The Journal of Rare DISORDERS

THE CONTINUING MEDICAL MYSTERY OF BALKAN ENDEMIC NEPHROPATHY

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ABSTRACT

Balkan Endemic Nephropathy (BEN) is a disease of subtle onset and insidious progression that typically occurs between the 4th and 6th decade in long-resident individuals in highly specific geographic locations of the Balkan region and affects 1 – 5% of the population. Though it does not follow typical Mendelian genetics, there is a familial pattern of occurrence. Although residents may live only a few kilometers apart, certain locations are highly affected while others close by, even as close as across the road, remain unscathed. Because of this geographic selectivity scientists have searched for an environmental cause. It is thought that exposure to the toxic plant Aristolochia clematitis is to blame. Genotoxic N-heterocyclic or polycyclic aromatic containing coal water leachates entering cultivated soil and drinking water are also a possible cause due to the proximity and predictive power of endemic foci to coal deposits. Evidence for Ochratoxin A fungal poisoning also exists. High levels of phthalates have been measured in BEN-endemic drinking water. BEN is a probably a multifactorial disease that may result from exposure through some of above-mentioned environmental sources, with genetic factors contributing. This review will discuss recent research concerning the etiology, potential therapies for the treatment of nephropathy, and unexplored research directions for this chronic kidney disease.

SCOPE AND HISTORY, TIME TRENDS AND PATHOLOGY

Balkan endemic nephropathy (BEN)[1] is a progressive degenerative disease of the kidney tubulointerstitium that was first described in 1956 in Vratza, Bulgaria[2-4] and has a prevalence of 0.5 – 4%.[5] Geographically and familially, it is highly restricted and oddly distributed. It is usually recognized later in life when kidney function is reduced by ≥80%, followed by kidney shrinkage, microproteinuria, jaundice of the palms and soles, weakness, and kidney failure necessitating dialysis.[6] The co-occurrence of tubulointerstitial nephritis and urinary tract tumors (UTT) was recognized early.[4] Tumors in endemic BEN areas (near tributaries of the Danube River in Bosnia, Bulgaria, Croatia, Romania and Serbia) occur more frequently in women and the elderly, are often bilateral, and more frequently accompanied by renal failure.[7] Of 766 UTT patients seen from 1970-97,[6] 68% were from BEN-endemic areas suggesting that geographic factors must play a role. In a comparative pathology study, the histologic features of 88 tumor specimens from endemic and non-endemic patients were examined in approximately equal numbers. BEN-endemic tumors were more aggressive by tumor grade, invasiveness, growth and metaplastic changes, and were significantly more solid than papillary. For nephropathy dialysis or transplantation are the available treatments and there is no cure, nor are the cause(s) known. In a cruel sequelum, one-third of transplant recipients will later develop tumors of the UTT, kidney, or renal pelvis.

The incidence of BEN decreased in Bulgaria through 1987,[8] however drinking water sources in the Vratza area were changed from shallow wells (**Figure 1**) to deep-aquifer extracted chlorine-treated waters in the early 1960's which could account for the later decline in BEN. Life expectancy prior to World War II was 40 to 50 years, possibly explaining why BEN was not diagnosed until after WWII.[9] In Serbia, BEN incidence decreased by an average of 10% annually through 1989 but non-significant increases of 3.9% per year occurred from 1989-2009.[9] Different factors may be responsible for Bulgarian vs. Romanian BEN (as measured by urinary markers)[10] and UTT (the type of p53 DNA transversions most frequently seen in tumors from different locations, see below). Therefore BEN may be a group of closely related diseases producing a similar outcome. The overall incidence is not high however UTT occurs in 33-50% of BEN patients, and is up to 100 times more frequent in endemic areas. These facts argue that BEN and UTT are related and preventable. It is important to study BEN because chronic kidney disease (CKD) is increasing throughout the world, portending future problems, and it may be prevented or reversed if

detected early. Because of the stability of the population and the unusual geographic distribution much can be learned about the fate, transport, and toxicity of the contaminants.

Figure 1. Public spring in a BEN endemic village from Romania, currently used as a drinking water supply. Many residents from endemic (as well as non-endemic) villages in Romania still rely extensively on the use of untreated shallow aquifers (springs and shallow dug wells) percolating Pliocene coal layers, as primary water sources, although treated water has been supplied to certain villages during the last several years. (Photo courtesy of C. Tatu, taken in September, 2012).

