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Evaluating Weight of Evidence in the Mystery of Balkan Endemic Nephropathy

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Balkan endemic nephropathy (BEN) is a chronic, progressive wasting disease of the kidneys, endemic in certain rural regions of the Balkan nations Croatia, Serbia, Bulgaria, and Romania. It is irreversible and ultimately fatal. Though this disease was first described in the 1950s, its causes have been a mystery and a source of much academic and clinical contention. Possible etiologic agents that have been explored include exposure to metals and metalloids, viruses and bacteria, and the dietary toxins aristolochic acid (AA) and ochratoxin A (OTA). AA is a toxin produced by weeds of the genus *Aristolochia*, common in Balkan wheat fields. *Aristolochia* seeds may intermingle with harvested grains and thus inadvertently enter human diets. OTA is a mycotoxin (fungal toxin) common in many foods, including cereal grains. In this study, we analyzed the weight of evidence for each of the suspected causes of BEN using the Bradford Hill criteria (BHC): nine conditions that determine weight of evidence for a causal relationship between an agent and a disease. Each agent postulated to cause BEN was evaluated using the nine criteria, and for each criterion was given a rating based on the strength of the association between exposure to the substance and BEN. From the overall available scientific evidence for each of these suspected risk factors, AA is the agent with the greatest weight of evidence in causing BEN. We describe other methods for testing causality from epidemiological studies, which support this conclusion of AA causing BEN.

KEY WORDS: Aristolochic acid; Balkan endemic nephropathy; Bradford Hill criteria; ochratoxin A; weight of evidence

1. INTRODUCTION

Balkan endemic nephropathy (BEN) is a chronic, wasting kidney disease; endemic in certain rural regions near the tributaries of the Danube river in Bosnia, Bulgaria, Croatia, Romania, and Serbia.[1,2] The disease, first described in Bulgaria during the 1950s, presents with signs of uremia—a buildup of nitrogenous waste products in blood, signaling renal failure—in patients aged usually in their 50s.[3,4] Features of the disease include focal occurrence in certain villages, a familial pattern of disease without genetic inheritance, a long incubation period with initial manifestation after residence in an endemic area for 15–20 years, an equal male-female distribution, similar incidence across ethnic groups, restriction to rural communities, and association with upper urothelial tract carcinoma (UUC).[4–6] Ultimately, this progressive, untreatable disease is fatal.[7] Because the potential risk factors for this disease have been a mystery for decades, we sought in this study to use a weight-of-evidence approach to analyze the likelihood of the prominent postulated risk factors in causing BEN.

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1.1. Background

The epidemiology of BEN has unusual facets. Within an endemic area in the Balkans, villages only a few kilometers apart may have very different patterns of risk, with some villages unaffected and others with high disease prevalence.[8] The disease has been found at different elevations in different nations: about 100 m in Croatia, 130 m in Bosnia, 200 m in Serbia, and 200–300 m in Romania.[8,9] A frequent history of flooding is common in all BEN-endemic areas except Bulgaria, which has endemic areas located on well-drained land.[8] The estimated prevalence of BEN has varied over time, and may depend on the criteria used to diagnose the disease. Studies place BEN prevalence estimates at 0.5–4.4% in endemic areas across the Balkans in general, and suggest the incidence may be decreasing with time.[10] On the other hand, in the Kolubara River region in Serbia, BEN prevalence was observed to increase from 6.4% in 1971 to 8.9% in 2002.[11,12] Current estimates of BEN prevalence in Croatia ranged from 0.3–2.3% with a mean of 1%.[13]

1.2. Historical and Current Speculations of the Etiology of BEN

The increase in average life expectancy in the Balkans post-World War II (WWII) has allowed BEN to manifest in the current population because of the relatively late onset of clinical symptoms. Previously, the average life expectancy of about 50 years in rural Balkan communities meant that individuals who might have developed BEN would likely have died earlier of other causes.[6] Hence, the etiologic agent for BEN may have been present in endemic areas well before WWII. Within the last 50 years, the cause of BEN has been much debated with a wide range of hypotheses. Some villagers in endemic areas still wear pendants and perform ritual ceremonies to be spared from the disease.[6] Poor epidemiologic data from many endemic areas, lost data due to social and political upheavals, and the relatively limited knowledge of nephropathies in the past have made research on finding the cause of BEN difficult in general.[6]

Multiple risk factors have been suggested as etiologic agents of BEN, including plant toxins, fungal toxins, toxin exposures resulting from hydrogeochemical factors, genetics, bacteria, viruses, metals and metalloids,[14] selenium deficiency,[15,16] and X-ray emitting compounds.[6,8,17] Long and Voice[18] had previously examined the role of environmental exposures in the mystery of BEN using an exposure analysis model. Since then, more evidence has emerged either supporting or refuting the role of exposures to key suspected risk factors.

1.2.1. Metals and Metalloids

Various studies have explored the hypothesis that either low or high levels of certain metals or metalloids may cause BEN. Among them, silica, lead, uranium, copper, cobalt, zinc, manganese, arsenic, titanium, barium, aluminum, chromium, strontium, cadmium, bismuth, molybdenum, nickel, tungsten, antimony, and tin have all been assayed in water and soil in BEN-endemic areas.

In two studies, BEN has been associated with high levels of these elements in water,[19] and tumor formation has been linked to exposure to silica and nickel.[20] On the other hand, metal assays in water and soil in BEN-endemic areas have found that measured levels all fell below the detection limits of the analytical methods used.[17] Selenium deficiency was further explored by Maksimovic *et al.*[16] and Maksimovic and Djujic,[15] whose studies concluded that there was inadequate evidence that selenium deficiency was a causative agent. In a later review of the evidence, Batuman reports that selenium was found to be uniformly distributed between endemic and nonendemic areas, and is highly improbable to be a cause of BEN.[4]

