Preface

Endemic nephropathy is a chronic progressive renal disease with the discrete, stable geographic distribution. Over the past 50 years it has attracted the attention of many scientists, yet its etiology remains a mystery: genetic influences were repeatedly investigated, but research mostly focused on the environmental factors, particularly the role of nephrotoxic agents. According to epidemiologic surveys, the mosaic distribution of endemic nephropathy has not changed significantly and the prevalence of the disease in some regions is similar to that reported decades ago. What is even more important, the prevalence of urothelial cancer remains several-fold higher in endemic regions as compared to nonendemic areas.

The abstracts published in the supplement of Collegium antropologicum represent the program of the International Symposium »Recent Advances in Endemic Nephropathy«. The Collegium's supplement collects the results of broad range of basic, translational, clinical and epidemiological studies. We hope that advances in basic research, development of new technologies as well as our understanding of biologic and environmental systems, that can be applied at the bench and the bedside, as well as environment, are a great challenge to translate the scientific innovations into clinical and epidemiological context. The necessity for the information flow between basic, translational and environmental research offers good prospects for advancing knowledge on endemic nephropathy.

The supplement covers the etiology of endemic nephropathy and its associated urothelial cancers. Recent studies on the role of nephrotoxic agents (ochratoxin A and aristolochic acid) are discussed in molecular toxicology, toxicogenomics, animal models and clinical setting. Some studies describe the clinical characteristics of endemic as well as aristolochic acid nephropathy; pathogenesis, clinical characteristics and epidemiology of urothelial cancer is presented. In several articles the epidemiology of endemic nephropathy from various regions is reported and environmental exposure evaluated.

We extend our appreciation to all the authors for their dedication in accomplishing this project. We specially acknowledge the help of Mrs. Annette Oesterreicher, MS, New York, for editing the abstracts.

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Radovan Pleština – *curriculum vitae*

Radovan Pleština was born in 1934 in Split, Croatia, where he completed grammar school education. In 1960, he graduated from the School of Medicine, University of Zagreb. Upon completing residency, he started to work in a hospital in Slavonski Brod where he frequently had patients presenting with a peculiar disease which at the time had no defined name but was referred to as a »Kobaš disease« according to a place where most of the patients came from. This part of his professional life encouraged his subsequent scientific interest and research activities at the Laboratory for Toxicology, Institute of Medical Research and Occupational Medicine, where he was employed since 1965. He won the master's degree in 1968, and defended the doctoral thesis in toxicology at the University of Zagreb in 1973. He habilitated at the Department of Pathophysiology, Zagreb University School of Medicine in 1976, where he was appointed associate professor in 1979 and a full professor in 1986. Apart from pesticide toxicology, his research activities at the Institute of Medical Research were since the early 1970's mostly focused on the study of Balkan endemic nephropathy, particularly a potential role of mycotoxins in the development of the disease with unclear etiology. Since 1978, Dr Pleština was active in the WHO as a counsellor on pesticide toxicology and an expert in vector biology, and in the 1987–1996 period he participated in the International Program for Chemical Safety in Geneva. After resigning from his duties in WHO and until retirement, he continued with his work at the Institute of Medical Research. In addition to numerous publications of scientific, professional and teaching character, it should be pointed that Radovan Pleština was the editor-in-chief of the journal *Archives of Industrial Hygiene and Toxicology* in the 1974–2004 period and in this manner, during thirty years, also contributed to research in health ecology and occupational medicine.

1. HISTORY

Kidney Disease among the Romans

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The signs, symptoms, and therapies for renal diseases were clearly described in 37AD by Cornelius Celsus in *De medicina*. The information provided in this volume included an account of blood or pus in the urine, vomiting in a long-term illness,[1] and therapeutic suggestions that enabled the patient to recover from most kidney problems (not including renal calculi), such as rest, sleeping in a comfortable bed, ensuring regular bowel movements, taking frequent hot baths, eating food that was warm in temperature, avoiding salt, but drinking considerable amounts of water and adding such items as pepper, leeks, fennel, and poppy. Ulcerated kidneys were to be cleaned out[2] and the use of herbal medicines were featured prominently,[3] much as they are 35 years later among the 130-odd substances prescribed for kidney problems in the *Natural History* by Pliny the Elder.[4] Turning to the Greek – from the same decade as Pliny's 37-book summary of everything in nature – *De materia medica* by Dioscorides of Anazarbus contains numerous remedies for kidney ailments, e.g. galingale (*Cyperus rotundus* L.)[5] for kidney stones, Celtic spikenard (*Valeriana celtica* L.) as a superb diuretic,[6] and about 60 additional remedies from all three natural kingdoms: vegetable, animal and mineral. Also surviving in Greek from the 2nd century of the Roman Empire is *Bladder and Kidney Diseases* by Rufus of Ephesus (fl. in the reign of Trajan [AD 98-117]).[7] This text also contains multiple remedies derived from botanicals designed to treat, for example, an inflamed kidney, which included easily obtainable ingredients such as celery, ivy, wormwood (Grk. *absinthios*), and marjoram (Grk. *origanon*).[8] Rufus is also well aware of the narcotic properties of mandrake[9] and he frequently prescribes such effective pain-killers in his treatments of renal problems.[10] Frequent and apparently successful surgeries for bladder stones are also described in some detail (one could 'make a living' in Roman imperial days as a *lithotomist*), and it appears that Roman treatments for kidney and bladder diseases might have had far more positive outcomes than what one discerns in the texts of late medieval and Renaissance Europe.

Significant, however, is Pliny's casual mention of birthwort as a remedy for kidney troubles,[11] which indicated widespread use by Romans and their Byzantine successors of birthwort for numerous ailments. Texts from Roman and Byzantine times show that there is likely a frequent occurrence of birthwort poisoning of the kidneys (an ancient »iatrogenic« disease) in recipes beginning with Cornelius Celsus, continuing through the precise pharmacology of Scribonius Largus' *Compositiones* (c. AD 43), and contained in the compound drugs of Oribasius of Pergamon (chief physician of Julian the Apostate [*regnat* AD 361-363]), Aetius of Amida (likely physician to Theodora [d. AD 548]) and a number of other sources and authorities. *Aristolochia* spp. were well known as inducers of childbirth and stimulators of

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[1] Celsus, *De medicina*, II, 7. 11: At si sanguis aut pus in urina est, vel vesica ven renes exculterati sunt. IV, 16. 17 (X), 1: At renes ubi adfecti sunt, diu male habent. Peius est, si frequens biliosus vomitus accedit.

[2] IV, 16. 17 (X). 2: Auxilio quoque his exulceratis sunt, si adhuc ulcera purganda sunt.

[3] *Ibid*., cucumeris semina detractis corticibus sexaginta, nuclei ex pinu silvestri xii, anesi quod tribus digitis sumi possint, croci paulum, contrite et in dias mulsi potions divisa, etc.

[4] Giovanni Aliotta and Antonio Pollio, »Useful Plants in Renal Therapy according to Pliny the Elder,« *American Journal of Nephrology*, 14 (1994), 399-411 [esp. Table 2: 'Index of Useful Plants in Renal Therapy Mentioned by Pliny in *Naturalis Historia*,' pp. 400-409]

[5] Dioscorides, (ed. Wellmann) I, 4. 2: kœperoj.

[6] *Ibid*., I, 8. 1: keltik n‹rdoj

[7] Alexander Sideras, ed., trans., comm.., *Rufus von Ephesos Über die Nieren- und Blasenleiden* (Berlin: Akademie Verlag, 1977 [*Corpus Medicorum Graecorum* III 1]), which supersedes Ch. Daremberg and Ch. Émile Ruelle, eds., and trans., »Traité des maladies des reins et de la vessie,« in *Oeuvres Rufus d'Éphèse* (Paris, 1879; rptd. Amsterdam: Hakkert, 1963), pp. 1-63

[8] Rufus, *Kidney and Bladder Diseases*, 15. 21 (ed. Sideras, p. 106).

[9] Especially prominent in Rufus' *Pain-Alleviating Potion*, as quoted by Galen (ed. Kühn), XIII, pp. 92-93

[10] E.g. *Kidney and Bladder Diseases*, 25. 15 (ed Sideras, p. 118): mandrake, opium poppy, and hyoscyamus for the pain of kidney stones. *Vid*. also *Ibid*., 40. 8 (ed. Sideras, p. 138)

[11] Pliny, *Natural History*, XXVI, 88: Calculos [either kidney or bladder] pellit malum erraticum radicis libra in vini congio decocta ad dimidias. Textual critics (beginning in medieval times) have assumed this is the same as the *malum terrae* of XXV, 95, thus *aristolochia*.