EXPOSURE PATHWAYS: INVOLVEMENT OF AIR, WATER, PAH AND NITRATES

Water is a complex mixture containing anthropogenic and naturally occurring constituents. Niagolova, et al.[11] tested drinking water from endemic and non-endemic BEN villages in Bulgaria and found no significant differences in levels of nitrates + nitrites, but levels exceeded U.S. drinking water standards. In addition, sodium, potassium and chloride were elevated and showed the order wells>springs>taps, which is in the same as the order of proximity to anthropogenic activities. Since those substances are related to anthropogenic activities, the excessive levels of nitrates + nitrites might be as well. More extensive testing could reveal differences between the endemic and non-endemic areas. Ma, et al.[12] tested drinking waters in Henan Province, one of the most populous areas in China, and found PAH levels were significantly elevated at several sites, indicating elevated cancer risk. Ratios of common coal constituents showed that the PAHs were anthropogenic.

Nitropolycyclic aromatic hydrocarbons (NPAH) are found in diesel and gasoline emissions, on the surface of airborne particulates (e.g. fly ash), in stream sediments, smoke and soot in homes where coal or wood is burned, and in grilled food.[13] Taga, et al.[14] assayed NPAH in particulate matter derived from coal-burning and found that several produced mutations, but 3-nitrobenzanthrone (3-NBA) alone was responsible for ~27% of the mutagenic activity. Chakraborty[15] found that water leached from fly ash was directly, significantly mutagenic in standard assays, raising the possibility of human and wildlife effects because slurry is collected in electric power plant holding ponds, which can leak or burst. Samples also contained high levels of metals that are genotoxic, neurotoxic, carcinogenic or developmentally toxic in several species and induced DNA damage in plants grown using leachate water. In that study PAH mutagenicity required metabolic activation with S9 rat microsomal fraction. Others[16-18] have successfully shown the mutagenicity of PAH, NPAH, and oxy-PAH and relationship to the risk of lung cancer. A recent meta-analysis[19] found significant empirical relationships between S9-activated S. *typhimurium* strain TA98 mutagenicity and soil PAH concentration, and significant direct-acting mutagenicity from soil dinitropyrene. PAHs have been found in fog, rain,[20] soil and river water.[21] In the latter study up to 50% of the total mutagenicity of extracts was due to NPAH, phenylbenzotriazole, or 4-amino-3,3'-dichloro-5,4'-dinitrobiphenyl. Lubcke-von Varel, et al.[22] found that fractions from three polluted sites of the river Elbe, (Germany)basin containing NPAHs were direct and indirect-acting mutagens. This polar fraction was eluted using a novel procedure, revealing both non-polar and polar constituents of environmental samples were potent mutagens, estrogens, AhR-

inducers, trans-thyretin-binders, and gap junction intercellular communication-inhibitors. Dichloromethane extraction recovers mainly non-polar constituents and may be less likely to identify 3-NBA.

Exposure to air pollution increases the risk of lung cancer and NPAH have been found within the lungs of non-smokers with cancer[16-18] therefore NPAH may contribute to increased risk. 3-NBA can also form directly from the reaction of atmospheric benzanthrone, the parent PAH, nitrogen dioxide and ozone.[23,24] Rainwater and soils in Japan were found to contain 3-NBA.[25] 3-NBA and the 2-NBA isomer caused oxidative stress and formed DNA strand breaks, but 2-NBA was only about 1/3 as genotoxic.[26] Stiborova[27] recently compared reductive activation and adduct formation by the two isomers. 3-NBA caused mutations in two strains of *S. typhimurium* at the highest frequency ever measured.[23,28] 3-NBA also caused genotoxic micronucleus formation in B-lymphoblastoid and human hepatocellular carcinoma cell lines[29-31] and formation of a unique bulky adduct on DNA in rat lung, liver and kidney by oral, intratracheal, or intraperitoneal injection[32-34] that persisted. It most frequently caused GC→TA transversions on DNA. Compared to aristolochic acid (AA), 3-NBA formed 3 times more adducts, and compared to 2-nitrofluorene, 80 times more.[32] Both AA and 3-NBA are mono-nitroaromatics, and share several features of metabolism in common suggesting they could share a carcinogenic mechanism. Levova[35] found that NQO1 enzyme activity was associated with bioactivation of AAI, the form of AA thought to be responsible for most of the mutagenicity, using Cyp1a knockout and CYP1A-humanized mouse lines. 3-NBA administered intratracheally to rats induced DNA adducts and lung tumors in a carcinogenicity study.[26] Therefore irrespective of the route of exposure, 3-NBA can induce tumors of the lung in rats, but adduct formation in liver and kidney and colon, liver and bladder (mouse,)[36] raises the question of possible tumor formation in other species in these tissues as well. Bleeker et al.[37] compared the mono-nitrogen-containing azaarenes to homocyclic PAHs and determined that typical acute:chronic ratios underestimate chronic toxicity and basing risk assessment of azaarenes on that of homocyclic PAH fails to protect against possibly stronger or other toxic effects, and recommended that chronic toxicity data be collected.

