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BALKAN ENDEMIC NEPHROPATHY IN THE BOSNIA AND HERZEGOVINA

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Review

CONTEMPORARY APPROACH TO BALKAN ENDEMIC NEPHROPATHY

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Abstract

Balkan endemic nephropathy (BEN) is a progressive disease with insidious course inevitably leading to terminal uremia. Histology reveals focal tubular atrophy, focal non-destructive, hypocellular interstitial sclerosis, focal segmental and global glomerulosclerosis of collapsed type and intimal hyalinosis of arterioli and interlobular arteries. Association of upper urothelial tumors (UUT) with BEN is striking. It seems that the most likely underlying pathological mechanism is the slow intoxication in genetically predisposed subjects. Hypothetical agents act upon tubular epithelial and endothelial cells. It exerts pathological effects via interfering with metabolism and through direct genotoxicity thereby disturbing cell cycle and initiating apoptosis that appears to be the underlying mechanism of atrophic changes. Target cells express the genes that are normally inactive and subsequently produce cytokines and complement thereby transforming into proinflammatory cells. Affected cells seem to undergo transdifferentiation, i.e. expression of mesenchymal markers with subsequent production of collagen as extracellular matrix that triggers sclerosis. The mechanism of malignant alteration seems to be the same and agent to induce it also seems to be the BEN-inducing agent. Regarding etiology, epidemiological studies have revealed elevated concentrations of many putative nephrotoxins in BEN area, but their particular concentrations still do not reach the toxic ones. Nevertheless, it seems reasonable to keep insisting on pathogenesis of known models of toxic nephropathies, especially in the light of recent advances in molecular biology. Ochratoxin A (OTA), aristolochic acid (AA) and polycyclic aromatic hydrocarbons (PAH) represent such models. Further research will help us understand them better, understand their toxic metabolites that are supposed to contribute to development of the disease as well as to better understand other concomitant

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and synergistic factors from endemic areas that might participate in that development.
Key words: *Balkan endemic nephropathy, epidemiology, pathogenesis, etiology, ochratoxin A, aristolochic acid, polycyclic aromatic hydrocarbons, molecular biology*

Introduction

Balkan endemic nephropathy (BEN) is an unknown disease that ultimately leads to terminal uremia. It affects rural population of the following countries in the Balkan Peninsula: Croatia, Bosnia and Herzegovina, Yugoslavia, Romania and Bulgaria. Distribution pattern of the villages is mosaic, with affected and non-affected ones adjacent to each other. The same applies to households within affected villages. Main epidemiological criteria are: living in the endemic region, familial occurrence, farming and occurrence of upper urothelial tumor (UUT) in a family. Functionally, it is characterized with tubular proteinuria type and morphologically with focal proximal tubular atrophy, focal non-destructive, hypocellular interstitial sclerosis, focal segmental and global glomerulosclerosis of collapsed type and intimal hyalinosis of arterioli and interlobular arteries.

Main topics at International Workshop held recently in Belgrade (April, 2002.) pertained to epidemiological and pathogenetic aspects of this disease. On the basis of the most recent epidemiological studies it seems that the incidence is decreasing and the onset of the disease has been moved towards older ages. It was accounted for by considerably improved living standard and subsequent possible reduced exposure to hypothetical causative agent. Concerning pathogenesis, the prevailing opinion nowadays is in favor of slow intoxication in genetically susceptible subjects. Regarding patho-physiology, immunological aspect does not seem to play important role. The same applies to inorganic and viral causes from etiological standpoint. Much attention is nowadays being paid to organic compounds such as ochratoxin A (OTA), aristolochic acid (AA) and polycyclic aromatic hydrocarbons (PAH)

Pathogenesis

Genetic hypothesis

On the basis of their cyto-genetic studies, Bulgarian authors have been providing the evidence for almost last two decades that the long arm of one chromosome of the 3rd pair at 3q25 is shortened. Therefore, they consider BEN inherited disease and believe the type of inheritance is autosome-dominant (1). In accordance with the development of recombinant DNA technology they conducted PCR

study that showed significant positive association of alleles C4 and A6 with BEN at loci ACPP and D3S1282, respectively at 3q22.1-3q26.2 and significant negative association of the allele a2 with BEN at locus D3S1509. The idea was to search for genetic marker for BEN. They concluded that their former cyto-genetic results on the significance of 3q24-3q26 region for BEN have been confirmed by this study, but proposed further research (2).