[12] esp. *Nature of Woman*, 32 (ed. Littré, Vol. VII, pp. 356 and 358). Dioscorides, III, 4 (Wellmann) is the standard Greek text on *aristolocheia* (three kinds); unlike Pliny, Dioscorides does *not* recommend birthwort for kidney problems

[13] Theophrastus, *Historia plantarum*, IX, 20. 4.

uterine contractions and such use was first recorded by the Hippocratic writers (esp. in *Diseases of Women* and *Nature of Woman*) in the 4th or 3rd centuries B.C.[12] Theophrastus of Eresus had described its use for such purpose in c. 300 B.C.;[13] but Hellenistic, Roman, and Byzantine pharmacology and therapeutics presumed multiple uses, with texts showing precise measurements, careful preparation, and dosages administered over long periods of time. There can be little doubt that cancers were fairly common in antiquity, and I shall focus on some Latin and Greek texts that suggest that cancers likely resulted from the recipes offered by Scribonius Largus, *Compositione*, 70 (a compound remedy for quinsy),[14] and 153 ('A Remedy for Those who Suffer from Bladder Stones, a Swollen Spleen, and are Affected with Dropsy'),[15] as well as Proclus' 'Medicine for Gout'[16] and Aetius of Amida's 'Vaginal Fumigation.'[17]

[14] Sergio Sconocchia, ed., *Scribonius Largus Compositiones* (Leipzig: Teubner, 1983), p. 39

[15] *Ibid*., pp. 75-76

[16] Proclus *fl. c.* AD 50; Greek text in J. Raeder, ed., *Oribasii Synopsis ad Eustathium. Libri ad Eunapium* (Leipzig and Berlin: Teubner,1926 *Corpus Medicorum Graecorum* VI 3]), pp. 95-96

[17] Aetius of Amida, *Tetrabiblon* [*Sixteen Books*], XVI, 55; ed. and trans. [Latin] Jan Cornarius, *Aetii Medici Graeci contractae ex veteribus medicinae Tetrabiblos,* etc. (Basil: Froben, 1542), p. 890, and ed. (Greek) Skevos Zervos, *Aetii Sermo Sextidecimus et ultimus. Ersten aus Handschriften veröffentlicht* (Leipzig: Anton Mangkos, 1901), p. 76

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1. History

Croatian medical history – A short walk through long centuries

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Soon after their arrival in the region in the 7th century, Croats accepted Christianity and developed their specific culture, embracing various cultural influences and medical traditions. The existing towns included Greek colonies, such as ancient Epidaurum lying in the territory that later became the Republic of Dubrovnik. Roman colonies followed them after the Illyrians had been pacified. The nature of medical practices in these regions can be discerned from the rudimentary surgical instruments, the tombstones of midwives and medical professionals, and various articles from apothecary and medical practice, which have been found in many areas in Croatia. Throughout history, towns in Croatia were connected to eachother by Roman roads and were built with great expertise in the field of public hygiene. In accordance with Justinian's Byzantine hospitals, similar institutions were developed in Zadar, Poreč, Rab, Split and Dubrovnik. The oldest public pharmacies in Croatia were in existence in the 13th and 14th centuries. Importantly, the inefficiency and failure of all anti-epidemic measures prompted the rulers of Dubrovnik to introduce, in 1377, the first quarantine, which constituted the greatest contribution of Croats to Medieval health. Also in the 14th century, Dubrovnik passed the 1395 Insurance Law, the oldest such legislation in Europe.

In the 12th century, the Croats joined the scientific activity in Western Europe, with the first contributions being made by Herman Dalmatin. During the Middle Ages, Croats studied and were active participants in numerous European scientific centers. For example, Federic Grisogono of Zadar, an early-16th century physician, wrote a text on astrological medicine and formulated a theory of sea tides. Additionally, the famous physician from Kopar, Santorio Santorio, who conducted experiments in physics, conducted his medical practice in Croatia, while the most outstanding physician who applied the principles of mechanics to his study of the structure and function of living beings, Đuro Baglivi, was of Croatian (Dubrovnik) origin as well.

Medicine in the 18th century overcame the conservative and reactionary tendencies of the beginning of the century. On Croatian territory, health practices followed van Swieten's *Normativum Sanitatis,* whose main purpose was the containment of epidemics. This era also saw the publication of the first medical textbooks in the Croatian language. During the first half of the 19th century, efforts continued to establish Croatian scientific terminology and there was also progress in writing in Croatian. In 1806, the Lyceum was established in Zadar under the French administration and was given the status of a central school that included higher education curricula and had the right to confer academic degrees. It also offered courses in medicine, surgery and pharmacy.

The Croatian Academy of Sciences and Arts was established in 1866 and played a significant role in the development of natural sciences. A major change occurred in 1874 when Zagreb University was reopened and reorganized along modern lines. That year, 1874, constituted the most significant turning points in the development of Croatian medicine in the 19th century. It was the year of the founding of the *Croatian Medical Association*, the passing of the first public health act of Croatia and Slavonia, and the founding of the University of Zagreb. The first two Croatian medical journals were launched in 1877. These developments were major milestones in the path to institutionalization and professionalization of medicine, which culminated in the founding of the University of Zagreb School of Medicine in 1917.

2. THE ROLE OF OCHRATOXIN A IN KIDNEY DISEASE

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Improvement of the postlabeling method for detection OTA-DNA and AA-DNA adducts

Biomarkers of exposure to ochratoxin A in endemic (Balkan) nephropathy

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Several studies have implicated mycotoxins in the kidney disease geographically limited to the Balkan region (BEN). BEN patients in Bulgaria have been found to have a much higher prevalence of ochratoxin A (OTA), exceeding 2μg/L and OTA is found more frequently in the urine of people living in BEN-endemic villages. To confirm and quantify exposure to OTA in the Vratza district of Bulgaria, we enrolled healthy volunteers (20–30 years-old) from two villages associated with a high risk for BEN and monitored their OTA intake and their OTA levels in blood and urine during a one-month period. Food samples were collected daily and 7-day samples were pooled. Blood and urine samples were collected at the beginning of each week. The results showed that the average weekly intake of OTA varied from 1.9–206 ng /kg of body weight, which is twice the tolerable weekly intake recommended by JECFA The concentrations of OTA in blood are in the same range as previously reported in this region, reaching 10μg/L. The weekly intake of OTA from food did not directly correlate with blood and urine concentrations. To further study this lack of correlation, we studied the relation between an increase in oral OTA intake and blood and urine concentrations of OTA in the rat and found that that relatively high OTA concentrations in blood were found in rats fed a low amount of OTA. Also, elimination of OTA in urine was not directly correlated with OTA intake. Differences between results in male and female rats can be explained by the fact that the expression of these transporters is sex- and species-dependent. It is well known that OTA is a cumulative toxin and thus, for steady-state conditions, the plasma concentration is fairly constant. It should be remembered that the circulating fraction of a toxin is the only one that could exert a biological effect. Biomarkers of biological effects, such as DNA adducts, have been detected in patients with urinary tract tumors, who have a high consumption of OTA and high plasma and tissue levels of OTA. Similarly, we found DNA adducts in the study of rats even among those with low OTA intake. It would be of interest to determine whether these adducts can also be detected in exfoliated cells in human urine. Such a result would indicate a good marker of kidney damage, which may lead to tumors.

All these data suggest that OTA is implicated in BEN and urinary tract tumors.