1.2.2. Bacteria and Viruses

Viruses and bacteria have also been implicated in contributing to BEN.[21] Apostolov and Spasic[22] suggested that coronaviruses could be transmitted to humans from pigs as a possible cause of BEN. Antibodies for the papilloma viruses BK and SV40 were analyzed in both BEN patients and healthy controls.[23] Results indicated the presence of antibodies for the BK virus in the endemic area; however, antibodies were also frequently found in the healthy population. Shindarov *et al.*[24] failed to establish the presence of viruses in cultures prepared from kidneys of fatal BEN cases. Another viral explanation by Radovanovic[25] speculates on the post-WWII use of DDT in the Balkans and its relation to the cat population. As DDT use increased, the cat population decreased, causing an increase in rodents that could have vectored viruses potentially involved in causing BEN. However, the presence

or absence of viruses, both specifically and generally, have not been found to be significantly different in BEN-endemic versus non-BEN-endemic populations. Some bacteria such as *Escherichia coli* have been suggested as a cause of BEN due to a similar nephropathy induced in exposed laboratory animals.[26] In humans, however, no bacteria was found in most BEN patients' urine samples.[27] The body of evidence is inconclusive about the role of viruses and bacteria in BEN etiology.[28]

1.2.3. Hydrogeochemical Factors: Pliocene Coal

Some BEN-endemic areas are located on large deposits of Pliocene lignite; thus, chemicals contained in this lignite have been hypothesized to potentially cause or contribute to BEN. Groundwater used for drinking water is thought to contain polycyclic aromatic hydrocarbons (PAH) and aromatic amines from the low-rank coals. Although the PAH concentrations may be low, long-term exposure and bioaccumulation in body tissue may lead to adverse health effects, including symptoms of BEN.[6,17]

Analyses by Feder *et al.*[29] indicated the presence of PAH and aromatic amines in groundwater samples from endemic villages. Goldberg *et al.*[30] reported that water samples from BEN households in endemic areas had excitation-emission matrix spectra not found in samples from nonendemic villages. Two water samples from endemic areas and one from a nonendemic area in Serbia showed high concentrations of naphthalene, fluorine, phenanthrene, and pyrene.[31] This study was followed by the findings that levels of aliphatic and aromatic compounds were higher in water samples in BEN-endemic areas than in those from nonendemic areas in Romania.[32] In contrast, a more recent study[33] found no detectable levels of any of the 16 priority pollutants designated by the U.S. Environmental Protection Agency, including these chemicals in water samples throughout the Balkans, and no difference in waterborne concentrations of these pollutants between BEN-endemic and nonendemic villages.

1.2.4. Genetics

Studies have identified a specific marker on a chromosome (3q25) frequently involved in spontaneous aberrations, chromosome fragility, and radiation-induced abnormalities.[34–36] It has been proposed that any 3q25 aberration could explain the familial development of BEN in the absence of exposure to a BEN environment. From an epidemiological standpoint, however, there are many factors that make the genetic explanation unlikely. These include similar disease prevalence across different ethnic backgrounds living in the Balkans; disease occurrence in immigrants to BEN-endemic areas; lower rates or absence of the disease among people who left the BEN-endemic area; and decreasing incidence in some traditional focal areas for the disease, although the same family lines (i.e., genetically related individuals) continue to live in those areas.[8]

1.2.5. Ochratoxin A: A Foodborne Fungal Toxin

Ochratoxin A (OTA) is a naturally occurring foodborne mycotoxin found worldwide in a wide variety of agricultural commodities, ranging from cereal grains and dried fruits to wine, chocolate, and coffee. It is produced by several different fungi, including *Aspergillus ochraceus, Aspergillus carbonarius, Aspergillus niger*, and *Penicillium verrucosum,*[37] under conditions in which food is improperly stored (excessive moisture and temperature). Based on the similarity between the histopathology of BEN and OTA-induced porcine nephropathy, it was hypothesized that OTA might be involved in the etiology of BEN.[38]

The kidney is the main target organ for OTA. OTA is a potent renal carcinogen[39,40] and is also hepatotoxic, teratogenic and immunotoxic in several animal species.[41–44] While OTA's carcinogenic mechanism is unknown, Pfohl-Leszkowicz *et al.*[45] hypothesize that two possible mechanisms may exist: covalent DNA adduction of OTA and oxidative stress-related DNA damage. More recently, Mally[46] described a carcinogenic mechanism based on OTA-mediated disruption of mitosis leading to genetic instability and increased proliferative drive.

Both Krogh *et al.*[47] and Pavlovic *et al.*[48] found 8–12% of cereal samples from endemic areas in the Balkans containing OTA. Food surveys in Bulgaria in the 1980s and 1990s found that staple foods contained more OTA in BEN-endemic than nonendemic areas, as well as occasional high levels of OTA (>2 ng/mL) in serum samples from affected families.[49–53] Many other previous epidemiologic studies conducted in multiple countries throughout Europe and Northern Africa[54–63] have correlated higher serum OTA levels in patients with kidney and urinary disorders, compared to healthy controls.[64] However, serum OTA has not been validated as a biomarker of OTA exposure.[65]

While it appears there is suggestive evidence pointing to OTA as an etiologic factor in BEN,

Grollman *et al.*[5,66] and others provide several arguments against OTA's involvement in this disease. OTA has been detected in human blood and serum samples throughout Europe while BEN is only limited to villages in endemic Balkan regions, and chemically characterized OTA-DNA adducts do not form under physiologic conditions in rodents or cultured cells treated with high levels of OTA.[46,67] A recent systematic review of epidemiological studies on OTA found no evidence of an association with BEN. Nephritic syndrome was the only health effect significantly associated with OTA exposure.[68]

1.2.6. Aristolochic Acid: A Plant Toxin

Aristolochic acid (AA) is a toxin found in certain plant species of the genus *Aristolochia*. For thousands of years, *Aristolochia* plants have been used for medicinal purposes, particularly for women in childbirth as a means by which to hasten labor and delivery. Nearly 200 years ago, the toxicity of certain *Aristolochia* species was elucidated in humans and various animal species.[69] However, it is still an ingredient in certain Chinese herbal medicines consumed worldwide. In the early 1990s, about 1,800 Belgian women who consumed weight loss supplements developed rapidly progressive renal interstitial fibrosis leading to chronic renal failure.[70] Associated with these cases was the ingestion of *Aristolochia fangchi*, an ingredient in herbal medicines used as part of the weight-loss program. Of those who ingested the herbal supplement, 5% developed end-stage renal disease (ESRD). This combination of health effects was first named Chinese herbs nephropathy (CHN). Based on the finding of aristolactam DNA adducts (AL-DNA) in the renal tissues of diseased patients,[71] along with animal studies showing carcinogenic effects of AA on rabbits,[72] CHN was renamed aristolochic acid nephropathy (AAN).