RELATIONSHIP OF BEN WITH CIGARETTE SMOKING AND INCREASED RISK OF BLADDER CANCER

42% of men worldwide smoke cigarettes. In Bosnia and Herzegovina prevalence is 54.2%, in Croatia, 30.6% and in the Czech Republic, 37.1%.[38] Women generally smoke less than men, and the rates quoted do not include smokeless tobacco. In Europe, tobacco smoking and alcohol consumption data match the pattern of overall cancer incidence.[39] Zeegers, et al.[40] performed a meta-analysis and concluded that the odds ratios for bladder cancer in current and former smokers compared with nonsmokers were 3.33, 2.63-4.21, and 1.98 (95% C.I.) and that in Europe approximately half of urinary tract cancer cases among males and one-third among females could be attributed to cigarette smoking. Since 1988[41] it has been known that the risk for renal pelvis and ureter cancers is significantly elevated in smokers, and in workers in the chemical, petrochemical and plastics industries and those exposed to coal, coke, asphalt or tar. NPAH and PAHs are carcinogens present in tobacco smoke, and functional polymorphisms in NAT2 and GSTM1, genes coding for enzymes that metabolize PAH/NPAH are associated with significant increases in bladder cancer risk.[42] Changes caused by a single nucleotide alteration in the metabolic enzyme may increase or decrease the toxicity of the parent PAH/NPAH. Samanic, et al.[43] and Zeegers, et al.[44] found significant associations between bladder cancer and smoking. Zeegers, et al. did not find any association between nitrate intake in food and water and bladder cancer risk in the Netherlands[45] but Ferrucci et al.[46] found a modest association between elevated risk and ingestion of nitrate/nitrite or NPAH/PAH from processed or cooked meat (resp.) in a US population. Vineis, et al.[47] found statistically significant associations between genetic susceptibility and smoking status. Gene deletions, null mutations, transversions, variant alleles, or single nucleotide polymorphisms in GSTM1, NQO1, p53, GSTP1, SULT1A1 and 2, CYP2E1, AKR1C3, ARNT, CYP1A1 and B1 genes have been examined and associations found between bladder cancer risk and smoking incidence in populations of Argentina, Kashmir, Taiwan, and Korea.[42,48,49, 50-52] The NAT2 acetylator genotype affects the pathogenesis of human bladder cancer upon occupational exposure to the aromatic amines benzidine, 4-aminobiphenyl, and 1-naphthylamine, where acetylation is detoxifying. GSTM1 genetic polymorphisms found in association with human bladder cancer support the association of PAH exposure with this cancer[53] because GSTM1 is known to metabolize PAH and its absence would significantly affect detoxification. Aside from one study that excluded smoking status as a factor, all of the above references showed conclusively that smoking

greatly increases the risk of bladder cancer. Exposure to secondhand smoke or ingestion of PAH/NPAH in drinking water increases the risk. There is growing evidence that women are more susceptible to cigarette-smoking-related cancers and COPD than men[112] leading to speculation that hormones play a role.