There is nowadays the prevailing consensus that genetic predisposition to develop BEN plays an important role in BEN development, but only in conjunction with hypothetical factor from the environment. Thus, it has been suggested that the various metabolic activities of xeno-biotic enzymes such as cytochrom P450-dependant mono-oxygenase (CYP) and glutathion-S-transferase (GST) might render selected subjects candidates to develop BEN. Various activity rates of these enzymes indicating genetic predisposition to develop BEN was demonstrated on a drug debrisoquine as a substrate (3). That activity would determine particular persons to be either fast or slow oxidizers of the putative environmental factor. The factor, itself does not necessarily even need to be toxic in its native form, unlike it's metabolite that might accumulate at a high rate. It has been demonstrated that BEN patients are fast oxidizers of the drug debrisoquine, thereby suggesting increased activity of their xeno-biotic enzymes (4). A significant polymorphism of the genes encoding for the synthesis of these enzymes has been demonstrated. Thus, CYP2D6 alleles with complete deficit or ultra-fast enzyme activity are associated with oncogenesis (5).

Intoxication

Slow intoxication seems to be the most likely pathogenetic mechanism in BEN development.

Illustrative example to demonstrate this is an ochratoxin A (OTA) nephropathy induced in rats. OTA produces following effects in rats:

1. inhibits aerobic respiration in mitohodria thereby lowering ATP
2. inhibits an enzyme tRNA synthetase thereby decreasing protein synthesis
3. increases lipid peroxidation
4. is genotoxic; evidence on genotoxicity is the finding of OTA-DNA adducts in mice treated with OTA (7). OTA-DNA adducts represent co-valent complex of OTA and guanine base in the DNA molecule and they initiate cancerogenesis (8).

Toxic effect of hypothetical agent produces genomic disorder whereby cell cycle becomes subject to change (proliferation vs.

apoptosis). Apoptosis has been demonstrated in the pathogenesis of toxic nephropathies other than BEN, i.e. analgesic (10), ochratoxin A (11) and cyclosporine (12), but it has been shown in 1998. to play a significant role in BEN, too (13). The finding was confirmed three years later and demonstrated to take place in tubular epithelial cells (14). In general nephropathology it has been shown that endothelial cells also undergo apoptosis, which then induces ischemic changes. Subsequent hypoxia accelerates tubular epithelial cells apoptosis that results in tubular atrophy and interstitial sclerosis (15). Moreover, damaged tubular cells become activated to produce complement, cytokines and extra-cellular matrix as a result of the expression of genes that are otherwise inactive in normal conditions (16, 17).

Expression of various intermediary filaments of the cytoskeleton is characteristic for the particular types of cells. Thus, *keratin* is the marker normally found in tubular cells whereas *vimentin* can be found in mesenchymal cells. It has been widely accepted that damaged tubular cells can express various markers during their regeneration. Thus, co-expression of keratin and vimentin can occur irrespective of a causative agent (18).

It has been demonstrated that tubular cells express in the early phase BEN vimentin along with keratin (19). Given mesenchymal cells produce *collagen* as an extra-cellular matrix and given tubular cells produce *laminin*, it has been suggested that transdifferentiation of tubular cells into myofibroblasts might be responsible for interstitial fibrosis in BEN (20).

Significant contribution of vascular lesion with this respect has been suggested by Ferluga and Vizjak who believe the same process might simultaneously affect endothelial cells and result in interstitial sclerosis (21, 22). They found in BEN patients collagen IV and laminin over-expression in thickened tubular basement membrane of atrophic tubules as well as in interstitial capillaries. They believe that an unknown agent could simultaneously act upon tubular epithelial cells and upon vascular endothelial cells thereby inducing their hyperactivity with overproduction of extra-cellular matrix that results in sclerosis, characteristic for BEN. Moreover, vimentin was expressed in non-sclerosed glomeruli, interstitium, blood vessels as well as in co-expression with cytokeratin in epithelial cells of the damaged tubuli whereas negative in intact tubuli. Also, they found complement C3 deposits that correlated with the most intense tubulo-interstitial histological changes as well as with high degree of proteinuria. They concluded that injured and activated tubular epithelial cells could produce complement (23).