Because of the similarities in BEN disease outcomes to AAN, AA remains a suspected to be a cause of BEN. One possible exposure route in these Balkan populations, which do not typically use Chinese herbal medicines, is that *Aristolochia* weeds growing in grain fields disperse seeds containing AA that commingle with the grains.[73] Hence, the postulated human route of exposure is inadvertent ingestion of AA-contaminated grain-based products such as bread.[5] After AA is consumed and metabolically activated, it reacts with DNA to form DNA adducts. It has been proposed the mutagenic AL-DNA adducts initiate A:T→T:A transversions in the p53 tumor suppressor gene.[5]

1.2.7. Additional Environmental and Geological Considerations

With regard to all of these possible etiological agents in BEN, it is important to consider the larger context in which they occur; namely, the environmental and geological settings in the Balkans that may or may not contribute to BEN prevalence. Various environmental conditions in these settings may enhance or reduce the presence of certain risk factors. As succinctly described in Long and Voice,[18] more flooded areas with poor soil conditions and less cultivation had been associated with greater abundance of *Aristolochia clematitis*. Additionally, growers' methods of harvesting, threshing, and bundling the grains, which could commingle *Aristolochia* with cereal grains, would increase the likelihood that plant tissues containing AA could contaminate the food supply. Of course, some of these conditions, such as flooding, could also increase the likelihood that OTA would develop in grains. Finally, there is the possibility that multiple risk factors work together to increase BEN risk.

2. METHODS

In this study, we performed a systematic review to evaluate the weight of evidence for each of the most prominent hypothesized risk factors implicated in BEN using the Bradford Hill criteria (BHC). The BHC, or Hill's Criteria for Causation, were described in 1965 as a set of conditions that provide a general guideline to determine evidence of a relationship between a suspected agent and a disease, from an observed association to the verdict of causation.[74] A brief summary of the nine criteria, defined by Sir Bradford Hill, which we use to evaluate the weight of evidence for each possible risk factor of BEN, are outlined in Table I. Our analysis is similar to those carried out by Rhomberg *et al.*[75] and Perrio *et al.*[76]

Each suspected cause was evaluated on the following nine criteria. First, *strength* of association between each suspected risk factor and the disease BEN was evaluated where possible. A strong association is more likely to have a causal component than is a modest association. Second, the *consistency* of the relationship was determined. A relationship between an agent and a disease that is observed repeatedly over time, and replicated in different studies by different investigative teams, is more likely to indicate

Table I. Summary and Description of the Bradford Hill Criteria

Criterion	Summary/Description
Strength	A strong association is more likely to have a causal component than is a modest association; higher correlation means there is a higher association between the agent and its effect
Consistency	A relationship is observed repeatedly; results are replicated in different studies using different methods
Specificity	A factor influences specifically a particular outcome/population; a single cause produces a specific effect
Temporality	The factor must always precede the outcome
Biological gradient/ dose-response relationship	The outcome increases monotonically with increasing dose of exposure; increasing exposure increases risk
Plausibility	The observed association can be plausibly explained by biological explanations
Coherence	A causal conclusion should not fundamentally contradict present knowledge
Experiment	Causation is more likely if evidence is based on randomized experiments
Analogy	For analogous exposures and outcomes, an effect has already been shown

Source: Refs. 74 and 154.

a causal association. Third, *specificity* was evaluated based on the criterion that the association is limited to specific populations and to a particular disease. Ideally, there is no association between the agent and other diseases, although Bradford Hill states that this point should not be overemphasized, as it is entirely possible for an agent to cause two different types of cancer or for a foodstuff to be a vector of multiple types of infectious agents, for example.[74]

Fourth, a suspected cause was deemed *temporally consistent* if exposure to the risk factor preceded the outcome (the disease BEN). The fifth criterion evaluated was *biological gradient/dose-response relationship*: whether higher exposures to the suspected agent are associated with higher incidence of disease in populations. Sixth, *plausibility* was evaluated: that a biologically plausible mechanism may exist for an agent to cause the disease.

Seventh, a suspected cause was considered *coherent* if the causal conclusion does not contradict generally known facts of the natural history and biology of the disease. *Experiment*, the eighth criterion, was scored high if by removal or absence of the risk factor, the disease risk was reduced in a population. Ninth, and finally, *analogy* is the criterion for which the body of evidence for each suspected cause was evaluated. For this criterion to be satisfied an effect must already be shown for analogous types of environmental exposures and outcomes.

In our study, we performed a systematic review for each of the suspected causes of BEN, and evaluated each for weight of evidence in causing the disease using each of the criteria described above. Each evaluation per agent/criterion pair is labeled "No Score," "Low" for low likelihood of association, "Moderate" for a moderate association, or "High" for a strong association; and an explanation is given based on the body of literature relevant to each evaluation.

Additionally, we investigated the possible use of other causality frameworks for the evaluation of the etiology of BEN. Epidemiologic models[77]—the epidemiologic triad, wheel of causation, web of causation, and causal pie model—were researched and determined if they would provide a more objective guide to causation compared to the BHC. Other causal inference designs, specifically, those listed in Cox,[78] were examined; and those for which a sufficient literature exists regarding BEN and its possible causes were applied to the question of which etiological factor was most likely to be the cause of BEN.

