly due to a lack of significant genetic contributions to the region from outside populations.

Exposure pathway is unlikely

The exposure pathway of the Pliocene lignite hypothesis is as follows: organic compounds in endemic area Pliocene lignites→leached by groundwater→organic compounds in well and spring drinking water→almost exclusive use of this water→long-term exposure→development of BEN. However, some researchers that found no significant differences in water supply samples from endemic and nonendemic villages in Bulgaria^{35,36} deemed the exposure pathway unlikely because “PAHs and many other coal-derived compounds are not very water-soluble and sorb strongly to geologic materials.”³⁶ In addition, they asserted that PAHs “do not leach to any great extent, nor are they particularly mobile in groundwater,”³⁶ and therefore in terms of involvement in BEN development “PAHs can be eliminated from consideration.”⁴¹

However, their conclusions were based on studies^{35,36} in which a maximum of only 16 PAHs and 16 unknown organic compounds were analyzed for—whereas hundreds of organic compounds are relevant. For example, significant quantities of dissolved and colloidal organic matter were solubilized from Pliocene

lignite samples by water,²⁸ and in experiments that best modeled long-term exposure, many hundreds of organic compounds (including phenols, PAHs, benzenes, and lignin degradation compounds) were also leached from Pliocene lignites by water.³⁹ Therefore, many hundreds of organic compounds (including PAHs) from Pliocene lignite samples from BEN areas are indeed water-soluble, water-leachable, and water-extractable. Furthermore, as many of the coal-derived organic compounds have been identified in endemic (but not nonendemic) area water supply samples, at least some compounds have most likely been transported from the coal via groundwater to the drinking water in endemic areas.³⁹

Organic compound concentrations are too low to cause disease

Some researchers have concluded that the Pliocene lignite hypothesis was not supported by their results in part because of the 16 PAHs they analyzed endemic area water samples for in Bulgaria, the maximum concentration of the only PAH [i.e., benzo(a)pyrene (BaP)] for which the World Health Organization has assigned a drinking water guideline was far below the guideline.³⁶

However, their results are consistent with the hypothesis, where low concentrations of organic compounds are indeed important, as long-term exposure to the BEN environment is a required⁷³ link in the exposure pathway (see the Exposure Pathway is Unlikely section). For example, there is a positive correlation between increasing residence time in an endemic area and a greater number of suspected BEN cases,⁴ indicating that long-term exposure is a necessary factor for development of the disease. Also, the residence time data⁴ are consistent with the 10–30 years or more subclinical incubation period³² of BEN.

Continuous exposure to the low concentrations of organic compounds or accumulation of the compounds in body tissue over time may produce the tubulointerstitial effects characteristic of the disease.²⁸ The low concentrations of organic compounds may also be linked to the high association of BEN with urinary tract carcinomas,^{24,28} which have a slower development than BEN, and possibly to the higher incidences of these cancers in endemic vs nonendemic areas.^{2,20,72} In the subset of individuals exposed to low concentrations of organic compounds over long periods of time in endemic areas, some would be susceptible to and develop BEN, others to urinary tract carcinomas, others to both diseases, and still others may not show any indications at all of being impacted by these compounds. In addition, other adverse human health effects may also possibly result from the exposure (see the Identified Compounds do not Account for BEN and Tumors section).

Identified compounds do not account for BEN and tumors

Organic compounds in well and spring water from endemic areas contain a greater number of aliphatic and aromatic compounds and in higher concentrations ($>10\times$) compared to water samples from nonendemic areas.⁶ Many organic compounds in the identified compound classes, e.g., biphenyls, aromatic amines, terpenes, and N-, S-, and O-containing heterocyclic compounds, of endemic area water samples are known or suspected of being nephrotoxic or carcinogenic.⁷⁷ In addition, PAHs have been detected in higher concentrations in well water from endemic vs nonendemic villages, and some PAHs (e.g., benzopyrene, benzanthracene, benzofluoranthene) are known or suspected carcinogens, and aromatic amines have been linked to tubulointerstitial nephropathies and urinary tract cancer.²⁴

Nonetheless, the currently identified organic compounds in the well water from endemic areas have been deemed as insufficient to account for BEN and the associated tumors.⁴⁴ For example, of the 14 aromatic organic compounds listed from well water samples from endemic villages,²⁴ none were reported to cause cancers of the kidney in laboratory animals.⁴⁰

However, not only do water samples from endemic villages contain hundreds of organic compounds, most compounds have not been identified or have only been tentatively identified.³¹ In addition, they have not been assessed on a compound-by-compound basis for nephrotoxicity and/or carcinogenicity, and certainly most have not been tested for, e.g., carcinogenicity in laboratory animals via the necessary route of exposure, i.e., drinking water. Moreover, even though a single compound may not be known to cause adverse health effects, the combined exposure from, e.g., additive or synergistic effects between the many organic compounds present in the drinking water may result in nephrotoxicity and/or carcinogenicity.