Pathogenesis of BEN and BEN-associated tumors

Striking association between BEN and UUT has become particularly interesting in the light of advances in molecular biology. Gen p53 and its protein are important mediators of cell cycle. They oppose cell proliferation and initiate apoptosis. The rate of apoptosis is a biological marker of tumor progression because of horizontal spreading of oncogenes from an apoptotic body of one tumor cell which is taken up by the other tumor cell (24). Savin and Petronic found an increased expression of p53 in epithelial tumors from BEN area and suggested p53 mutation. This finding is certainly not BEN-specific since half of human tumors have p53 affected, but they also found less frequent apoptosis in BEN-associated UUT from BEN region vs. UUT outside that region and concluded that this accounted for former less invasive tumors (25).

As for the striking association between BEN and UUT, Bulgarian researches keep reaffirming the genetic background of both diseases. Toncheva et al. found loss of heterozygosity in one out of 3 analyzed patients affected with BEN-associated tumors at locus D3S1299 at 3q24 thereby supporting their previous data on that region being associated with BEN. Loss of heterozygosity test is the method to prove an increased incidence of tumor occurrence in certain families and method that discovered the existence of suppressor genes. They suggested the existence of a new tumor-suppressor gene at 3q24 (26).

Etiology

A lot has been done so far studying various inorganic substances (heavy metal, ionizing substances, microelements, selenium deficiency etc.) as well as viruses, but no causative relationship with BEN has been established. In the light of previous, but also recent researches of organic compounds such as: ochratoxin A (OTA), aristolochic acid (AA) and polycyclic aromatic hydrocarbons (PAH), much attention has been paid to them, accordingly. All three are nephrotoxic and oncogenic which fits BEN clinical presentation, too.

Ochratoxin A (OTA)

OTA is secondary metabolite of fungi Penicillium and Aspergilus. It is natural contaminant of food and feed. According to studies conducted so far, population has been exposed to OTA to a greater extent in BEN than in non-BEN region, but still in insufficient amounts to produce toxicity in humans (27).

There is an established animal model of OTA-related nephropathy in pigs which is called Porcine nephropathy, discovered in Denmark by Krogh and characterized with proximal tubular atrophy and

interstitial sclerosis. It can be encountered in West and Mid Europe. Krogh happened to spend some time in the Balkans and then started to work on the issue of BEN bearing in mind already existing porcine nephropathy in Denmark. His research on BEN brought him to the solution of porcine nephropathy enigma in his country (Ozegovic* - personal communication). On the other hand, it seems that pigs from former Yugoslavia do not suffer from OTA-related nephropathy (28).

OTA-related nephropathy patho-histology in mice differs from the ones in pigs as well as from BEN. It is characterized with "post-proximal nephron" injury, with impaired concentrating capacity (29), unlike the latter ones that are characterized with proximal tubular injury and related Fanconi syndrome.

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OTA is carcinogenic in mice. It induces renal parenchymal cancer by creating OTA-DNA adducts (7). OTA-DNA adducts were also found in human UUT (30), but there is still not enough evidence of OTA–related pathology in humans. There were only two cases in Tunisia suspect on OTA-related nephropathy, then a case of acute renal failure in Italy after inhalation exposure and the cases of a brother and a sister in France with high blood ochratoxin A levels and karyomegaly (31).

Aristolochic acid (AA)

Ivic found AA in wheat flour in BEN area in 1970. He presumed that AA originated from the plant *Aristolochia clematitis* that can be encountered in BEN region, but is ubiquitous, too. He was feeding thereafter rabbits with Aristolochia clematitis seeds. The result was tubulo-interstitial nephropathy very similar to BEN (32).

It was demonstrated then that AA was cancerogenic in rats (33) and that AA activates gene *ras* in experimental animals tumors (34).

Thereafter, AA has been virtually forgotten as a serious candidate for BEN elucidation until an incident that took place at one Belgian weight-loss clinic in 1992. As many as 80 women undergoing slimming regimen with Chinese herbs containing AA developed rapid progressive terminal renal failure (35), accompanied by UUT in 40% of them (36).

Morphologically, it was tubulo-interstitial nephropathy, tubular proteinuria was found and no hypertension was observed thereby resembling BEN very much. AA-DNA adducts were found in a controlled study in the same patients (37). Then, rabbits and rats were fed with AA and the results were interstitial fibrosis and UUT. The conclusion was that AA caused the disease.