3. RESULTS

Table II summarizes the main suspected risk factors for BEN, and the weight of evidence linking each to the disease according to each of the nine BHC. Included among the evaluated etiological factors in Table II are AA, OTA, pliocene-coal-associated chemicals, and bacteria/viruses. Not included in Table II are metals/metalloids and genetics. Metals and metalloids have been eliminated as a cause of BEN due to the multiple studies over decades that have found that levels of heavy metals in BEN-endemic areas were below the limit of detection.[4,17,79] Limited studies exist that examine the relationship between genetic predispositions and the development of BEN. An equal risk for people from different ethnic backgrounds, disease occurrence in immigrants to endemic BEN areas, and absence of disease among the progeny of people who have left endemic

Table II. Evaluation of the Suspected Causes of Balkan Endemic Nephropathy, Using the Bradford Hill Criteria

	Aristolochic Acid	Ochratoxin A	Pliocene Coal/PAHs	Bacteria/Viruses
Strength	AL-DNA adducts in BEN patients renal tissues taking CHMs; no adducts in patients in nonendemic areas[5] AA consumption in Mu-Tong[86] >60 mg: OR 1.6 (1.3–2.1) >150 mg: OR 1.4 (1.1–1.8)	OTA-DNA adducts identified in UTTs of humans[7] OTA in maize and beans detected at highest levels in endemic areas[52] OTA in blood and urine detected at highest levels in endemic areas[89]	PAHs detected in groundwater samples in endemic villages[28] Toxic substances found in water collected in BEN villages at higher levels than nonendemic areas[33]	Coronaviruses isolated from pigs associated with BEN[28] Antibodies for papilloma viruses isolated from BEN patients[6] Also in healthy subjects in BEN areas[6,23]
	High	Moderate-high	Moderate-low	Low
Consistency	Data consistent over geographic regions Belgium, Taiwan, India[70,95,97]	Inconsistent over geographic areas OTA detected at high levels in areas with no history of BEN[65,121,122]	Inconsistent data Endemic areas not associated with Pliocene coal[33] Pliocene coal found in nonendemic areas[6]	Inconsistent data Viral particles not isolated in all BEN patients[123]
	High	Low	Low	Low
Specificity	The health outcomes for AA are specific to the urinary systems in humans AA has been associated with other adverse health outcomes such as ESRD, UTTs, and other transitional cell carcinomas[5,69]	The health outcomes for AA are specific to the urinary systems in humans OTA has been associated with other adverse health outcomes such as ESRD, UTTs, nephritic syndrome, and other transitional cell carcinomas[118,126]	The health outcomes for PAHs are not specific to BEN PAHs such as pyrene, naphthalene, fluorine, and phenanthrene, which have been discovered in well water associated with Pliocene lignoites, have been associated with other adverse health outcomes; these outcomes include hemolytic anemia, kidney damage, liver damage, and jaundice, among others[130]	The health outcomes for bacteria and viruses are not specific to BEN Leptospira infections, coronaviruses, and papova viruses have all been associated with other adverse health endpoints other than BEN[129,131]
	High	High	Low	Low
Temporality	Exposure precedes outcome unless genetic predisposition is proven	Exposure precedes outcome unless genetic predisposition is proven	Exposure precedes outcome unless genetic predisposition is proven	Exposure precedes outcome unless genetic predisposition is proven
	High	High	High	No score
Dose-response relationship	Linear dose-response relationship between UTTs and dose of AA in humans[86] Benchmark dose modeling shows exposure to 162.8 g of AA results in 10% risk of renal disease to population[86]	Higher levels of OTA in blood and urine in BEN patients compared to healthy individuals[89]	No dose-response data exists in humans Higher levels of PAHs in BEN-endemic areas compared to nonendemic areas[33]	No dose-response data exists in humans
	High	Moderate	Low	No score

(*Continued*)

Table II. Continued

	Aristolochic Acid	Ochratoxin A	Pliocene Coal/PAHs	Bacteria/Viruses
Plausibility	AA forms adducts with DNA[5] Mutations in p53 tumor suppressor gene[5] 78% A:T to T:A mutation rate in tumors[5]	OTA promotes adduct formation and increase DNA mutations[7]	No specific mechanism proposed due to variety of PAHs found in water contaminated from Pliocene coal	Biologically plausible, no mechanistic data exists
	Moderate-high	Moderate	Low	Low
Coherence	Coherent with the biology of BEN as a renal disease, AA and OTA specifically exert their toxicological effects in the renal system	Coherent with the biology of BEN as a renal disease, AA and OTA specifically exert their toxicological effects in the renal system	Not renal specific, but does not mean that the biological explanation that they may cause BEN is not coherent	Not renal specific, but does not mean that the biological explanation that they may cause BEN is not coherent
	High	High	Moderate	Moderate
Experiment	Laboratory evidence exists for animals; epidemiologic evidence exists for humans exposed to AA[69]	Laboratory evidence exists for animals;[44] epidemiologic evidence exists for humans exposed to OTA[118,155]	Only epidemiologic evidence exists correlating Pliocene coals, PAHs, and BEN[30,32]	Varied laboratory data on animals exists; epidemiologic evidence shows some correlations between BEN and viruses/bacteria[6,22,157]
	High	Moderate	Low	Low
Analogy	AA definitively linked to the CHN/AAN cases in Belgium[74–76] and Taiwan[88,95,96] that are very clinically similar, if not identical, to BEN	Adverse renal effects have been observed in laboratory animals administered OTA; there is no human disease similar to BEN that has definitively been linked to OTA exposure[7,17,88,158]	Benzo[a]pyrene shown to cause liver and renal cancer in mice[145]	Certain viruses have been linked to urothelial carcinoma[147]
	High	Moderate-high	Moderate	Moderate
Overall score	High: 8 Moderate-high: 1 Moderate: 0 Moderate-low: 0 Low: 0 No score: 0	High: 3 Moderate-high: 2 Moderate: 3 Moderate-low: 0 Low: 1 No score: 0	High: 1 Moderate-high: 0 Moderate: 2 Moderate-low: 1 Low: 5 No score: 0	High: 0 Moderate-high: 0 Moderate: 2 Moderate-low: 0 Low: 5 No score: 2

AA = aristolohic acid; AL-DNA = aristolactam-DNA; BEN = Balkan endemic nephropathy; CHM = Chinese herbal medicines; ESRD = end-stage renal disease; OTA = ochratoxin A; PAH = polycyclic aromatic hydrocarbons; UTT = urinary tract tumors.

areas make it unlikely that genetics play a key role in BEN development.[8,80,81]

3.1. Strength

As BEN is strongly associated with upper UUC, evidence of DNA damage in the upper urothelial area caused by a specific agent would lend strength of evidence to that agent's association with BEN. AL-DNA adducts specific to AA exposure have been found in humans—specifically, those with BEN—and replicated in animal studies.[5,71,82–84] A strong association between exposure to AA and development of BEN exists through the finding of AL-DNA adducts in the renal tissues of patients taking Chinese herbal medicines known to contain AA, but not in patients with renal diseases residing in a non-BEN-endemic village.[5] Nortier[85] found that cumulative dose of AA was a significant risk factor for UUC. This was confirmed by Lai *et al.*,[86] who found that prescriptions of Chinese herbal medicines such that lifetime cumulative AA exposure exceeded 150 mg

was associated with higher risk of developing UUC (OR = 1.4; 95% CI = 1.1–1.8). Linking these findings back to high-risk Balkan populations, Jelakovic *et al.*[87] found AL-DNA adducts in 70% of UUC patients residing in a BEN-endemic village, but found no AL-DNA in adducts in 10 renal patients who resided in nonendemic areas. Because of the strong association of the exposure to the specific disease and the evidence of AA-specific DNA damage, AA was rated as "high" based on the BHC evaluation.