Furthermore, as metabolites may be more potent than their parent compounds,¹⁰⁹ organic compound metabolites may affect whether BEN and the associated urinary tract cancers develop. Metabolic oxidation of organic compounds may form nephrotoxic and carcinogenic metabolites, and the kidney would be particularly susceptible as a result of its high blood supply and ability to concentrate metabolites.⁹³ For example, of the sixteen PAHs designated as priority pollutants by the U.S. Environmental Protection Agency, though only BaP has been classified by the International Agency for Research on Cancer (IARC) as a known human carcinogen, PAHs in general are extensively metabolized,¹¹⁰ and many become mutagenic and carcinogenic following

metabolism into more biologically active metabolites.¹¹¹ Thus, residents of BEN endemic areas are exposed to drinking water containing mixtures of hundreds of organic compounds, and their potentially more toxic and carcinogenic metabolites, that moreover, may have additive or synergistic effects.

Therefore, no compound-by-compound evaluation could model the true potential risks to endemic residents' health. But data necessary in order to adequately assess human health risk are unavailable for the vast majority of chemicals even in commerce, even among the 2750 high production volume organic chemicals (that are produced in volumes of greater than 1 million pounds per year).¹¹² Even for chemicals that have been determined as hazardous, there is limited exposure data.¹¹² For the majority of contaminants of emerging concern, even the most basic information is unavailable.¹¹³

Notwithstanding, compared to a single exposure, more adverse human health effects can be expected from combined exposures to multiple factors, which of course represent real-world exposures. For example, complex mixtures of PAHs were more likely to be classified by IARC as definite carcinogens than single PAHs.¹¹⁴ Residents of endemic areas, therefore, are exposed to complex mixtures of hundreds of organic compounds at varying concentrations and their metabolites, in conjunction with other environmental factors, e.g., other compounds and their metabolites, plus other factors, like genetic profiles, all of which may have additive or synergistic effects, and any combination of, for example, a particular dose of certain organic compounds with another factor, may account for BEN and its associated tumors.

Finally, adverse human health effects from exposure to organic compounds via drinking water would not be expected to be limited to nephrotoxicity and urinary tract tumors, nor only to BEN patients. However, these effects have naturally been the focus in the literature, and few studies have addressed, e.g., cancer at sites other than that of the urothelial tract in BEN endemic village populations. For example, residents from villages with high and moderate incidence of BEN had increased incidence of cancers at sites other than that of the urinary tract vs those from villages with low and no incidence of BEN; males had an increased incidence in 5 out of 5 other cancer sites (e.g., prostate) evaluated, and females had an increased incidence in 5 out of 7 (e.g., skin).⁹⁸ In addition, though BEN patients are well known to be at high risk for developing urinary system tumors (they had the highest risk of all groups studied¹¹⁵), the second highest risk group was the population living in endemic areas but not affected by BEN.¹¹⁵ Nonaffected individuals from BEN villages also had a higher risk of developing cancer at sites other than

the urinary system than both BEN patients and the population from control villages.¹¹⁵ Furthermore, age-adjusted mortality rates for nonurinary system cancers were considerably higher in BEN foci vs the control population in the Vratza district, Bulgaria; and nonurinary cancer mortality was slightly higher and total cancer mortality considerably higher in BEN vs control municipalities in Serbia.¹¹⁶

The data from these studies are consistent with, for example, the organic compound spectra of water samples from non-BEN households located in endemic villages showing characteristics of water from BEN households, but with less intensity, and unlike water samples from nonendemic villages.²⁹ Thus, the organic compounds in the water supplies may at least contribute to the development of additional (from other than BEN) morbidity and mortality in endemic areas.

Epidemiological data do not support the hypothesis

Some researchers have concluded that there is no “relationship between exposure to Pliocene coal and the etiology of BEN” and that the hypothesis “cannot be supported,”⁴⁷ based on results of their epidemiological study that had intended to evaluate the impact of toxic compounds from Pliocene coal on Romanian miners.

However, occupational diseases are caused by exposures, not jobs.¹¹⁷ Though according to one miner’s anecdotal account, some past miners from the region had drunk large amounts of water directly from mine springs between coal layers,⁴⁷ for the group being studied, there is no information about the mine water consumed (if any, as some miners had brought water from the surface). That is, no questionnaire or interview or assessment of any kind was conducted in order to determine whether the specific exposure purported to have been evaluated had ever occurred in the group being studied, much less to what extent.