Ochratoxin A in Human Kidney Disease

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In the 1970s, a hypothesis was put forward that the mycotoxin ochratoxin A (OTA) was involved in the etiology of endemic nephropathy (EN). EN is a human interstitial bilateral noninflammatory kidney disease with a fatal outcome and a prevalence that peaks when persons are in their early 70s. A high incidence of otherwise rare urothelial tumors, occurring about ten years after the age of peak prevalence, was first noticed in the endemic area of Bulgaria and then in other countries. The fact that the appearance of EN and these tumors in farmers is geographically limited suggests there may be a unique, and most likely natural, cause of both diseases. OTA has been found in the dust of grain and coffee in food production facilities, but the main source of human exposure is food, as OTA contaminates food of vegetable and animal origin worldwide. Large studies were performed in Croatia and Bulgaria to see whether the population of the EN regions was exposed to higher OTA concentrations than other populations. In both countries, mean blood OTA concentrations in the endemic populations were higher than in control populations. However, OTA was also found in food, feed and human blood in countries where EN has not been detected. Regional differences in OTA blood concentrations in healthy population have been established in Canada, Croatia, France, Sweden, Switzerland, and Tunisia. In Croatia, blood samples obtained from blood banks showed seasonal variations in OTA concentrations, with the highest number of OTA positive samples and the highest mean OTA concentration being found in samples collected during the summer. OTA was also found in breast milk (Italy, Norway, and Sweden). In the Czech Republic, Italy, Spain, and Turkey, dialysis patients were found to have higher OTA concentrations in blood than patients with other renal diseases or healthy persons. Several attempts have been made to link exposure to OTA in patients suffering from the end-stage kidney disease in North Africa with nephropathy

Molecular Mechanism of ochratoxin A Toxicity and carcinogenicity

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Ochratoxin A (OTA) is a mycotoxin that may contaminate a variety of agricultural products, resulting in chronic human exposures. OTA is nephrotoxic and induces renal tumors in rodents, exhibiting significant sex and species differences. Male rats are most susceptible to OTA carcinogenicity and repeated administration of low doses of OTA (up to 210 μg/kg b.w.) for 2 years results in high incidences of renal adenomas and carcinomas arising from the straight segment (S3) of the proximal tubule epithelium. In OTA-exposed rats, kidney tumors develop with a relatively rapid onset and are characterized by their malignant and aggressive behavior. Interestingly, no increase in tumor incidence was observed following treatment with 21 μg/kg b.w, suggesting a non-linear dose-response for renal tumor formation by OTA. However, the mechanism of tumor formation by OTA in the kidney is not well defined and controversial results regarding mode of action have been published. OTA is not mutagenic in *Salmonella typhimurium*, but weak genotoxic effects have been observed in some mammalian cell systems. Conflicting results have been obtained in studies designed to evaluate the potential of OTA to covalently bind to DNA. While experiments using radiolabeled (^{3}H or ^{14}C) OTA and liquid scintillation counting or accelerator mass spectrometry indicate lack of formation of covalent DNA-adducts, spots detected by ^{32}P-postlabeling have been attributed to treatment with OTA. However, these putative DNA-adducts have not been shown to contain OTA or any part of the OTA molecule and no structural information has been provided to date. Consistent with the absence of DNA-binding of radiolabeled OTA, studies on biotransformation in vivo and in vitro indicate that OTA is poorly metabolized and does not form reactive intermediates capable of interacting with DNA. Recently, the structures of a carbon- and an oxygen-bonded OTA-deoxyguanosine adduct, formed by photoirradiation of OTA in the presence of deoxyguanosine, have been reported and suggested to be involved in OTA carcinogenicity. However, formation of these potential adducts in relevant activation systems in vitro or in rats treated with OTA in vivo could not be confirmed by LC-MS/MS or ^{32}P-postlabeling, consistent with results of the DNA binding studies. In agreement with the lack of metabolic activation and low genotoxicity, these data indicate that DNA binding of OTA is not likely to be involved in the mechanism of OTA-induced tumor formation. More recent evidence suggests that OTA may interfere with microtubular dynamics and function of the mitotic spindle, resulting in apoptosis or – in the presence of survival signals such as stimulation of the NFκB pathway – premature exit from mitosis. Aberrant exit from mitosis resulting in blocked or asymmetric cell division may favor the occurrence of cytogenetic abnormalities and may therefore play a critical role in renal tumor formation by OTA.

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Gene expression profile and cell signalling in the kidney of rats fed a low dose of ochratoxin A

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The gene expression profiles were studied in rats fed OTA for 7 days to 12 months at a dose that produced a significant incidence of renal carcinoma after 2 years. In the kidney, several genes known to be markers of kidney injury and cell regeneration were found to be significantly modulated by OTA, suggesting slight cytotoxicity. The expression of genes known to be involved in DNA synthesis and repair, or genes induced as a result of DNA damage or apoptosis, were only marginally modulated. We observed a disruption of pathways regulated by the transcription factors hepatocyte nuclear factor 4 alpha (HNF4α) and nuclear factor-erythroid 2-related factor 2 (Nrf2). The disruption of the Nrf2 pathway was characterized by an inhibition of Nrf2 binding to the Antioxidant Responsive Element (ARE) promoter, resulting in a reduction in the expression of downstream genes known to play a major role in cellular defense against antioxidants. These effects resulted in oxidative damage as evidenced by the formation of abasic sites. These data suggest that reduction of cellular defense against oxidative stress may be a plausible mechanism of OTA toxicity and carcinogenicity. Samples were further analyzed for various cell signaling proteins known to be involved in chemical carcinogenicity. OTA was found to increase the phosphorylation of PKC-zeta, which was correlated with a slight, but selective, downstream activation of the MAP-kinase extracellular regulated kinase isoforms 1 and 2 (ERK1/2) and of their substrates ELK1/2 and p90RSK. Moreover, analysis of effectors acting upstream of PKC indicated a possible mobilization of the Insulin-like Growth Factor-1 Receptor and Phosphoinositide Dependent Kinase-1 system (PDK1). These findings are potentially relevant to the understanding of OTA nephrocarcinogenicity. PKC and the MEK-ERK MAP-kinase pathways are known to play roles in cell proliferation and survival as well as in renal cancer development. Taken together these data support an epigenetic mechanism for OTA carcinogenicity.

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Endemic nephropathy and exposure to mycotoxins

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The occurrence of endemic nephropathy (EN) in populations living in nonindustrialized rural areas suggests a naturally occurring toxic compound as a causative agent. The mycotoxin theory of EN pathogenesis is based on the similarities of pathological kidney lesions in patients with EN and that of pigs in Scandinavian countries. This fact focused EN research on human exposure to the mycotoxin ochratoxin A (OTA), the causative agent of pig nephropathy. OTA is a nephrotoxic and carcinogenic compound that correlated with an unusually high incidence of upper urothelial tumors in patients with EN. In several studies, OTA was found either more frequently or in higher concentrations in various commodities and in blood of persons in endemic region. However, OTA was also found very frequently in healthy humans outside of the endemic region. Various mycotoxins are produced by the same genera of molds, but even one strain may produce different mycotoxins. The co-occurrence of OTA, citrinin and fumonisin B_1 (FB_1), was found in commodities from endemic regions in Bulgaria and Croatia. Therefore, the possibility of the exposure of humans in the endemic region to various mycotoxins and their combinations seems real. The literature data on combined toxic effects of OTA and other nephrotoxic mycotoxins, such as FB_1, penicillic acid (PA), and citrinin, are rather scarce. We have studied the effect of OTA and FB_1 on oxidative DNA damage in rat kidney measured with single cell gel electrophoresis (comet assay). In the kidney of rats treated with either OTA or FB_1 or their combinations, tail length and tail intensity of the comet assay clearly showed their synergistic genotoxic effect. This effect was seen even after the treatment with the combination of such low doses of OTA and FB_1 that are equivalent to those in daily human exposure in Europe (5 and 200 ng/kg b.w., respectively). Taken together these results confirm that the simultaneous exposure to different mycotoxins may have impact on human health.

P-1.1

Effects of flavonoids in ochratoxin A-induced toxicity

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Ochratoxin A (OTA) is a widespread mycotoxin produced by several species of the fungi *Aspergillus and Penicillium*. OTA has been found in relatively high concentrations in sera from people living in areas where Endemic Nephropathy (EN) occurs. Since the occurrence of high serum OTA is not restricted to EN areas, the link between OTA exposure and EN remains to be verified. However, animal experiments show that OTA is a potent nephrotoxic and carcinogenic agent as well as having adverse effects on blood coagulation and the immune response. OTA-induced oxidative stress may explain its genotoxic effects and apoptosis, which, in turn, are likely to play important roles in the development of chronic tubulointerstitial nephritis. The aim of this study was to investigate the impact of flavonoids on cellular defenses in OTA-treated cell lines. MDCK, LLC-PK1, HeLa and K562 cell lines were exposed to 1 M of quercetin, kaempferol, myricetin naringenin and apigenin and/or selected doses of OTA (0.1–10 M OTA/mL of medium) for 24 hrs. We studied the effects of OTA and flavonoids on cell viability and the type of cell death (apoptosis or necrosis), total antioxidative status, reduced-oxidized GSH-GSSG levels and intracellular ATP concentration. The free-radical scavenging ability of flavonoids with different substitution patterns was also tested. We used the DPPH radical-scavenging assay. A decreased total antioxidant status during exposure to OTA would emphasize the role of cellular antioxidants in the cells' defense mechanisms against oxidative stress. We found that all investigated flavonoids possessed scavenging activity against DPPH radicals, but to different degrees. The maximum antiradical activity was recorded for myricetin and quercetin. Thus, we conclude that the administration of antioxidative adjuvants such as flavonoids could be used as a preventive action or as a therapeutic intervention in OTA-induced toxicity.