Some debate exists about the existence of OTA-DNA adducts.[7,17,38,66,88] ^{32}P-postlabeling analyses of DNA obtained from urinary tract tumors (UTTs) of humans[88] and tissues from animals treated with OTA[7] identified such adducts; moreover, two food survey studies[49,51] found higher OTA levels in food samples from BEN-endemic areas than nonendemic areas. Urinary and serum OTA has been found at higher levels, and more often, in affected families living in BEN-endemic areas compared to people living in non-BEN-endemic villages,[50,52,89] although serum OTA is not a validated biomarker of exposure. In other studies, however, researchers could not detect OTA-DNA adducts in cells or rodents treated with OTA.[46,67] Pfohl-Leszkowicz and Manderville[7] suggest that differences in methodology may explain the interlaboratory differences in not finding OTA-DNA adducts in humans and/or animals. Some associations appear to exist based on epidemiological, biochemical, and geographical studies between OTA and BEN.[50,89,90] Based on the BHC evaluation, OTA was scored as "moderate-high."

It is important to point out the similarities and differences in methodologies associated with both AL-DNA and OTA-DNA adducts, due to the debate surrounding their identification in both humans and animals. Detection of both OTA-DNA and AL-DNA adducts in humans and animals have used ^{32}P-postlabeling/PAGE techniques after DNA isolation and digestion. ^{32}P-postlabeling is commonly used to detect environmental exposures in humans and animals because only microgram quantities of DNA are required for analysis.[91] Specifically, for AL-DNA adducts, Grollman *et al.*[5] obtained renal tumor tissue from women with end-stage renal failure, confirmed their diagnosis with a histopathologic examination, and used the ^{32}P-postlabeling assay to detect AL-DNA adducts. Similarly, the ^{32}P-postlabeling process is used for detection of OTA-DNA adducts in humans and animals. The issue with this process is the reproducibility of results. Several laboratories have been unable to detect OTA-DNA adducts in animal studies.[46,92] Researchers who have successfully isolated OTA-DNA adducts argue that the interlaboratory differences are due to varying details in methodologies.[7]

For Pliocene coal PAHs, the strength of association with BEN is "low." No data exist on these agents causing DNA damage specific to the characteristics of BEN, either in the Balkans or in other parts of the world. Moreover, studies on differential exposure to these agents in endemic and nonendemic areas are limited and conflicting. Goldberg *et al.*[30] reported differences between BEN-endemic and nonendemic villages' excitation/emission matrices of water, and two studies found potentially toxic substances in water collected at BEN-endemic areas at higher levels than in nonendemic areas.[31,32] More recently, however, Voice *et al.*[33] found low PAH levels in both BEN-endemic and nonendemic areas, and no higher levels of dissolved organic compounds that leach from coal in BEN villages compared with nonendemic villages. They concluded that there was no basis to connect PAH exposures, or other exposures associated with Pliocene coal, to the etiology of BEN.

A "low" strength of association exists between bacteria/viruses and BEN. Since BEN has commonly been found in farming communities, close contact with pigs had been postulated to have an association with increased BEN risk.[6,22,92] In turn, coronaviruses have been isolated from pigs and suggested as a cause in BEN; however, evidence supporting this hypothesis is lacking.[6] As for other viruses, antibodies for the papillomaviruses BK and SV40 were isolated from BEN patients; however, antibodies for these viruses were also isolated in healthy subjects from endemic and nonendemic areas.[6,23] Furthermore, broader searches for these viruses in BEN patients have not been successful.[6,24]

Strength of association was highest for AA, due to the number of studies indicating the presence of AL-DNA adducts in individuals with BEN or BEN-like diseases who consumed products containing AA. Furthermore, there is a lack of studies contradicting these results. In the case of OTA, the evidence for adducts that may be related to BEN is more contradictory. Some researchers were not able to detect OTA-DNA adducts, nor to associate them with BEN,[46,93] while others were,[46,92] which led to its lower score when compared to AA. Pliocene coal/PAH etiology in BEN was scored low, due to a lack of information explaining DNA damage specific

to BEN, and conflicting reports on differential exposures in BEN versus non-BEN areas. Finally, data supporting bacteria and viruses were lacking, with some studies unable to detect viruses in BEN patients and others finding viruses in otherwise healthy individuals. For these reasons, bacteria and viruses were scored low as well in strength of association to BEN risk.

3.2. Consistency

Consistency in human data was another criterion that set AA apart from other suspected causes. The presence of BEN/AAN/CHN has been reported consistently worldwide in human populations known to be exposed to AA. CHN, later described as AAN, was first detected in Belgian woman in the early 1990s in women using medicines containing AA.[94] Epidemiologic data from Taiwan showed a strong association between chronic kidney disease (CKD) and the use of herbal medicines known to contain AA.[95,96] Medicines containing AA have also been associated with high CKD rates in India.[97] Exposure to AA in BEN-endemic regions was confirmed by Ivic[98] and Hranjec[73] when they found *A. clematitis* to co-occur with wheat in the Balkans, and the seeds of the weeds to contain AA. Ingestion of wheat contaminated with AA over a period of 8–10 years results in exposure similar to the Belgian women, resulting in BEN.[73] Data are also consistent across animal species.[72,83,99–117] AL-DNA adducts have been found in humans and the carcinogenic and nephrotoxic effects were confirmed by treating rabbits with low doses of AA.[72] These data meet the criterion for consistency at a "high" level.