So, the new disease was discovered and was designated as Chinese Herbs Nephropathy (CHN), even though minimal nephrotoxic doses in rats were still several times higher than those found in slimming pills. Moreover, doses administered in slimming pills did not differ from the ones used to be prescribed in traditional Chinese medicine, with no adverse health effects (39, 40). Therefore, it has been suggested that it was not only AA to result in CHN nor in UUT development. The supplemental slimming regimen consisting of sympathomimetics (appetite suppressors), purgatives and diuretics (acetazolamide) could have contributed to the pathology (41, 42).

Due to similarities between CHN and BEN, the idea of relating AA to BEN was proposed again (38).

Polycyclic aromatic hydrocarbons (PAH)

Chemical analysis of water samples from BEN and non-BEN villages in Romania showed the presence of nephrotoxic and cancerogenic organic compounds (naftilamin, anilin, aminofenol and PAH) in much higher concentration in endemic vs. non-endemic villages (43).

Low-rank Pliocene lignite correlates with BEN areas. Weathered lignite deposits contain the above mentioned compounds that are hydro-soluble and thus transported by the local ground water flow system thereby contaminating shallow water wells of the BEN-affected households.

Feder et al. tested methanol lignite extracts from BEN and non-BEN areas. In BEN area benzene and naphthalene were found and they were rich in functional groups: methoxy, acetyl, keto and hydroxy. Those compounds are hydro-soluble and some of them are nephrotoxic and cancerogenic. In the same samples terpane/steranic groups were found. They are the markers of the poor quality of the coal that resembles very much a fossil wood. Extracts from non-BEN areas neither contained toxic functional groups nor were hydro-soluble (44). Therefore, the authors proposed explanations for the geographical restriction of BEN. Two of them appear most attractive. First, BEN area lignite has to have specific features and has to differ from non-BEN area one. Pliocene lignite location in the Balkans corresponds to the southeastern and southern margins of the Tertiary Panonian Basin and overlaps with BEN areas. A specific coalification process, which has been incomplete, could have taken place under local climatic and geological conditions in the Balkan Peninsula, thereby leaving partially decayed compounds such as *terpenoids* – biomarkers of incomplete degradation of fossil fuels. Some of those compounds, such as *terpineol*, are mutagenic and others are nephrotoxicants. They were found in coal samples of BEN unlike in non-BEN area.

The another explanation for BEN geographical restriction pertains to ground waters that leach the toxic organic compounds from the rocks and transport them to shallow wells/springs thereby determining endemic villages as well as BEN-affected households within an endemic village. Those waters determine affected households with their flows and various concentrations of the toxic compounds, which depends on local soil characteristics (permeability, rainfall etc.) This explanation nicely accounts for both the existing mosaic distribution of endemic villages in the Balkan Peninsula as well as for mosaic distribution of particular affected households within an endemic village.

Conclusion

Genetic predisposition to develop BEN in susceptible subjects who are exposed to hypothetical factor from the environment is nowadays prevailing approach to this disease. It has been suggested that the susceptibility might be the result of the various metabolic activities of xeno-biotic enzymes such as cytochrom P450-dependant mono-oxygenase (CYP) and glutathion-S-transferase (GST). The rate of activity of these enzymes would qualify particular persons to either fast or slow oxidizers. It has been suggested that BEN-susceptible candidates are fast oxidizers of the agent that is not necessarily nephrotoxic and/or oncogenic in its native form, but its metabolite that is being accumulated at a high rate might be so (3).

Regarding pathogenesis, it seems that slow intoxication of metabolism and/or direct genotoxicity of the hypothetical agent/s in genetically susceptible subjects are the underlying mechanisms of BEN.

The outcome of toxic activity of a hypothetical toxic metabolite is a gene control disorder of the cell cycle (proliferation vs. apoptosis) which results in the expression of a gene responsible to trigger apoptosis via an enzyme that initiates atrophy of the target tissue. Target tissue is postulated to be renal epithelial and endothelial cells (13-17). Cells initially damaged express during their regeneration various markers that are otherwise not characteristic for that tissue (transdifferentiation) and produce mediators such as cytokines, complement and extra-cellular matrix which gives rise to interstitial sclerosis (16, 17).