GENETIC VARIATION IN RELATION TO BEN RISK

Mantle, et al.[10] used 1H NMR to examine urinary metabolites in Bulgarian and Romanian BEN cohorts and compared the results to healthy people from both regions. Using principal component analysis (PCA), they found an unexpected pattern of separation between the cohorts indicating these may be 2 distinct diseases. Alternatively the patients from the 2 groups may have been in different stages of the disease. While BEN is frequently accompanied by UTT, the reverse is not true, therefore the etiology of the 2 conditions may differ or else the UTT represents a condition that stems from BEN but occurs later. Therefore it should not be assumed the UTT seen with BEN is caused by the same agents that produced changes in kidney size and function. Andonova[54] found that the GSTM1 wild type allele associated positively with BEN in patients from the same endemic region, and concluded that the null genotype of GSTM1 could be protective while by contrast Toncheva, et al.[55] presented data that neither GSTM1 null or NQO1 genotype influenced the risk of BEN in Bulgarian patients. But in bladder cancer patients that have the genetically-determined 187Ser allele of the NQO1 gene, p53 transversions and end stage disease are more common.[101] Others[56] showed that the CYP3A5*1 allele, a marker for CYP3A5 expression in human kidney, was associated with an increased risk for BEN in a Bulgarian cohort, while CYP3A4*1B and CYP2D6 genotypes were not. Spontaneous aberrations and chromosome lesions were more prevalent in BEN patients with folic acid deficiency or exposed to x-rays.[57] The same group[58,59] investigated gene amplification in urinary bladder cancer in Bulgarian or other patients and found that erbB-2 (Bulgarian patients) and a group of genes including CCND1, FGF4, FGF3, and EMS1 (others) were amplified in 68% of the 123 tumors with amplifications. Factors of genotypic variation and chromosomal aberration and breakage warrant further study. A recent epigenetic study by Staneva, et al.[60] examined the differentially-methylated regions throughout the whole genome of BEN patients compared with healthy individuals and found common changes in all patient-control pairs. SEC61G, IL17RA, HDAC11 genes contained hypomethylated CpG islands, suggesting that immunologic dysregulation may be important in BEN. This exciting new area of inquiry is promising.

ARSENIC IN DRINKING WATER AND BLADDER CANCER

Some evidence exists for the association of low-level arsenic exposure with bladder, but not kidney cancer. Arsenic has been definitively associated with an increased risk of bladder cancer[61,62] but others[62,63] found that levels of <100-200 µg/L were not associated with an increased incidence of UTT. A meta-analysis by Mink et al.[63] found insignificantly elevated summary relative risk estimates for 'never + ever' smokers while for 'ever' smokers in some sub-groups the risk was significantly elevated (OR = 1.24) but detailed smoking histories were needed. By contrast, a 1999 study in Finland of well-water drinkers found that even when comparing 0 - 0.1 µg arsenic/L and above, relative risks for bladder cancer were significantly increased >0.1 µg arsenic/L.[64] The same study found no association with kidney cancer, and a recent study found no association between the presence of arsenic in blood or urine, and kidney size or function[65] in the adult offspring of BEN patients. Since one study showed that kidney size and function were affected in the adult offspring of BEN parents (particularly, maternal),[66] but not related to arsenic, arsenic in drinking water is not a factor in BEN risk for adult offspring although it is associated with bladder cancer at large.

PHTHALATES

Plasticizers are found ubiquitously throughout the environment and the levels of phthalates were found to differ markedly in concentration between endemic vs. non-endemic villages.[67] Phthalates are compounds with partially aromatic chemical structures that are known endocrine disruptors,[68] teratogens and are developmentally and reproductively toxic.[69-71] Phthalates induce oxidative damage (lipid peroxidation and DNA damage) and apoptosis[72-75] and are classified as Group B2, Probable Human Carcinogen (US EPA), Group 3, Not Classifiable as to Its Carcinogenicity to Humans (IARC), and Reasonably Anticipated to be a Human Carcinogen (US HHS, NIH NTP).[76] Recent evidence links laboratory exposure to phthalates and the occurrence of systemic lupus erythematosus (SLE) in mice[77] in which autoantibodies attack kidney, heart, and lung DNA. Lim[78] found that four strains of mice exposed to di-(2-ethylhexyl) phthalate (DEHP) developed autoantibodies, however one strain (NZB) also developed nephritis, kidney failure and early death. SLE is 5

times more prevalent in women than in men and exposure to phthalates is extensive, especially among women aged 20-40.[77] Mice exposed for 90 days to Nanjing, China municipal drinking water containing PAHs, phthalates, and other organic pollutants developed perturbations in glucose-alanine cycling, branched-chain amino acid and energy metabolism and kidney function.[79] Levels described[67] for two endemic villages were 2 - 2.5 times that of a nearby control village for the phthalates dimethyl, diethyl, di-n-butyl, and di-ethyl-hexyl-phthalate. In 2 BEN villages, actual drinking water exposure levels were many times higher than the Nanjing study and several times higher than in the BEN control village. People living in Balkan villages ingest a much higher amount of phthalates than OSHA standards allow.[76]