By contrast, the presence of BEN has not been reported consistently in populations exposed to OTA. While OTA has been found at higher levels in populations affected with various kidney diseases in Egypt,[118] France,[119] Portugal,[120] and Italy,[55] the diseases are not similar to BEN, AAN, or CHN. OTA has also been found at high levels in nonendemic regions not associated with any adverse health effect. These countries include Hungary,[121] Italy,[122] and the United Kingdom.[65] Nor has it been noted in the studies where CHN or AAN have been reported (Belgium, Taiwan) that OTA exposures are higher in diseased individuals than in nondiseased individuals, or in the populations at large to which they belong. In animal studies, OTA-DNA adducts have been identified in rodents and pigs via ^{32}P-postlabeling analyses;[7,88] however, other laboratories have failed to detect OTA-DNA adducts in cells or rodents treated with OTA.[46,67] These data score a "low" rating compared to the available AA data.

Data for both Pliocene coal/PAHs and bacteria/viruses are inconsistent. Pliocene lignite deposits in Slovenia and Croatia are not located in or near BEN-endemic villages.[33] Pliocene lignites are also known to occur in regions outside the Balkans with no association with BEN or a BEN-like disease.[6] As for bacteria or viruses, many studies have not found a bacterial or viral presence in BEN patients. Manev *et al.*[123] did not demonstrate specificity for leptospira infection in the endemic region, while Russev[124] failed to isolate viral particles from cattle bred in the endemic area. Both suspected causes of BEN scored "low" for consistency.

Hence, in the category for "Consistency," AA is the only potential etiological agent of BEN for which no contradictory reports exist, and a consistent body of evidence links AA exposure with BEN or related diseases. For the other potential etiological agents, reports of their association with BEN have been inconsistent.

3.3. Specificity

Specificity was evaluated based on the association being limited to specific populations and to features of the specific disease in question. Both AA and OTA were given scores of "high," as both agents are known to exert their toxic effects primarily on the renal system. AA has been associated with BEN[5] along with various urothelial cancers and transitional cell carcinomas in humans and animals.[72,85,125] Similarly, OTA has been associated with BEN and various adverse effects to the human and animal renal systems, including nephritic syndrome, renal cancers, and ESRD.[118,126–128] Pliocene PAHs and bacteria and viruses were scored "low" because of their potential to cause adverse health effects in virtually all systems of the body, rather than effects specific to the renal system.[129–131]

3.4. Temporality

A score of "high" was given for temporality for all suspected causes of BEN except bacteria and viruses, which were given "no score." Temporality is satisfied if exposure to the suspected agent precedes the onset of disease. Because the onset of BEN occurs late in life, typically between the ages of 50 and 60, it is likely for Balkan residents to have been

exposed to AA, OTA, and Pliocene coal PAHs at chronic low levels all of their lives.[17,132] It is plausible that exposure to implicated bacteria or viruses could precede diagnosis of BEN, but no evidence exists to suggest that earlier exposures to the specific bacteria and viruses implicated, and subsequent infections, lead eventually to onset of BEN.

3.5. Biological Gradient/Dose Response

AA rated "high" for the dose-response criterion. Many studies examining dose-response relationships for AA in a variety of animals have shown increasing risk of adverse health effects corresponding to increasing doses of AA.[72,83,99–117] In humans, Muniz Martinez *et al.*[133] analyzed the impact of *A. fangchi* total ingested doses on the progression rate of AA nephropathy among AAN patients in Belgium and determined that higher intake of *A. fangchi* was associated with an increased risk of renal dysfunction in AAN patients. In addition, Lai *et al.*[86] documented a monotonically increasing dose-response relationship between consumption of Chinese herbal products containing AA in Taiwanese populations and the risk of ESRD.

Multiple animal studies have examined the dose-response relationship between OTA and various health effects, particularly renal toxicity.[37,38,134–143] The dose-response relationship for OTA causing adverse effects in humans, however, has not been studied as vigorously as AA. Higher levels of OTA in blood and urine are found in patients with BEN or UTTs compared to healthy individuals; however, not all results reached statistical significance.[50,52,89] No human studies have looked at exposure levels of OTA and BEN as a health endpoint. Hence, OTA is rated "moderate" in this criterion.

For Pliocene-coal-related chemicals, no dose-response data exist for humans in regard to their association with BEN or UUC. Previous studies have shown higher levels of PAHs in BEN-endemic areas compared to nonendemic areas;[29–32] however, no studies have examined health effects based on varying doses. It should be noted that the studies available are based on sample sizes of no more than 17 endemic and nonendemic villages sampled, which makes determining statistical significance difficult.[33] Hence, the Pliocene chemicals are rated "low" for this criterion. No dose-response data exist for any bacteria or viruses with respect to BEN or related diseases; thus, this risk factor was given "no score."

In summary, for the criterion of dose-response relationships, only AA exposure has been shown to result in increased risk of a BEN-related disease with increasing doses, at more than one dose group in an experiment, in both animal and human studies. For each of the other potential etiological agents, dose-response data are more lacking. Several studies show increased renal toxicity with increasing OTA doses in animals, but such studies are lacking for BEN-related diseases in both animals and humans.

3.6. Plausibility

The criterion "Plausibility" would ideally cover both toxicological and exposure plausibility. From the toxicological perspective: Does a reasonable mode of toxicity for the agent exist that is consistent with the disease pathway? From the exposure perspective: Does a reasonable explanation exist for how individuals or populations are exposed to the agent?

While there is debate as to the disease pathway of BEN, mutagenic DNA adducts are a plausible precursor. The hypothesized mode of AA toxicity is that after metabolic activation, AA reacts with DNA to form covalent AL-DNA adducts. Specifically, mutational analysis of the p53 tumor suppressor gene shows that patients in the BEN-endemic areas had A:T→T:A mutations at a rate of 78%: a mutation specific to AA exposure.[5] On the other hand, the postulated exposure route has not yet been definitively proven. Although, as described earlier, *Aristolochia* plants were found growing in Balkan grain fields and their seeds were found to contain AA, no AA has actually been detected in food in the Balkans. Hence, the plausibility of AA's role in causing BEN is rated as "moderate-high" in the plausibility category.