P-1.2

Comparative genotoxicity of ochratoxin A and aristolochic acid in human kidney cells: Interpretation of ongoing analyses of food, blood, urine and kidney tissue from serbia.

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Ochratoxin A (OTA) is a ubiquitous mycotoxin, considered for 30 years to be a potential etiological agent of Balkan endemic nephropathy (BEN) and associated urothelial tract tumors. OTA is nephrotoxic in all animal species tested so far and is carcinogenic in the rodent. Its toxic effect is enhanced by citrinin, another mycotoxin often produced by the same molds. More recently, it has been speculated that aristolochic acid (AA) could also be responsible for a nephropathy similar to BEN. The aim of this study was to compare the cytotoxicity, using the MTT test, and the genotoxicity, using ^{32}P-postlabeling methods, of OTA and AA, in the range of contamination by these toxins detected in food samples. For this purpose, human kidney cells (HK-2) were incubated in the presence of increasing amounts of each toxin (from 0.1 to 100 μM) for 24h and evaluated for cytotoxicity. DNA adducts were analyzed on DNA extracted from HK-2 cells treated for 7, 24 or 48 hours with increasing amounts (0.1 to 5μM) of OTA, OTHQ (quinone derivate of OTA), and various components of *Aristolochia fangchi*. OTA was found to be more cytotoxic than all components of *Aristolochia* tested. The total amounts of DNA adducts were in the same range for AAI and OTA. In parallel, we analysed food, blood, and urine collected during one month from three families in Serbia. Two of the families have at least one BEN patient and the third contains no person with nephropathy. Large amounts of OTA and citrinin were detected in the food and several OTA metabolites were found in urine. In addition, analyses of the kidney from a BEN patient shows specific OTA-related DNA adducts identified so far by co-chromatography.

P-1.3.

Citrinin enhances the toxic and genotoxic effects of ochratoxin A *in vitro* and *in vivo*

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During storage of cereals, some fungi grow and elaborate Ochratoxin A (OTA) or citrinin (CIT), nephrotoxic mycotoxins that are suspected of being involved in Balkan endemic nephropathy and associated urothelial tumors. Both chemicals are frequently found in food, notably in the Balkan region, and simultaneous administration of OTA and CIT has been found to enhance the incidence of renal tumors in male mice. The aim of this study was to determine the cytotoxic and genotoxic effects of the combination of CIT and OTA in (i) cell culture and (ii) in vivo on Dark Agouty rats fed for 3 weeks with ground wheat enriched with OTA and/or CIT. When the mycotoxins were present simultaneously, cytotoxicity was found to be considerably enhanced and expression of biotransformation enzymes (CYP, COX, LIPOX) was modulated differently. After the rats were fed the combination for 3 weeks, the DNA adduct patterns in their kidneys were similar to those obtained on cell cultures. The main OTA DNA-adduct, identified as C8 dG-OTA and found in human tumors, is increased by the simultaneous presence of CIT and OTA.

P-1.4.

Ochratoxin A induces oxidative stress in rats

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The mycotoxin ochratoxin A (OTA) is produced by *Penicillium* and *Aspergillus* spp. that frequently contaminate cereals and other commodities in tropical countries as well as in countries with a mild climate. OTA has hepatotoxic, genotoxic and carcinogenic effects in all tested animal species, and, due to its pronounced nephrotoxicity, was considered to be involved in the development of endemic nephropathy. The mechanisms of its toxicity and genotoxicity are not understood. The proposed mechanisms of OTA toxicity involve inhibition of protein synthesis, disturbance of gluconeogenesis, and production of reactive oxygen species. In this study, we tested the importance of oxidative stress in the mechanism of OTA toxicity in rats. Adult male Wistar rats were treated orally with multiple daily doses of OTA for 15 days and sacrificed 24 hours after the last treatment. The doses of OTA were: 5 ng/kg b.w., 0.05 mg/kg b.w. and 0.5 mg/kg b.w. The lowest dose used in this study corresponds to the estimated daily intake for OTA for humans in Europe. To determine the effect of OTA on lipid peroxidation and oxidative damage of proteins, concentrations of malondialdehyde (MDA) and protein carbonyls were measured in liver and kidney homogenate. In the liver, the two higher doses of OTA significantly increased the concentrations of MDA and protein carbonyls ($p<0.05$). In kidney, even the lowest OTA dose increased the concentration of MDA and protein carbonyls ($p<0.05$). The concentrations of MDA and protein carbonyls increased with increasing OTA dose. The catalytic activity of the antioxidative enzymes catalase and superoxide dismutase (SOD) was measured in liver and kidney homogenate. Only the highest OTA dose significantly decreased catalytic activity of catalase and SOD in liver and kidney ($p<0.05$). Taken together, these results indicate that oxidative stress is involved in OTA toxicity.

P-1.5

Improvement of the postlabeling method for detection OTA-DNA and AA-DNA adducts

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A recent EU-funded research program on the mechanisms of renal carcinogenicity of ochratoxin A has addressed the genotoxicity debate that was raised because of other laboratories' inability to reproduce results on toxin-DNA adducts that were demonstrated by ^{32}P postlabelling methodology in French laboratories during the past 20 years. DNA adducts of some genotoxic chemical carcinogens are readily demonstrable in many laboratories, but demonstration of the low maximum incidence associated with exposure to ochratoxin A (~$100/10^9$ nucleotides) is dependent on adherence to strict protocols for all the stages of DNA extraction, purification and the complex steps and specific reagents in ^{32}P postlabeling. Demonstration of adducts in renal DNA from rats exposed to very small amounts of ochratoxin A, more representative of natural exposure than of the doses necessary for maximum expression, require an even greater attention to analytical detail. DNA from rats treated with OTA, synthetic dG-OTA adduct and DNA from rats treated with aristolochic acid have been analyzed by the ^{32} P-postlabeling technique (contact transfer or multidimensional) under a variety of DNA extraction and chromatographic conditions. This study allows us to demonstrate that the lack of OTA--DNA adduct detection by some teams is due to methodological problems, including incomplete hydrolysis and inappropriate solvent of migration.

3. MOLECULAR TOXICOLOGY OF ARISTOLOCHIC ACID

INVITED LECTURES

Grollman AP (United States)
Evidence for the role of aristolochic acid in endemic (Balkan) nephropathy

Arlt V (United Kingdom)
Aristolochic acid mutagenesis

Stiborova M (The Czech Republic)
Metabolic activation of aristolochic acid

Dickman K (United States)
Renal metabolism and transport of aristolochic acid

Rosenquist T (United States)
Mouse model of aristolochic acid nephropathy

Josic Đ (United States), Clifton JG, Wang A, Li X, Hixson D, Dong H, Rosenquist T
Proteomics of aristolochic acid nephropathy

Cosyns J-P (Belgium)
Aristolochic acid nephropathy: a pathological clue to Balkan endemic nephropathy?

Zavadil J (United States)
Toxicogenomics of endemic (Balkan) nephropathy

Jelaković B (Croatia)
Endemic (Balkan) nephropathy and aristolochic acid. Lessons from Croatia

Xiaomei Li (China)
Aristolochic acid nephropathy in China

Nortier J (Belgium)
Revisiting aristolochic acid nephropathy in Belgium

Toncheva D (Bulgaria)
Genetic factors predisposing to Balkan endemic nephropathy. Studies in Bulgaria

POSTERS

P-1.6
Scarborough J, Grollman AP, Fernandes A
Lessons from antiquity: chemical analysis of birthwort

P-1.7
Gruia A, Moldovan F, Ilie D, Ordodi VL, Orem WH, Lerch III HE, Pavlovic N, Tatu CA, Paunescu V
Aristolochia clematitis **and Balkan endemic nephropathy in Romania: is there a connection?**

P-1.8
Hranjec T, Kovač-Pejić A-K, Kos J, Dika Ž, Brzić I, Mao W, Chen JJ Grollman AP and Jelakovic B
Dietary exposure to *Aristolochia clematitis* is a risk factor for endemic nephropathy