PROXIMITY OF BEN TO PLIOCENE LIGNITE COAL DEPOSITS

High- and low molecular weight organics (humic-like substances, aromatic amines, N,S,O-heterocyclics, terpenoids, etc.) and/or minerals may leach from Pliocene coal into drinking water sources in BEN areas, causing disease. **Figure 2** shows an active surface Pliocene lignite coal mine in a BEN-endemic area. Feder, et al.[80] pointed out that all except one of the clusters of BEN villages are located near Pliocene coals. The exception, in Bulgaria, was later found to be near a previously-unknown coal seam[80,81] and BEN occurrence was predicted and later confirmed in areas where low rank coals are found. U.S. Geological Survey studies of water samples collected at least twice yearly from 2000 – 2006[82-89] found that well and spring water samples from BEN villages contained greater numbers and concentrations of low molecular weight extractable hydrocarbons (biphenyls, aromatic amines, terpenes, and N-, S- and O-containing heterocyclic compounds) compared with non-BEN villages.[83] A recent study[90] determined that a greater number and concentration of organic compounds (including phthalates) occurred in lignite coal samples from endemic BEN areas in Romania and Serbia than lignite and bituminous coals from non-endemic regions in Romania and the US. Bunnell et al.[91] showed that well water from BEN villages increased cell proliferation in human kidney cells up to a threshold, beyond which there was toxicity. Pavlovic et al.[92] investigated the role of lecithin acyltransferase (LCAT) and found that water from BEN villages significantly inhibited the activity of human plasma LCAT compared with non-BEN villages or lab controls, and related this to a previously-observed secondary LCAT deficiency found in some BEN patients.[93] Gluhovschi et al.[94] compared glomerular filtration rates (GFR) and proteinuria from patients who had worked for many years in coal mines in endemic and non-endemic villages with active Pliocene mining, non-Pliocene mining, or inactive mining and found no correlation with kidney function or proteinuria. Data were lacking concerning contaminant lev-

Figure 2. Active surface Pliocene lignite mine in the BEN endemic area from Romania. Extensive Pliocene coal layers underlie or are located in the vicinity of the BEN villages in the affected Balkan countries, and they are shallow enough to be percolated by wells used as drinking water supplies. (Photo courtesy of C. Tatu, taken in September, 2011).

els in drinking water sources, years of residence, and family history of BEN. If a correlation had been measured it would not have proved that coal leaching into water was the cause of BEN. Studies providing a link between ingestion of drinking water and BEN should measure components of household water over several decades and record subsequent diagnoses in the same households.

A study of drinking water components by source[11] was discussed above. In a study of PAHs, Voice, et al.[95] examined the Pliocene lignite hypothesis by testing 56 spring, well and tap water samples in Bulgaria during spring, 2000 using a broad definition of endemicity. Levels did not show significant differences; other heterocyclic or aromatic organics such as were found in BEN areas near low rank Pliocene lignites were not tested. PAH levels were within WHO and USEPA regulatory guidelines. The authors stated that analytical methods might not have identified the constituents responsible for BEN due to methodology or uncertainty of targets. Voice, et al. states that: 1) multiple large Pliocene lignite deposits exist in other parts of the world without endemic nephropathy, 2) coals are not highly water-soluble (however low-rank Pliocene coals are more water-soluble and would be likely to release organics),[96] 3) lignite coals are complex and contain constituents that are commonly found in other materials so that a marker of exposure would be difficult to identify (however see 12), and 4) more samples should be collected to decisively prove differences. Studies were conducted to identify other BEN clusters in the US. Bunnell, et al.[97] studied the incidence of RPC in five parishes of northwestern Louisiana close to lignite coal deposits using data from the Louisiana Tumor Registry and found that increased tumors were significantly correlated with organic compounds, fungal Zygomycetes, and 13 chemical elements (phosphates, ammonium and known carcinogenic elements such as chromium, arsenic and bromine). Pathogenic Leptospires, known to induce chronic nephritis in pigs,[98] were found in half of the samples. Large rural populations located near low rank coals where well water is more often drunk, have high rates of UTTs. A recent online article[99] noted that age-adjusted mortality rates for kidney and renal pelvic cancers are above-average in Texas counties with lignite deposits. Other areas containing such coal deposits are North and South Dakota and Wyoming, states that are in the top ten highest in the US for RPC. The Powder River Basin (within WY, ND, SD) contains higher rank sub-bituminous coals that are less soluble but contain significantly higher levels of PAHs in formation and produced water, as well as aromatics and heterocyclics such as were found in BEN villages. Based on the above, this theory could account for the unique specificity of BEN for the kidney, the development of RPC and geographic specificity, and shows partial linkage to UTT or familial occurrence. A critical data gap in the understanding of BEN is the glaring lack of animal models or *in vivo* data for experimental induction of UTT after administration of any of the supposed etiologic agents by the routes that have been hypothesized, including local water sources.