OTA's plausibility was scored as "moderate." Mally[144] has recently proposed a mode of action for the renal carcinogenicity of OTA, involving genetic destabilization and increased proliferative drive due to OTA-mediated disruption of mitosis, in which the site specificity of tumor formation is caused by selective renal uptake of OTA into the proximal tubule epithelium. But because human populations exposed to similar levels of OTA in world regions other than the Balkans have no documentation of BEN incidence, and because there is no evidence of high OTA exposure in world regions where CHN/AAN patients were found, the plausibility of an association between OTA and BEN is questionable.

Pliocene lignite coals are chemically complex and may produce varying constituents in drinking water. Toxicological mechanisms are known for some PAHs, like benzo[a]pyrene; however, the levels to which humans are exposed to various PAHs in the endemic areas are unknown. A "low" rating was given for PAHs because it remains biologically plausible that they may cause BEN, but mechanistic data and epidemiological evidence specific to the disease are lacking. Finally, it is biologically plausible that a virus or bacteria can cause BEN; however, no mode of action or epidemiological evidence exists linking a virus or bacteria to BEN. For this reason, bacteria/viruses were given a "low" rating.

3.7. Coherence

The link between a suspected etiological agent and a disease considered *coherent* if the causal conclusion does not contradict generally known facts of the natural history and biology of the disease. Based on what has been described above in Section 3.3 on specificity of the agent to the disease, AA and OTA both score "high" on the criterion of coherence. Coherent with the biology of BEN as a renal disease, AA and OTA specifically exert their toxicological effects in the renal system. Moreover, regarding the history of the disease in the Balkans, it is entirely plausible that certain Balkan populations have been exposed to both AA and OTA in their diets for at least several decades. The fact that Pliocene-coal-related chemicals and bacteria and viruses are not renal specific does not mean that the biological explanation that they may cause BEN is not coherent, but that the weight of evidence is relatively weaker. Hence, these two potential risk factors are scored "moderate" on the criterion of coherence.

3.8. Experiment

The "experiment" criterion, by Bradford Hill's definition,[74] is fulfilled if, by removal or absence of the risk factor, the disease risk is reduced in a population. No studies exist on *removal* of any of the postulated risk factors of BEN and resultant reduction in BEN incidence in high-risk Balkan populations. However, experiments do exist comparing the incidence of BEN and its associated diseases, such as urinary tract cancers, in populations in the presence versus *absence* of certain risk factors.

In the Balkans, the AL-DNA adduct, a biomarker of exposure to AA, was present in 70% of a cohort in UUC patients in a BEN-endemic population and in 94% of upper urothelial carcinoma patients with the specific TP53 mutation for AA. In contrast, neither the adducts nor the specific DNA mutation leading to UUC was detected in the UUC patient cohort from non-BEN-endemic regions.[89] Similarly, it was clearly found that UUC risk was significantly higher in Taiwanese individuals who were prescribed over 150 mg AA in Chinese herbal medicines than those who had been prescribed less than this cumulative amount.[88] Hence, AA scores "high" in the criterion for experiment.

Similarly, studies on the differential exposures to OTA in BEN-endemic versus non-BEN endemic villages exist.[54,56,91] However, there are also the countervailing issues that other relatively highly OTA-exposed populations around Europe and other parts of the world do not have reported BEN incidence, and the populations that do report CHN, AAN, or UUC are not known to have high OTA exposure. Hence, the body of evidence is conflicting, and OTA scores "moderate" on this criterion. For Pliocene-coal-related chemicals and bacteria and viruses, the experiment score is "low," as there is no evidence that removal or absence of these risk factors corresponds to a lower risk of BEN.

3.9. Analogy

In the final Bradford Hill criterion, an agent is ranked highly in "analogy" if it can be shown that it, or a similar agent, causes analogous effects to the disease of interest. In this regard, AA ranks "high." AA has been definitively linked to the CHN/AAN cases in Belgium[74–76] and Taiwan[88,95,96] that are very clinically similar, if not identical, to BEN. Moreover, the mechanism by which AA causes these renal effects has been characterized.[69] OTA ranks "moderate-high" in analogy because although adverse renal effects have been observed in laboratory animals administered OTA, there is no human disease similar to BEN that has definitively been linked to OTA exposure. Bui-Klimke and Wu[68] describe a statistically significant association between OTA exposure and nephritic syndrome; however, this syndrome has a wide variety of symptoms, and does not have features unique to BEN. Among the chemicals associated with Pliocene coal, benzo[a]pyrene has been shown to cause liver and renal cancer in mice.[145] A quantitative review of occupational exposures to PAHs showed a higher risk of bladder cancer in PAH-related occupations.[146] Similarly, certain viruses have been linked to urothelial carcinoma.[147] As these diseases have some, but not

all, features similar to BEN, and effects are not similar across all PAHs and viruses, both categories receive a "moderate" score for analogy.

3.10. Other Causal Inference Models

Causal inference models other than the Bradford Hill Criteria provide alternative methods for considering weight of evidence. In a review on epidemiological evidence for a bacterium causing Crohn's disease, Uzoigwe *et al.*[77] describe four epidemiological models for causation: the epidemiological triad, wheel of causation, web of causation, and causal pie model. The epidemiologic triad model examines host, environment, and agent factors of a particular disease: factors that are already described in the above analyses. The wheel of causation model places genetic factors in the center of the wheel, and varies the size of biological, social, physical, and host components depending on their influence. In this specific instance of BEN, such a model would only be useful if it were presupposed that BEN were related to genetic factors, but evidence exists to the contrary.[8,80,81]

Finally, causal inference methods outlined by Cox[78] were also considered, and several for which sufficient studies existed on BEN were applied to examining BEN etiology. Cox's article describes formal methods to model and test causal hypotheses, of which three are relevant for this analysis: conditional independence tests, counterfactual and potential outcome models, and negative controls.

Under *conditional independence tests*, by which dependence of disease risk on location can be explained by variations in measured exposures and disease rates at different locations, AA is the only etiological agent described above that passes the conditions. Consistently, where AA exposure is higher, human disease is higher; and where it is lower or nonexistent, there is no reported BEN or related diseases. The other agents (OTA, Pliocene coal/PAHs, and bacteria/viruses) are inconsistent in whether human exposures differ in BEN-endemic versus nonendemic regions of the Balkans and of the world. For example, OTA exposure is as high or much higher in other regions of the world than the BEN-endemic areas,[68] yet BEN or a similar disease is not prevalent in other highly exposed populations.