Nevertheless, assuming that the miners were exposed to the mine water in order to validate the conclusions of the epidemiological study authors, that they found no relationship between exposure of miners and BEN may be due to the healthy worker effect, i.e., the working population is healthier than the general population due to, e.g., selective attraction or rejection of new workers depending on physical demands of a job.¹¹⁸ For instance, in a random sample of the total Finnish population, those entering the workforce had a standard mortality ratio (SMR) of 70, showing a healthy population selection effect, but of all the occupational categories studied, the one that included mining workers had the most pronounced selection effect (SMR=40), and the total healthy worker effect (which included additional

selective factors) was also the largest for the category that included miners.¹¹⁹

In the case of the Romanian miners’ jobs, “due to hard working conditions”⁴⁷ the healthy worker effect may have likewise been a significant factor, and may therefore also explain the lack of the expected higher degree of renal functional impairment in the former Romanian miners. Finally, other methodological shortcomings limit the usefulness of the epidemiological study’s results, e.g., because of fear of being ostracized by the society due to BEN, a subset of individuals refused to participate in the screening, even when requested by the nephrologist,⁴⁷ who probably would not make such requests without cause. Therefore, the lack of data due to the refusing subset would have also likely skewed the study’s results in favor of not finding an association between exposure to Pliocene lignites and the etiology of BEN.

Why does not the Roma population develop BEN?

Some researchers have asserted that the Pliocene lignite hypothesis “cannot explain the ethnic character” of the disease,⁴⁷ i.e., why the Roma population does not develop BEN. However, in the exposure pathway of the Pliocene lignite hypothesis (see the Exposure Pathway is Unlikely section) long-term exposure by way of a settled population is required for development of the disease, which is not consistent with nomadic gypsy life.

However, long before a discussion on the 10–30 year exposure duration to organic compounds for disease development in the Roma is needed, we must be cognizant of the many factors that would influence the accuracy of the known incidence and prevalence rates of BEN for this unique population. For example, the Roma have substantial barriers to accessing health services, such as extreme poverty and high illiteracy, geographical isolation, cultural differences, discriminatory practices by health care providers, and mistrust of health care workers especially among elderly Roma.¹²⁰ These and other factors would function to lower known incidence and prevalence rates of BEN from that of true rates. Assuming, however, that known incidence and prevalence rates of the Roma population are found in the future to accurately reflect the burden of the disease (and they have met the additional exposure requirements of the Pliocene lignite hypothesis), their lack of BEN may be a result of their distinctive genetic profile due to intergroup endogamy.¹²¹

Conclusions

All hypotheses for the origin of BEN have limitations, and the Pliocene lignite hypothesis is no exception. However, for the first time in the literature, the limitations of the Pliocene lignite

hypothesis have been addressed in a systematic manner. Of the three environmental hypotheses for which there is the most compelling evidence, the Pliocene lignite hypothesis best accounts for both the geographic distribution and constancy of BEN, and results from many studies have shown that the association between Pliocene lignite deposits and endemic BEN areas is not simply due to chance.

As more fieldwork has been conducted, more lignite deposits, including Pliocene lignites, are being discovered in and around endemic areas not previously associated with lignites. In addition to accounting for BEN on the regional level, the hypothesis has the potential to account for BEN incidence and prevalence on the village level, household level, and individual level, including the potential to explain the gender difference in incidence and prevalence. The Pliocene lignite hypothesis is also consistent with: a genetic predisposition for BEN, the association of BEN with increased rainfall, incidence rates as a result of water treatment, and BEN not being a new disease, nor found in other regions of the world. Studies have shown that the proposed exposure pathway of the Pliocene lignite hypothesis is likely, and that the low concentrations of organic compounds in endemic area water supplies are also consistent with the hypothesis. Organic geochemistry and epidemiological studies with conclusions contradictory to those in support of the Pliocene lignite hypothesis have suffered from methodological shortcomings that have impacted the results on which their authors' interpretations were based.

Though residents of endemic areas are exposed to complex mixtures of hundreds of organic compounds at varying doses and their potentially more toxic (including nephrotoxic) and/or carcinogenic metabolites, that moreover, may have additive or synergistic effects, a multifactorial etiology of BEN appears most likely. Notwithstanding, adverse human health effects from exposure to organic compounds via drinking water may not be limited to nephrotoxicity and/or urothelial cancer, nor only to BEN patients. Additional fieldwork, laboratory, and epidemiological research is needed, e.g., well-by-well evaluations in endemic and nonendemic households within the same endemic villages, and person-by-person evaluations of total water exposure in endemic households, for a more complete understanding of the role of organic compounds in the etiology of BEN.

Conflicts of Interest

None

Acknowledgements

Two anonymous journal reviewers are gratefully acknowledged for their expertise and time.

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