ARISTOLOCHIC ACID (AA)

Aristolochia spp. are forms of a wild plant and AA is a nitro-phenanthrene derivative (therefore an NPAH) found throughout the world and shown to be a complete urinary tract carcinogen[100] in humans and rodents. It grows in the Balkans (**Figure 3**), Europe, and the world, and probably causes UTT 101. The mutational signature of AA from the human TP53 gene[102-106] and whole exome sequencing[107] has defined a signature of exposure to AA that is found at greater frequency in BEN-endemic patients from Bosnia, Croatia or Serbia who have UTT. The development of UTT after kidney transplantation correlates significantly with AA exposure as evidenced by the presence of specific adducts. In these UTT, AT→TA transversions represented 68 to 78% of the TP53 mutations. Most human tumors of all kinds show mutations in the p53 gene,[108] many of which are AT→TA transversions, however for UTT the overall prevalence is 5%. The occurrence of AA adducts in healthy residents in any tissue or UTT from non-BEN areas of the Balkans is limited; one study showed one UTT patient of 5 from a non-endemic area had AA adducts.[102] There is a strong link between exposure to AA and UTT occurrence based on the foregoing. However, there is no evidence (aside from one self-reported exposure questionnaire)[109] of the route of exposure, or correlation with clinical disease prior to UTT based solely on AA exposure. We still do not know whether BEN and UTT are of the same pathology. This suggests that 1) AA plays a role in UTT, and possibly BEN and 2) it cannot be the sole causative factor of BEN. NQO1 or other allelic status or smoking (see above) may also influence p53 transversions. This theory leaves unexplained the familial and geographic patterns of BEN.

OCHRATOXIN A

OTA is a known carcinogen.[100,110] OTA DNA adducts were

first identified in kidney and urinary tract tumors of Bulgarian BEN patients in 1993.[111] OTA was shown to be nephrotoxic and carcinogenic in pigs, rats, mice and poultry,[5,111-114] causes RPC and nephrotoxicity with a long latency period and produces specific DNA adducts. Available in vitro and *in vivo* evidence indicates that ochratoxin A acts as a carcinogen through formation of DNA adducts via oxidative stress, as well as direct quinone formation resulting in genotoxic lesions. Whole-life porcine studies, however, may not be an adequate model for carcinogenicity due to the relatively brief lifespan of the pig.

Figure 3. Aristolochia clematitis plants growing in a BEN village in Romania: (A) in a cornfield in an endemic household and (B) in a wheatfield in a non-endemic location. A higher density of A. clematitis plants in endemic vs. non-endemic locations might provide an explanation for the differential exposure to toxic levels of aristolochic acids. (Photo courtesy of C. Tatu, taken in September, 2012).

Increased levels of OTA have been found in cereals and smoked meats[115,116], and OTA, fumonisin b1 markers, and citrinin (CTN) were found in the urine and blood of BEN village inhabitants in Croatia and Bulgaria.[5,117,118] A recent study found OTA and CTN in the food and urine of 3 BEN families, but not AA.[119] OTA adducts were found in kidney and/or urinary bladder tumors from BEN-endemic Bulgarian and Croatian patients.[120,121] The mechanism of action involves the CYP2C9 metabolic enzyme which enhances the mutation rate, forms a nephrotoxic GSH conjugate, and depletes GSH resulting in oxidative stress,[5] a common pathway of carcinogenesis. OTA is a contaminant in many foods but the population of BEN regions is more heavily exposed than non-affected parts of Europe due to conditions of high humidity and diet[118] and exposure through air (breathing spores), water (mold in stored or still water), or ingestion of mold-contaminated foods has been described. Genetic differences may render some individuals more or less susceptible and differences in water and food storage may explain how adjacent families could experience different outcomes. A study of the combined effects of OTA and AA could be illuminating.