The association with UUT is striking and its elucidation might contribute to better understanding of BEN through molecular genetic studies. Thus, the finding of AA-DNA adducts in patients that were taking AA and developed consequently CHN and UUT was quite sufficient (along with successful animal experimentation) to relate CHN and UUT to AA (36, 37). OTA-DNA adducts were related to cancerogenesis in mice (8) and were found in human UUT (30).

So, in some patients a hypothetical metabolite can be nephrotoxic inducing atrophy and fibrosis due to activation of normally inactive genes. Those genes encode for biosynthesis of proteins that trigger apoptosis and extra-cellular matrix production, respectively. In other patients carcinogenesis might take place via either activation of proto-oncogenes into oncogenes, inactivation of tumor-suppressor genes or via damaging the genes responsible for reparation of DNA replication errors in S stage of cell cycle. The mechanism might be point mutation, chromosomal rearrangement or gene amplification/deletion. Accordingly, p53 over-expression was found in BEN-associated UUT and its mutation was suggested (25). Also, loss of heterozygosity (LOH) was found in BEN-associated UUT and presence of a new tumor-suppressor gene at 3q24 was suggested. It was found in the same chromosomal region which Bulgarian authors considered chromosomal marker for BEN (26). It has been also suggested that the same agent induce both BEN and UUT. As in a number of UUT associated with toxic nephropathies, such as analgesic, CHN and OTA (the latter one confirmed in animals, only) apoptosis plays an important role in tumor progression in BEN, too (25).

No results have been achieved after a half of a century of thorough investigations searching for a single BEN-causing agent. Much effort has been made working on OTA, AA and PAH.

OTA intake is higher in BEN vs. non-BEN area, but it still fits into safety limits and the lowest toxic OTA doses in animals are still 4-5 times higher than those found even in hyper-endemic areas. The latter applies to AA, too, but such findings should not be discouraging for further research. First, further work on OTA, AA and PAH-related nephropathies, respectively will bring us closer to better understanding of pathogenesis of toxic interstitial nephropathies, which will be helpful to possibly get closer to BEN enigma solution, itself. Second, it is not necessarily that the parent compound from the environment, irrespective of its concentration, could be responsible for the pathology, but its metabolite that is accumulated at a high rate in susceptible subjects. Good example to illustrate this provides model of OTA intoxication in rats. Preliminary results suggest that oxidative pathways in OTA metabolism generate genotoxic metabolite via ko-oxidation of the metabolic pathway of prostaglandin synthetase (6). Administration of prostaglandin-synthetase inhibitors such as Aspirin or Indomethacin significantly reduced the amount of those adducts (9).

Third, patho-histological presentation of OTA-related nephropathy in animals differs from BEN, but on the other hand, OTA-related rat nephropathy differs from porcine nephropathy, too.

Fourth, OTA-related porcine nephropathy in Denmark differs at ultrastructural, mitochondrial level from the one in Bulgaria. Thus,

electron-dense formations in the nuclei, surrounded by a small electron-dense mass as well as myelin figures in the cytoplasm and mitochondria were not demonstrated in Denmark (unlike in Bulgaria). The authors suggested that described differences were probably due to some interference between ochratoxin A and other mycotoxins so that some synergistic effects between ochratoxin A and various other nephrotoxic mycotoxins produced by the same ochratogenic fungi might have taken place (45). Such other nephrotoxic mycotoxins could be: citrinin, penicilic acid, rubratoxin A, B, viomellein and xanthomegnin (31). Accordingly, Abouzied et al. suggested that OTA might not cause BEN alone, but in synergistic action with other toxicants in genetically predisposed subjects (27). That is why mycotoxin hypothesis for BEN elucidation should not still be necessarily ruled out.