Evidence for the role of aristolochic acid in endemic (Balkan) nephropathy

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In 1969, Ivic suggested that seeds of *Aristolochia clematitis* might harbor the etiologic agent of endemic nephropathy (EN). He based this novel hypothesis on his observation that, during harvesting, seeds from this plant frequently co-mingled with wheat grain and that flour containing such seeds were nephrotoxic and carcinogenic in experimental animals. In a pilot molecular epidemiologic study in Croatia (*Croat Med J* 46: 116, 2005), we gathered data that strongly support Ivic's hypothesis. Additionally, the clinical and histopathologic features of aristolochic acid nephropathy (AAN), described in a group of women in Belgium who accidentally had ingested *Aristolochia fangchi*, were strikingly similar to those reported for EN. These observations suggest a common etiologic agent for AAN and EN and constitute the guiding hypothesis for our investigations. Using P^{32}postlabeling techniques and HPLC, we detected 7-(deoxyadenosin-N^6-yl)- and 7-(deoxyguanosin- N^2-yl)-aristolactam DNA adducts in renal tissues of patients with EN and in upper urothelial cancer tissues of residents of the endemic region of Croatia. Approximately one-third of these cancers were p53 positive; sequence analysis of the p53 gene revealed that A:T → T:A mutations, found only rarely in urothelial carcinomas, comprise more than half of the mutations detected in this cohort. To explore the possibility that one or more genes involved in the biotransformation of AA and/or repair of AA-DNA adducts might account for the varying degree of nephrotoxicity observed among persons exposed to AA, we used high-throughput genotyping methods to analyze the pattern of single nucleotide polymorphisms in genes of individuals enrolled in our pilot epidemiologic study. In turn, this information was used to establish haplotypes/genotypes associated with susceptibility to EN and to identify genes most likely to be associated with this disease. Taken together, our studies strongly support the central hypothesis, namely, that chronic dietary poisoning by aristolochic acid explains all of the known characteristics of EN, including its unique geographical distribution, the specific pattern of renal tubulointerstitial fibrosis, and the associated increased risk of upper tract urothelial cancer.

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Aristolochic acid mutagenesis: a molecular clue to the etiology of balkan endemic nephropathy-associated urothelial cancer?

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Aristolochic acid nephropathy (AAN) is a unique type of renal disease that predisposes patients to a high risk of urothelial malignancy. There is clear evidence that aristolochic acid (AA), a known human nephrotoxin, is a mutagen and rodent carcinogen forming covalent DNA adducts. The most abundant DNA adduct that we detected in urothelial tissue of AAN patients and AA-treated rats is 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI). In AAN patients, urothelial atypia is associated with overexpression of the P53 protein, suggesting that p53 is mutated in AAN-associated tumors. Interestingly, in one AAN patient whose urothelial tumor cells were available for analysis, we found a characteristic A to T transversion mutation in the p53 gene in these cells. To examine AA-induced p53 mutation spectra in the human p53 gene in laboratory animals, a human p53 knock-in (Hupki) mouse has been constructed. When we sequenced the Hupki p53 gene of immortalized cell lines derived from primary Hupki embryonic fibroblasts (HUFs) exposed to AA, we found that several mutations in HUFs carried a specific A to T transversion mutation. A to T transversions are typical mutations observed in the H-*ras* oncogene of tumors from rodents treated with AA and correspond with DNA adduct formation at adenine residues. These data may indicate the probable molecular mechanism whereby AA causes urothelial tumors. On both clinical and morphologic grounds, similarities between AAN and another fibrosing nephropathy, the Balkan endemic nephropathy (BEN), including the association with urothelial tumors, have been observed. It has been suggested that the mycotoxin ochratoxin A is associated with the development of BEN, however, the etiology remains still unclear. A role of AA in the etiology of BEN is under debate, because AA was found in flour used to bake bread (a dietary staple in the endemic region of Croatia) that was derived from wheat grain contaminated with seeds of *Aristolochia clematitis*. Using the ^{32}P-postlabeling method, we previously identified dA-AAI adducts in randomly collected kidney tissue from a small number of farmers (2 out of 3 cases) with end-stage renal disease and upper urinary tract malignancy living in endemic areas for BEN. These results provide new evidence that AA is a clear risk factor for BEN. AA mutagenesis may provide a molecular clue to the etiology of BEN-associated urothelial cancer.

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Metabolic activation of aristolochic acid

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Aristolochic acid (AA), a naturally occurring nephrotoxin and carcinogen, has been associated with tumor development in patients suffering from Chinese herb nephropathy and/or Balkan endemic nephropathy. Major DNA adducts [7-(deoxyadenosin-N^6-yl)-aristolactam and 7-(deoxyguanosin-N^2-yl)aristolactamĆ formed from AA after reductive metabolic activation were found in renal tissues of patients with both diseases. To assess an individual's susceptibility to this plant carcinogen, it is important to understand which human enzymes are involved in AA activation and/or detoxification.. Using the detailed enzymatic study employing the ^{32}P-postlabeling assay for detection of AA-DNA adducts, we were able to identify the major hepatic and renal enzymes responsible for AA-DNA adduct formation in humans and model experimental animals. We demonstrated that the phase I biotransformation enzymes play a crucial role in AA metabolic activation in species forming DNA adducts, while a role for the phase II enzymes in this process is questionable. AA is activated by both microsomal and cytosolic enzymes. Most of the activation of AA in human hepatic microsomes is mediated by CYP1A2 and/or 1A1, while NADPH:CYP reductase plays a minor role. In contrast to human hepatic microsomes, in human renal microsomes NADPH:CYP reductase is more effective in AA activation. In addition, prostaglandin H synthase (cyclooxygenase, COX) is another enzyme activating AA in renal microsomes. Among the cytosolic reductases, NAD(P)H:quinone oxidoreductase (NQO1) is the most efficient in the activation of AA in human hepatic and renal cytosols, although a role of cytosolic xanthine oxidase (XO) cannot be ruled out. Using the purified enzymes, CYPs, NADPH:CYP reductase, COX, NQO1 and XO, we confirmed their role in the formation of AA-DNA adducts. The orientation of AA in the active sites of human CYP1A1, 1A2 and NQO1 was predicted from molecular modeling and explains the strong reductive potential of these enzymes for AA detected experimentally.

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Metabolism and transport of aristolochic acid by the rat kidney

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Because the basis for selective renal toxicity of aristolochic acid (AA) is not well-established, we have characterized its metabolism and transport by the rat kidney.

Renal handling of AA was examined by measuring (1) urinary excretion of AAI and its metabolites by Wistar rats treated *in vivo* with AAI (10 mg/kg); (2) renal clearance of AAI and its metabolites by isolated, perfused rat kidneys (IPK); and (3) AAI metabolism and transport in isolated, purified rat proximal tubules.

HPLC analysis of 24-h urine samples collected from rats treated with AAI detected 5 metabolites, principally in the form of AAIa (70%), a demethylation product, and aristolactam Ia (25%), a demethylation and nitroreduction product, while the parent compound accounted for only 2% of total urinary AAI content. The urinary metabolite profile in IPKs perfused with 50 μM AAI was similar to that observed *in vivo*, with AAIa and aristolactam Ia accounting for 67% and 6%, respectively, of total AAI excretion. AAIa was the main metabolite detected in perfusate and increased in concentration over time. Excretion ratios (ER) indicated net reabsorption of AAI (ER 0.24), while AAIa underwent net secretion (ER 20), which was blocked by the organic anion transport inhibitor probenecid. GFR and fractional reabsorption of Na^+ and glucose were stable over 80 min, indicating the absence of acute toxicity. Isolated rat proximal tubules, the primary site of toxicity *in vivo,* extensively metabolized AAI by demethylation, with medium levels of AAIa exceeding those of the parent compound after 30 min incubation with 5 μM AAI. Transport assays with ^{3}H-AAI indicated that uptake into isolated proximal tubules was time- and dose-dependent, and could be inhibited by unlabeled AAI, indicating the presence of a carrier-mediated step. We conclude that renal excretion of AAI involves secretion of AAIa, a demethylation product formed by intrarenal metabolism.

Mouse model of aristolochic acid nephropathy

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Mice treated with aristolochic acid develop proximal tubule necrosis, interstitial fibrosis, and renal failure. While both AAI and AAII are genotoxic and form DNA-adducts in renal tissue in mice, it is the methylated form, AAI, which is responsible for the cytotoxic effects in proximal tubules. As a prelude to mapping genetic loci that contribute to this disease, we tested the relative sensitivity of the C57Bl/6, DBA/2, C3H/He, Balb/C, A/J, and 129S6/SvEv inbred strains of mice to AAI treatment. Although all strains exhibit proteinuria and glycosuria within 3–4 days of treatment, there is a marked gradation in the severity of the response and the recovery time following treatment. Similarly, histologic examination of renal tissue reveals that the strains vary in degree of renal injury. We have begun treating the BXD recombinant inbred lines derived from the DBA/2 (sensitive) and C57Bl/6 (resistant) strains to map loci contributing to AAN in the mouse.