VIRUSES

An infectious origin for BEN was first suggested in 1981[122] in a report describing coronavirus-like particles (a porcine virus capable of infecting humans) in renal biopsies from seven BEN patients. This hypothesis was followed up with a study of 3 and 20 patients.[123,124] An ultrastructural study of freshly-isolated virus from renal biopsies of BEN patients, grown in Vero cells showed it was not a coronavirus.[125] Several other viruses have been considered as potential co-factors or causes of BEN (SV-40, JC and BK polyoma viruses). SV-40 can be carcinogenic in rodents and humans, resulting in mesothelioma, lymphoma, brain and bone cancers.[126] Viral titers in the general population are low but persistent and both horizontal and longitudinal transmission occurs. All three viruses are opportunistically activated in the kidney and other tissues upon immunosuppression although normally latent.[127,128] Seroprevalence worldwide is high beginning in childhood and increases to nearly 100% by the later decades.[129] JC virus is neurotropic and causes multifocal leukoencephalopathy, a progressive and fatal demeyelinating disease, upon re-activation.[130] BK virus is urotheliotropic and reactivation causes interstitial nephritis and consequently, after kidney transplant, is frequently responsible for graft rejection during immunosuppres-

sive chemotherapy.[131] A recent study of patients with renal transplants, healthy controls, or CKD patients found the highest titer in transplant patients (30.5%), followed by healthy controls (22.4%) and those with CKD (3.9%).[132] The high titer in transplant patients seems self-apparent. However the observed low titers in CKD patients are puzzling. The authors explained that CKD patients had decreased urine volume and cell content and elevated urea excretion, therefore the results might not show true viral titers. A simple comparison of the viral load between groups would not provide a complete understanding of disease burden. JC or BK re-activation represents a serious potentially fatal complication of BEN. The three viruses frequently cross-react, so care must be taken identifying these viruses. Xiao, et al.[133] found that HPV-16 or -18 infection had no association to the development of urinary malignancies. The role of viruses in BEN is unknown.

NON-MENDELIAN INHERITANCE PATTERN

BEN shows a maternal inheritance pattern that could be explained by epigenetic inheritance, which does not require germline fixation. Recent studies have shown a link for development of smaller kidneys or renal insufficiency in adulthood to intrauterine stress and glucocorticoid exposure followed by low nephron development.[134] Coupled with other studies regarding the effects of pre-natal parental stress on offspring health[135,136] these data indicate that chromatin modification in parental cells could be transmitted to offspring and result in health effects later in life.[137] Children of adults with renal pathology could inherit kidney disease susceptibility without having been exposed, as shown in animal models.[138] Transgenerational susceptibility would subside gradually in subsequent generations [139] for instance if a person moved out of the endemic area. OTA was shown to cross the placental barrier and to cause DNA adduct formation in the developing rat,[140] demonstrating a possible prenatal exposure route. A 2008 study[141] investigated the correlation between lead, arsenic, cadmium and selenium concentrations in body fluids of BEN adult offspring and kidney variables. Neither cadmium nor arsenic was associated with kidney size or function. Lead had a small but significant effect on creatinine clearance and selenium showed a significant non-protective association with 3 of 4 kidney parameters. One study showed that adult offspring of BEN parents (particularly maternal) showed shorter kidney length and increased excretion of albumin, total protein, and beta2-microglobulin66, while another[142] showed no increased proteinuria in BEN family history patients. Increases in excretion of protein and beta2-microglobulin are the precursors of BEN. Further studies of adult BEN offspring are warranted.

PROSPECTS FOR TREATMENT OR CURE OF BEN

Drugs that show promise for the treatment of CKD include bardoxolone methyl, olmesartan medoxomil, sulodexide and avosentan.[143] These drugs are breakers- and receptor antagonists of glycation endproducts, NADPH oxidase inhibitors, growth factor inhibitors, antifibrotics, protein kinase C inhibitors and endothelin antagonists.[144] A recent animal study[145] found exogenous tetrahydrobiopterin and sepiapterin treatments attenuated advanced glycation end products (AGE)-induced hypertrophic effects in renal tubular cells. The above treatments were designed to treat diabetic nephropathy and may not all benefit BEN patients. At present there is no cure for BEN or CKD. Hematologic or peritoneal dialysis with or without kidney transplant is recommended. After transplantation approximately one third of BEN patients subsequently develop cancer in the pelvis, kidney (native, transplanted, or both), bladder, or upper urinary tract (i.e., UTT) as compared with <1% of non-BEN CKD renal transplant patients.[146] For this reason nephroureterectomy is sometimes performed. However, kidney transplantation lengthens life.