The same applies to aristolochic acid that has been proved to cause Chinese Herbs Nephropathy in humans, even though particular ingested doses have not been considered toxic to humans, but they still caused the disease in conjunction with dehydration (purgatives and diuretics) as well as peripheral vasoconstrictors (appetite suppressors) administered at a Belgian weight-loss clinic. BEN resembles CHN, too and AA has been identified as early as in 1970. as its possible causative agent. Since farming is one of the epidemiological criteria for BEN diagnosis and in the light of insufficient sole AA dose to cause CHN, it is worthwhile to note that farmers are sweating a lot in the field! The main difference between those two diseases is that CHN presents clinically as rapidly progressive tubulo-interstitial nephropathy after only 8 months of AA ingestion, as opposed to BEN which takes up to two decades. This could reflect a higher level of toxic exposure in CHN than in BEN patients (47). Therefore, the role of AA in BEN development can not still be ruled out if it was a matter of slow intoxication with minimal doses over the course of two decades and without additional rigorous slimming treatment applied at Belgian weight-loss clinic. The final demonstration that AA plays a role in BEN requires the evidence that patients with an unequivocal diagnosis of BEN have ingested foods containing AA, present the typical biological and morphological characteristics of CHN and harbour AA-DNA adducts in their renal tissue (46).

PAH-related nephropathy as the consequence of ground waters that leach the toxic organic compounds from the rocks and transport them to shallow wells/springs thereby determining endemic villages as well as BEN-affected households within an endemic village provides an attractive example of geographically restricted disease. It is more difficult to deal with OTA and AA in this regard because they are ubiquitous, but genetic predisposition, along with concomitant and synergistic effect of known nephrotoxins might account for BEN as

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opposed to non-endemic areas where sufficient amount of various nephrotoxins and/or their even more toxic metabolites in genetically predisposed subjects is possibly not reached. Schmeiser, the prominent molecular biologist from Heidelberg (Germany), has recently found DNA adducts of both AA and OTA in renal tissue in 2 out of 3 patients from BEN area that were suffering from UUT and ureteral stenosis. In the third case OTA-DNA adduct was not found and presumed AA-DNA adduct was rather faint for complete identification to be confirmed. This finding was still not conclusive in terms of causative relation to BEN because the study was neither controlled nor was it the matter of confirmed BEN cases (47), but we know that UUT is highly associated with BEN in BEN regions and this study could give us the guidelines for further research.

Therefore, in order to keep on trying to elucidate BEN enigma, the research on the described toxins should not be given up.

Apstrakt

Balkanska endemska nefropatija (BEN) je progresivno oboljenje podmuklog toka koje neumitno vodi u terminalno bubrežno zatajenje. Histološki se radi o fokalnoj tubularnoj atrofiji, fokalnoj nedestruktivnoj hipocelularnoj intersticijskoj sklerozi, fokalnoj segmentnoj i globalnoj glomerulosklerozi kolapsnog tipa i intimalnoj hijalinozi arteriola i interlobularnih arterija. Izrazita je udruženost sa tumorima gornjeg urinarnog trakta. Čini se da je najvjerovatniji patološki mehanizam u podlozi ove bolesti spora intoksikacija u genetski predisponiranih osoba. Hipotetički agens djeluje na tubularni epitel i endotel. On ispoljava patološki efekat interferirajući sa metabolizmom kao i direktnom genotoksičnošću zbog čega se remeti ćelijski ciklus i injicira apoptoza za koju se smatra da je u osnovi atrofičnih promjena. Ciljne ćelije istovremeno aktiviraju gene koji su normalno inaktivni te proizvode citokine i komplement transformišući se u pro-inflamatone ćelije. One trpe transdiferencijaciju, tj. Ekspresiju mezenhimskih markera zbog čega proizvode kolagen kao ekstra-ćelijski matriks, što je uvod u sklerozu. Čini se da je mehanizam maligne aliteracije isti, kao i da je agens koji ga pokreće isti. Što se tiče etiologije, epidemiološke studije su pokazale povišene koncentracije mnogih nefrotoksina na BEN terenu, ali njihove pojedinačne koncentracije još uvijek ne dosežu toksične doze. I pored toga se čini opravdanim i dalje insistiranje na patogenezi poznatih modela toksičnih nefropatija, naročito u svjetlu skorih dostignuća u području molekularne biologije. Ohratoxin A, aristolohijska kiselina i policiklički aromatski ugljeni hidrati predstavljaju takve poznate modele. Buduća istraživanja će doprinijeti njihovom boljem razumijevanju, zatim razumijevanju njihovih sekundarnih toksičkih metabolita za koje se pretpostavlja da bi mogli doprinijeti razvoju bolesti, kao i boljem razumijevanju drugih konkomitantnih i sinergističkih faktora sa endemskog terena koji tom razvoju doprinose.

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