Using *counterfactual and potential outcome models*, in which exposed individuals have significantly different disease probabilities than they would have had if they had not been exposed, evidence exists for AA passing this condition (i.e., the Belgian women who consumed the weight loss supplements containing AA versus those who used a different weight loss supplement). For the other etiologies, no such evidence exists.

Finally, using *negative control models*, under which exposures predict health effects better than they predict effects that cannot be caused by exposures, again the evidence is stronger for AA than for other risk factors. AA exposure in humans has specifically been linked with nephropathies of distinct characteristics and carcinomas in the upper urothelial tract. For the other possible etiologies, the effects are inconsistent and/or diverse to different body tissues and organs. Thus, applying epidemiological causality models and methods other than the BHC yields the same conclusions: that AA is the most likely etiological risk factor in BEN.

4. DISCUSSION

BEN has been a mysterious disease for decades because of the unusual spatial epidemiology and unclear roles of potential etiological factors. While the toxicology of certain suspected agents has been reasonably well-established, its combination with epidemiology into a comparative assessment across multiple potential hazards was relatively unexplored. In this study, we applied a systematic review of the literature and weight-of-evidence methodology to examine the likelihood of different risk factors in causing the disease BEN, using the BHC.

In summary, AA scored the highest in the BHC evaluation, rating highly across eight of the nine criteria. Hence, it has the strongest weight of evidence among the prominently debated risk factors in causing BEN. OTA ranked as the next most plausible etiologic factor, although certain inconsistencies across different studies make it unlikely to be a singly acting etiologic agent in BEN. PAHs and viruses and bacteria had low weight of evidence in a causal relationship with BEN.

Other causal inference methods were considered and applied to increase objectivity of our analyses. Epidemiologic models such as the epidemiologic triad or wheel of causation would lead to comparisons that would be similar to our current analysis. Other causal inference methods, such as those described in Cox[78], were also considered to objectively compare etiologic factors. These formal methods to test causal hypotheses all corroborated our conclusions from applying the BHC: that the weight of evidence points to AA as the most probable cause of BEN.

The toxicological and epidemiological evidence linking AA with BEN is convincing in terms of the strength of association, consistency, specificity, temporality, dose-response relationship, coherence, experiment, and analogy. The literature provides consistent and convincing evidence of the dose-dependent relationship between AA exposure and BEN/related diseases in multiple populations worldwide. A mode of toxicity has been proposed as well. We interpret the body of evidence as showing a clear and consistent link between AA exposure and BEN, with no studies to contradict these findings; hence, AA received "high" scores for eight of the nine BHC. The one area in which we assigned less than a high degree of association is plausibility because, to date, no AA has been detected in foodstuffs consumed in the Balkans. Future studies should focus on analyzing grain-based foods in the at-risk rural Balkan areas for AA; and, if possible, analyzing any existing food samples from past years for AA. A comparison should be done on AA content in foods from the BEN-endemic versus non-BEN-endemic regions to further increase the weight of evidence that AA is the causal risk factor for BEN. If AA cannot be detected in the foods, it does not necessarily mean that AA is not the etiologic agent in causing BEN, given the strong evidence provided by the presence of AL-DNA adducts in BEN patients versus no detectable AL-DNA adducts in patients from non-BEN-endemic areas. It may be that the exposure route is different from ingestion of locally grown and produced foods, that AA is currently impossible to detect in grains given available analytical methods, or that food today does not contain AA at the same levels as food in past decades, which may be causing BEN cases today.

In the body of literature available linking OTA and BEN, the associations are more tenuous upon our review. It is possible that OTA plays a contributory role in BEN, but is unlikely to cause the disease on its own; another risk factor such as AA must be present for the disease to be manifested. This is based on studies indicating that OTA exposure is higher in BEN-endemic areas than non-BEN-endemic areas; however, other parts of the world with equally high OTA exposures do not report BEN incidence, and other parts of the world with disease manifestations similar to BEN do not have notably high OTA exposure. Dose-response data linking OTA to BEN/related diseases are lacking in both animals and humans. Hence, OTA was generally ranked to have a "moderate" plausibility of contributing to BEN.

Various gaps in knowledge exist with respect to OTA's role in BEN or other human renal diseases. One of the most debated issues is the detection of OTA-DNA adducts and, relatedly, OTA's mode of toxicity. Some researchers believe they have established genotoxic modes of action for OTA by proving the ability of OTA to form DNA adducts.[7] OTA-DNA adducts have been found in the kidneys of both rats and mice,[148] as well as in the liver and spleen of others,[149] and similar adducts have been found in BEN patients' bladder and kidney tumors.[88] On the other hand, many researchers have been unable to detect OTA-DNA adducts in animals or humans[46,67,93] and argue that any adverse effects of OTA are likely a result of some indirect mechanism involving oxidative stress[150,151] or cytotoxicity.[93] Additionally, a lack of dose-response data in humans and no analogous examples of diseases that OTA may cause in humans are major gaps in knowledge regarding OTA.

There is little evidence in the existing literature implicating Pliocene coal chemicals or bacteria and viruses in BEN risk. Not only have studies contradicted each other in the differential exposures of these in BEN versus non-BEN populations, human health effects of exposure to these agents are diverse and nonspecific, and no dose-response data exist on exposure to these agents and BEN risk. Based on the weight of evidence, these agents are ranked "low" in their likelihood of a causative role in BEN.

A final gap in knowledge concerns the interactive effects of multiple potential risk factors in BEN. For example, it is not known whether individuals who are exposed to both AA and OTA have a synergistically higher risk of developing BEN than those exposed to either agent alone, or whether one agent may potentiate another that alone cannot induce BEN.

Beyond the inadvertent exposures to AA in certain Balkan regions, the risk of broader global AA exposure has recently been brought to light in two studies.[152,153] Because of the use of *Aristolochia* plants in a variety of herbal medicines, its associated human nephropathies may cause a greater burden of disease worldwide than was previously understood. What began as a study of an unusual disease that was thought to be geographically contained—BEN—has expanded to reveal the much larger potential role of

this environmental toxin in causing human disease worldwide.

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