UNEXPLORED/FUTURE RESEARCH DIRECTIONS

The epigenetics of BEN have been little studied. Since NQO1 is present in maternally-inherited mitochondrial DNA, a genetic predisposition to alter the metabolism of AA, 3-NBA, or other contaminants, could be further investigated. Interactions between cigarette smoking and other potential etiologic agents should be considered. Sources of PAH/NPAH should be examined for polar and non-polar molecules to determine the total burden of these pollutants. 3-NBA and AA similarities in metabolism suggest that one or both could be responsible for BEN together with OTA. Transgenerational susceptibility to BEN could be followed to determine the longitudinal exposure effects. SNPs in metabolic enzymes that have been associated with increased risk of UTT should be tested. The long-term effects of changes in water supply should be followed, with replacement water testing. AA is a risk factor for BEN but has not been shown to enter the food chain. Areas containing AA but no elevated CKD should be compared to areas where both occur. Determining the risk factors for BEN may depend on identifying absence

of behaviors, exposures, or genetic predispositions (**Figure 4 and Table 1**). It appears that no single factor is responsible for BEN, however a common characteristic of AA, OTA, PAH, NPAH and phthalates is aromaticity.

The absence of regional collaboration with respect to planning, funding, conducting studies and maintaining and sharing of health data and experimental results (such as; pathology data, tissue banks, demographic data, and sharing of equipment and expertise) has been and continues to cripple efforts to discover the origins and causes of BEN disease, which would enable the enactment of preventive measures by individuals, communities, or governmental authorities. We urge close collaboration and sharing of resources and results, in order to leverage otherwise scarce resources and achieve meaningful progress.

As previously stated, a major data gap in BEN investigations is the lack of *in vivo* data. Tatu et al. (personal communication) have undertaken whole life exposure studies that are in progress. We sincerely hope there are other sub-chronic or whole life studies underway and look forward to seeing peer-reviewed results that conclusively prove or disprove the carcinogenicity of any or all of the putative agents of BEN. Standard NTP-type studies are critically needed.

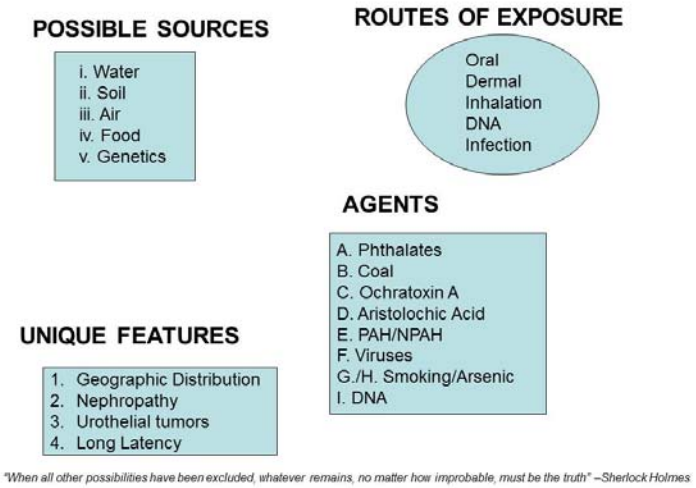

Figure 4. Major characters in the mystery of BEN: Possible Sources (upper left), Routes of Exposure (upper right), Unique Features (lower left) and Agents (lower right). See Table 1 for a list, by agent, of the features of BEN explained by each hypothetical agent.

ACKNOWLEDGEMENTS

Support for this work was provided by The Department of the Interior, U.S. Geological Survey, Energy Resources Program. The views expressed herein do not represent Agency policy.

Table 1. Possible Etiology of BEN

Route	Agent (source)	Features of BEN Explained by Agent	References
oral dermal	phthalates (cosmetics, water, plastics leaching into food)	(1) Geographic distribution (2) Nephropathy	67-79
oral inhalation dermal absorption	coal (soil, water)	(1) Geographic distribution (2) Nephropathy (4) Long latency	14,15,17,80-99
oral inhalation	ochratoxin A (food, soil, air)	(1) Geographic distribution (2) Nephropathy (4) Long latency	5,110-121, 140
oral	aristolochic acid (food)	(2) Nephropathy (3) UTT	35, 100-109
oral inhalation	PAH/NPAH (water, soil, food, smoking/tobacco)	(1) Geographic distribution (3) UTT	11-16, 18-34, 36, 37
infection	viruses (contact with humans/livestock)	(2) Nephropathy (3) UTT (4) Long latency period	122-133
inhalation oral dermal	smoking arsenic (soil, water)	(3) UTT (4) Long latency	38-44, 47 63,64
familial	genetics/epigenetics	(1) Geographic distribution (5) Increased susceptibility of women	48-61, 65, 66, 134-142

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