Proteomics of aristolochic acid nephropathy

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Starting with the hypothesis that endemic nephropathy (EN) is caused by chronic poisoning with aristolochic acid, we established the proteomic profile of serum and urine from mice sensitive and resistant to the effects of this nephrotoxic agent. Serum and urine from mice treated with aristolochic acid were taken at different time points and analyzed with SDS-PAGE, 2D-electrophoresis, and SELDI-TOF mass spectrometry (MS). We then compared protein profiles and characterized spots/bands representing proteins present or absent in body fluids of sensitive animals. Statistically significant differences were further analyzed and proteins identified by MS/MS using a QStar mass spectrometer. AAI-treated mice display proteinuria characteristic of proximal tubule defects. This signature is greatly reduced in AAII-treated mice. These and other recent proteomics studies in the mouse model of aristolochic acid nephropathy will be discussed.

Aristolochic acid nephropathy: a pathological clue to (balkan) nephropathy?

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Aristolochic acid nephropathy (AAN) is a rapidly progressive renal interstitial fibrosis caused by the consumption of herbs containing aristolochic acid (AA). It is characterized by extensive, dense, pauci- or acellular interstitial fibrosis and tubular atrophy decreasing in intensity from the upper to the inner cortex. End-stage kidneys are severely and symmetrically shrunken, presenting smooth outlines and weighing only about 10 g each. Multifocal urothelial atypia is found consistently and upper urinary tract malignancy develops in approximately 40%–46% of the patients. Balkan nephropathy is a chronic progressive interstitial nephritis characterized by multifocal to diffuse hypocellular interstitial sclerosis and tubular atrophy predominating in the outer cortex. End-stage kidneys are contracted with a smooth outline and weigh only 20–30g each. Upper urinary tract cancer and urothelial atypia develop in 41% of the patients. Morphometric analysis discloses that in both AAN and BN, the severe interstitial fibrosis is hypocellular and decreases from the outer to the inner cortex. This pattern is unlike that seen in other common end-stage renal diseases, such as chronic glomerulonephritis, benign nephrosclerosis, chronic idiopathic interstitial nephritis (without AAN or BN), and chronic pyelonephritis or reflux nephropathy. Nevertheless, the morphologic features of interstitial fibrosis are not pathognomonic of AAN or BN. Hypocellularity is indeed also present in the interstitial fibrosis caused by toxic substances including cadmium, lead, cyclosporin A, ifosfamide and herbs (possibly without AA content). However, it is not associated with urothelial cancer or with a decreasing cortico-medullary gradient (except for cadmium). In contrast, interstitial fibrosis due to analgesics (also associated with urothelial malignancy), lithium and nitrosureas, is hypercellular (without evidence of a gradient). Available data on the interstitial fibrosis caused by ochratoxin A (OTA), a nephrotoxic and carcinogenic mycotoxin produced by several species of Aspergillus and Penicillium, are too scarce to assess cellularity and a possible cortico-medullary gradient.

Thus, available pathological data seem to incriminate AA as the etiologic agent responsible for the combination of urothelial malignancy and the severe, hypocellular, mainly outer cortical interstitial fibrosis characterizing AAN and BN.

Toxicogenomics of endemic nephropathy

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The aims of this research were (1) to determine distinct gene regulation patterns and pathophysiologic responses to AAI vs AAII in aristolochic acid nephropathy (AAN), and (2) to identify key gene regulatory mechanisms underlying tubular injury and tubulointerstitial fibrosis (TIF) in AAN. To study pathogenesis of AAN *in vivo*, C3H/He mice were injected intraperitoneally with 1.8 mg of AAI or AAII per kg of body weight, daily for 11 days. To identify the molecular bases of AAN, we combined toxicogenomic and histopathologic temporal analyses of AA-treated C3H/He renal cortical epithelia as well as cultured human primary and immortalized renal proximal tubular epithelial (PTE) cells. AA-DNA adduct formation was assayed by ^{32}P-postlabeling/PAGE analysis. PTE injury and TIF were analyzed by (immuno)histochemistry, and temporal mRNA and microRNA expression analyses were performed using Mouse Genome GeneChip 430A 2.0 (Affymetrix) and microRNA labeling and detection system mirVana™ (Ambion), respectively. MicroRNA: mRNA interactions were predicted by computational algorithms PicTar, TargetScan and miRBase. Both AAI and AAII rapidly formed AA-DNA adducts in renal PTE cells and initiated the DNA damage response and DNA repair genetic programs. In contrast, only AAI caused severe nephrotoxic and fibrogenic effects in C3H/He mice, which were marked by induction of the fibrogenic markers FSP-1 (S100A4), vimentin, a-smooth muscle actin, and collagen-I and -III. Correlation of microRNA and mRNA expression profiling with histopathologic analysis implicated AAI-regulated genetic programs of necrosis and apoptotic cell death, inflammatory/immune response and fibrogenesis. By comparing the AAN model with the TGF-ß transgenic mouse model of progressive renal injury, a central role of TGF-ß in AAN was established based on overlapping TGF-ß-specific target gene and microRNA expression signatures. In conclusion, AAI and AAII elicit distinct gene regulation programs that are responsible for the severity of nephrotoxic effects in the mouse model of AAN. We have identified key gene regulatory and signaling pathways underlying AAN, which provide potential molecular targets for therapeutic approaches to renal epithelial injury and fibrosis.

Endemic (Balkan) nephropathy and aristolochic acid. Lessons from Croatia

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Although the first surveys on EN in Croatia were performed in 1957, oral tradition and church records suggest that EN was present many decades earlier. In fact, EN was verified post-mortem in one patient from a Croatian endemic village who died in 1948. If EN was present before, why was a dramatic number of patients noticed specifically 40–50 years ago? Is this a consequence of better diagnosis of renal failure (RF) or related to greater exposure to the causative agent as a result of changes in agriculture habits after melioration? In 1959, Radošević proposed the name EN (accepted by WHO, 1965) and Radonić published the first findings on renal biopsy. During recent decades, in addition to several epidemiological surveys, investigations in Croatia focused on early diagnosis. In addition, many earlier hypotheses were not confirmed by subsequent studies and remain unproved. In the last several years, investigators have been studying *A. clematitis* (AC). Croatian scientists have been aware of the toxicity of AC for many decades, beginning with Dumić (1954) and Martinčić (1957) who described the toxic effects of AC on the horses' kidney. The prevalence, clinical course and pathological findings in horses poisoned with AC strikingly resemble the findings in human aristolochic acid nephropathy (AAN) and EN. It is hard to understand why these obvious similarities didn't result in further investigations. In 1969, Ivić published important work in a Croatian medical journal describing his field investigations in an endemic focus in Serbia as well as his findings on the toxic effects of AC on the rabbit kidney, which were in line with results of Dumić and Martinčić. Again, unfortunately, the importance of this work went unrecognized. Finally, the Chinese herb nephropathy tragedy in Belgium and Cosyns analogy between AAN and EN led to intense investigations of the role of AC in EN. Using a ^{32}P postlabeling assay and 2D-thin layer chromatography, Arlt et al (2002) detected spots in DNA of patients from the Croatian EN region, which corresponded to those found in AAN patients in Belgium. Our group has detected AA-DNA adducts in the renal tissue of several EN patients as well as in patients with EN-associated upper urothelial cancer (UUC). Further, in a majority of our patients, the mutation spectra of p53 was typical of an AA-induced mutation (A to T transversion). In this paper, the clinical course of three patients from one Croatian EN family will be presented – one with RF, another with bilateral UUC and later development of RF and a third with RF and later development of UUC. The clinical course, pathohistologic findings and mutation spectra of p53 in those patients support AC not only as a marker of exposure but as a causative agent of EN and associated UUC.

Progression of Chronic Aristolochic Acid Nephropathy: Follow Up of 43 Chinese Patients

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Aristolochic acid nephropathy (AAN) is a progressive interstitial nephropathy, although it has been observed that the clinical features of this disease differ among Chinese patients. The aim of this study was to explore the progression pattern of this disease and the risk factors related to deterioration of renal function. In this study, the clinical characteristics of 43 patients with AAN were followed regularly for an average of 21.817.9months. 91% of patients suffered from AAN because of ingesting Chinese herbal medicine containing AA often over the course of many years. Parameters that might contribute to progression of renal function deterioration were recorded, including anemia, hypertension, proteinuria, the cumulative dose of AA-I in the medication and any concomitant diseases. Based on known routine therapies for CKD, the progression rate of renal failure was monitored by the decline in eGFR, calculated by the MDRD formula. The endpoint of this follow-up was considered reached when the eGFR declined less than 15 ml/min. The data were analyzed by correlation analysis and Cox regression analysis. The results revealed that at the time of diagnosis the level of eGFR (34.810.6ml/min) was not related to the cumulative dose of AA-I in the medication, but was correlated with hemoglobin level ($R=0.539$, $P<0.05$). The deterioration velocity of eGFR in these patients averaged 3.19 ml/min per year. At the end of the study, 20 patients reached the eGFR endpoint after being monitored for 4–59.8 months (median 9.7 months). Only a few of the patients deteriorated rapidly within six months. Analysis by Cox survival regression showed that accompanying coronary heart disease and the level of hemoglobin were important factors affecting outcome of AAN, the relative risk ratios being 15.5 and 0.763, respectively. In conclusion: Under effective routine therapy for CKD, renal function decline in Chinese AAN patients appears to have a relative slow progression, which is unlikely related to the exposure dose of toxic AA drugs. Effective therapy of CKD, especially anti-anemia and intervention in cardiovascular disease, may improve the outcome of AAN.

Aristolochic acid nephropathy in Belgium: A clinical and epidemiological update

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A new renal disease called »Chinese-herb nephropathy« (CHN) has been reported to occur in Belgian women who ingested slimming pills containing powdered extracts of the Chinese herb *Stephania tetrandra* (ST). Moderate- to end--stage renal disease (ESRD) developed in these women, requiring renal replacement therapy by dialysis or transplantation. Phytochemical analyses revealed the presence of aristolochic acids (AA) instead of tetrandrine, suggesting the substitution of ST (Han fang ji) by *Aristolochia fangchi* (Guang fang ji), containing nephrotoxic and carcinogenic AA. Histological characteristics of AAN are a progressive interstitial fibrosis leading to severe atrophy of the proximal tubules, as documented by the urinary excretion rates of markers of tubular integrity. As of December 31, 2005, CHN – also called aristolochic acid nephropathy (AAN) – had been diagnosed in 112 patients in our Nephrology department and 53 of them had reached the stage of ESRD. Among these patients, removal of the native kidneys and ureters revealed a high prevalence of urothelial carcinoma of the upper urinary tract (>40%). Tissue samples were found to contain AA-related DNA adducts, which are not only specific markers of prior AA exposure, but are also directly involved in tumorigenesis. In renal transplanted AAN patients, endovesical follow-up resulted in the detection of 14 cases of bladder urothelial carcinoma. We were able to reproduce the main features of AAN (chronic renal failure, interstitial fibrosis, urothelial dysplasias) in a short-term animal model by injecting AA to male Wistar rats, bringing the experimental proof of AA involvement in human AAN. Despite the US Food and Drug Administration alert and IARC recommendations, botanicals known or suspected to contain AA are still available over-the-counter in many countries and on Web sites. Health professionals should be aware that in traditional Chinese medicine, *Aristolochia* spp. are considered interchangeable with other ingredients and are sometimes mistaken for ST, *Akebia*, *Asarum*, *Clematis* spp. and *Cocculus* spp. in herbal remedies.

Genetic studies in ben and associated urothelial cancers

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BEN is a primary, chronic tubulointerstitial nephritis with early onset and slow progression to terminal renal failure. About 30%–48% of BEN patients develop epithelial cell tumors of the upper urinary tract. The etiology of BEN remains unclear. Two groups of factors may contribute to the etiology and may also explain the endemic distribution of BEN – environmental agents and hereditary factors. The combination of polymorphic genes with various environmental factors may result in an increased risk for the disease. A large-scale investigation in BEN patients on the role of genetic polymorphism in 9 genes from a detoxification system was done: *CYP2D6 (*3, *4, *5, *L), CYP3A4 (*1, *V), CYP3A5 (*6986G>A, 14690G>A, 27289C>A, 3705C>T, 3709-10insG), *NQO1 (*1, *2, *3)*, *NAT1 (*C559T, G560A, T640G, T1088A, C1095A), *NAT2 (*T341C, C282T), *GSTT1 (*1, *0), GSTM1 (*1, *0).* The data revealed a higher risk for BEN (OR 2.41) in individuals carrying the *CYP3A5*1* allele G6986. We explored the effect of various combinations of genotypes on BEN risk and found an increased risk when the *CYP3A5* genotype G6986/A6986 (OR 2.5) is combined with active *GSTM1*, *NAT1* genotype rapid/slow and null *GSTT1*. Polymorphisms in transporters (*MDR1* gene*)* may also contribute to inter-individual differences in the response to environmental factors. For example, the haplotype pair 11/22 is associated with BEN (OR 2.5). TGFß1 was suspected to be a factor in renal fibrogenesis in BEN, since interstitial fibrosis is the major feature of the disease. The variant allele *263Ile* was significantly rare in BEN cases compared to the controls. The established lower frequency of the variant allele *263Ile* in BEN may indicate that active TGFß1 protein is more frequent in BEN patients than in the general Bulgarian population. It was also found that the genotype *2/*2 *NQO1* predisposed BEN patients to the development of UTT (OR=13.75, 95%CI 1.17–166.21).

Neopterin was used as a marker to detect activation of the cellular immune system in the course of viral infections, inflammatory diseases and cancer. Data obtained demonstrate a markedly increased N/C ratio in 50% of BEN patients (263.1 mol/mol of creatinine) as compared to the normal ranges (197–229 mol/mol of creatinine in males, 229–251 mol/mol creatinine in females).

P 1-6.

Lessons from antiquity: chemical analysis of ancient herbal recipes

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For several millennia, birthwort (*Aristolochia clematitis*.) has been used for medicinal purposes, including by midwives as an aid in childbirth. The first written records of its use come from Ancient Greece, although there is limited evidence of use among earlier cultures of the ancient Near East. Recently, the nephrotoxic and carcinogenic properties of *Aristolochia sp.*, used for medicinal purposes in China and elsewhere and, inadvertently, as a dieting adjunct in Belgium, have been amply documented. Despite these reports, *Aristolochia sp.* continue to be used throughout the world as an integral component of traditional medical practices. In view of this widespread usage based on traditional texts, we have explored the potential toxicities of ancient recipes containing birthwort. One of us (JS) translated 16 recipes from the original Greek, Latin, Coptic, and Arabic, noting specific weights, measures, and methods of administration. Four of these recipes were selected for subsequent laboratory analysis. The texts include Scribonius Largus (Latin) and Galen quoting earlier authorities (Greek), an anonymous Coptic recipe (c. A.D. 900), and an Arabic recipe in the Small Dispensatory of Sabur ibn Sahl (c. A.D. 900), the earliest known tract of medieval Islamic pharmacology. Although the ingredients are often exotic, one of us (AF) obtained all substances necessary to replicate these ancient recipes. We found that the aristolochic acid content of these herbal mixtures, as determined by HPLC, is sufficient to produce nephrotoxicity and, potentially, urothelial cancer. We conclude that the long-standing failure of practitioners of herbal medicine to recognize the profound toxic effects of *Aristolochia sp.* reflects the extended period of time that elapses between the ingestion of this herb and the onset of aristolochic acid nephropathy and its associated urothelial cancer.

P 1-7

Aristolochia Clematitis and Balkan endemic nephropathy in Romania: is there a connection?

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The purpose of our study was to correlate the toxicity of plant extracts in cell cultures after aristolochic acids were identified and quantitatively analyzed by high-performance liquid chromatography and gas-chromatography with mass spectrometry.

In order to obtain the extracts from *Aristolochia clematitis* we used two methods: reflux and ethanolic maceration. We then tested the obtained plant extracts on mesenchymal stem cells and a kidney cell line. To assess the toxicity of these extracts we used a standard MTT assay. The cytotoxic effects of plant extracts obtained by reflux and maceration ethanolic solutions were compared and statistically analyzed.

Aristolochia clematitis is a nephrotoxic and carcinogenic plant species due to its content of aristolochic acids. We showed that aristolochic acid, a possible cofactor of Balkan endemic nephropathy, can induce toxic effects in the cell lines studied in both aqueous and ethanolic extracts.

P 1-8.

Dietary exposure to *Aristolochia clematitis* is a risk factor for endemic nephropathy

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In 1969, Ivić suggested that seeds of the plant *Aristolochia clematitis* (*vučja stopa)* might harbor the etiologic agent for endemic nephropathy (EN), namely, aristolochic acid. In support of his hypothesis, he cited reports that horses consuming hay containing *vučja stopa* developed signs of renal failure and that histopathologic examination of tissues from these animals revealed renal tubular disease and chronic interstitial nephritis with minimal glomerular damage. To explore the possibility that chronic dietary poisoning of humans by aristolochic acid could account for the clinical and epidemiologic features of EN, including the increased risk of developing upper urothelial cancer, we conducted a case--controlled epidemiologic study of 88 subjects residing in an endemic region of Croatia. The results of this study, reported in 2005, provide strong evidence of potential exposure to aristolochic acid in the form of home-baked bread, a dietary staple in the endemic region where harvesting practices enable the wheat grain used in preparing flour to co-mingle with seeds derived from *A. clematitis*. Chemical analysis established that seeds from plants in the endemic region contain 0.65% aristolochic acid. Thus, dietary exposure representing 8–10 years of daily ingestion of one-half a loaf (0.5 kg) of bread prepared from flour contaminated by a single seed of *A. clematits* is equivalent to the exposures reported for women in Belgium and in China with documented aristolochic acid nephropathy. These observations prompted a detailed review of harvesting and milling practices in the endemic region over the past 80 years. Throughout this survey, we identified practices that could account for the unique geographical distribution of EN. In conjunction with the confirmation of our pilot study results by the ongoing *perlustracija* and the unequivocal identification of aristolactam-DNA adducts in target tissues of patients with EN, our investigations will help to identify individuals at risk of this devastating disease. These data may also help to persuade public health authorities to implement measures that effectively remove the sources of contaminated grain.

4. EPIDEMIOLOGY OF ENDEMIC (BALKAN) NEPHROPATHY

INVITED LECTURES

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Endemic (Balkan) nephropathy in Bosnia and Herzegovina: current status

Dimitrov P (Bulgaria)

Current research on Endemic (Balkan) nephropathy in the Vratza region of Bulgaria

Miletic-Medved M (Croatia)

Prevalence of endemic nephropathy and urothelial cancers in Croatia

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A study of adult offspring in families with Balkan endemic nephropathy

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Health effects of toxic organic substances from coal: pandemic nephropathy

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Prevalence, treatment and control of hypertension in a Croatian focus of EN – comparison with Epidemiology of hypertension (EHUH) study results

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Serum paraoxonase activities in endemic nephropathy patients on long term hemodialysis

P.-2.13

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Endemic nephropathy: should the diagnostic criteria be revised?

Endemic (Balkan) nephropathy in Bosnia and Herzegovina: current status

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To evaluate the trend in prevalence of Balkan endemic nephropathy in Bosnia-Herzegovina during the years 1977-–2006. The prevalence of Balkan endemic nephropathy (BEN) was tracked in a prospective study from 1977 through 2006. An epidemiological survey was conducted in 1977 in six endemic municipalities (Samac, Brcko, Orasje, Odzak, Bijeljina and Modrica) and was repeated by our medical team in 2002 and 2006 in the same municipalities, plus Doboj and Domaljevac. Data on the total population and BEN patients were obtained in the 1977 study and data on the total population, number of BEN patients, number of BEN patients on dialysis and total number of dialysis patients were determined in the studies in 2002 and 2006. A comparison of proportions test was applied using MedCalc for Windows, version 8.1.0.0 (MedCalc Software, Mariakerke, Belgium). Data from the surveys are shown in the table below:

Survey Year	Inhabitants	BEN patients	BEN on dialysis	Total dialysis pts
1977	244,016	1730		
2002	354,657	1890	253	337
2006	434,180	2026	265	573

When the number of BEN patients on dialysis and total dialysis patients from Modrica and Doboj in 2006 were excluded from the calculation (since they did not have data to compare from 2002), these numbers were 1890:2026, 253:248 and 337:543. Thus, the total BEN burden in 2006 appears unchanged from 2002, although the actual number of BEN patients may have been higher in 2002, as data from Orasje, Domaljevac, and Odzak were missing. In 1977, 2002, and 2006, the BEN prevalence rates were 7.09%, 6.2% and 6.4%, respectively. There was no significant difference between 1977 and 2002, 2002 and 2006, and 1977 and 2006 ($p=0.81$, >0.95 and 0.90, respectively). The prevalence of BEN patients on dialysis was 0.71% and 0.61%, respectively ($p=0,09$). There has been no change in the prevalence of BEN in Bosnia and Herzegovina from 1977 to the present.

Current research on Balkan Endemic nephropathy in the Vratza region of Bulgaria

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Our aim was to describe the epidemiologic characteristics of BEN in the Vratza region of Bulgaria and to rigorously test hypotheses regarding its etiology and clinical development. The epidemiologic characteristics of BEN were determined from register and hospital records of BEN patients. In 2003, with funding from the US National Institutes of Health, we started a new follow-up study in the Vratza region with a study population of 102 adult BEN offspring and 99 controls. Currently, we have taken three measurements, one year apart. Our first study showed a decline in the incidence and prevalence of BEN in the Vratza region of Bulgaria. The incidence declined from 1.7/1000 (1965–1975) to 0.8/1000 person-years (1976–1987). However, we also found an underreporting of BEN cases and a less complete case identification, especially after 1979. Our new study showed that BEN offspring had a greater risk of developing early reductions in kidney size and function, in particular when it was the mother who had BEN. The study also found increased blood pressure related to kidney size and maternal history of BEN. However, it is not clear whether hypertension is a clinical precursor of BEN, will disappear in the course of the disease, or whether BEN patients have increased blood pressure. The study also found that BEN is not likely to be caused by exposure to metals or metalloids: urine excretion of cadmium and arsenic was not greater in the offspring of BEN patients and was also not related to kidney function nor kidney size. Selenium was not protective, but rather was related to increased beta2-microglobulin excretion. Surprisingly, we found that C-reactive protein, a marker of inflammation, was increased in adult BEN offspring, but only if the mother had BEN.

To understand etiology and clinical development of BEN, we need more rigorous studies. As much of the risk appears to be related on the maternal side, we suggest focusing on the intrauterine development of BEN and on exposures during pregnancy. In addition, research into BEN may serve as a model to better understand the etiology of other kidney diseases.

Prevalence of endemic nephropathy and urothelial cancers in Croatia

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The estimation of the specific dynamics of endemic nephropathy (EN) in the endemic area of Brodska Posavina is facilitated by medical surveillance of the population over several decades, frequent large-scale field screening, almost unvaried diagnostic criteria and uniform files in this region. In the last 25 years, approximately 70% of inhabitants from 13 Croatian endemic villages (10,865 individuals) were examined several times using the WHO criteria. In the period 1980–1991, the mean prevalence for the entire region was 1.3%, and ranged from 0.4–2.3% for each village. Between 1991 and 2002 the average overall mortality in the endemic region was 10.3/1000 and the specific mortality for patients with EN was 0.65/1000. The average age at death of patients with EN was 69.2 years, which is similar to the rest of the population in the county (67.8). The specific mortality of all patients with cancers of the pyelon and ureter was 14 times higher in the endemic region than in Brodsko-Posavska county and 55 times higher than in Croatia overall. In the spring of 2005, 1,081 inhabitants of three endemic villages (Kaniža, Bebrina, Banovci) and the control nonendemic village of Klakar were examined using the same criteria, and persons were divided as follows: having the disease of EN; suspected of having EN; at risk of EN; and other inhabitants. Among the 1,081 persons, we found 25 with EN (12 men and 13 women). This group consisted of autochthonous Croats as well as Ukrainian immigrants. The prevalence of EN was 0.6% in Kaniža, 2.1% in Bebrina, and 2.3% in Banovci, while the prevalence of suspected EN was twice or three times higher. One fifth of the inhabitants were at risk of having EN. The disease has not vanished in any of the endemic villages. This epidemiologic survey found that the prevalence of EN in three endemic villages in Croatia is approximately the same as it had been during the previous survey(s) and remains a serious public health problem in Brodska-Posavina county. In addition to medical and scientific examinations, we will need help in elucidating the etiology of EN as well as in improving the treatment of this poor rural population.

A study of adult offspring in families with Balkan endemic nephropathy

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BEN is a chronic tubulointerstitial disease with insidious onset and slow progression to end-stage renal failure. The disease shows familial clustering and develops only in certain areas in the Balkans. The aim of this study, in the South Morava Region of Serbia, was to determine whether clinical markers of BEN are altered in adult offspring of BEN patients compared with non-BEN offspring or in new members of endemic families. A total of 98 BEN offspring, 25 immigrants to endemic families, and 50 control subjects participated in this study. All cases and controls resided in the villages of Brestovac and Kutles. There were no statistically significant differences in age or gender between the BEN offspring and controls. The mean length of the kidneys in the offspring of BEN patients was 112 mm and in the control offspring it was 112.5 mm. The minimum cortex width was 12 mm in both BEN offspring and in the control offspring. With regard to parental history of BEN, having a mother with BEN was related to a significant reduction in kidney length.

The CCR analyzed according to Cockcroft and Gault did not show any statistically significant difference for the two risk factors under consideration: being an offspring from a BEN family or having a mother and/or father with BEN. The total urinary protein excretion in BEN offspring was higher than in the control offspring, with median values of 9.8 and 9.1 mg/mmol creatinine, respectively. Total urine protein excretion was higher among offspring whose fathers had BEN than among offspring with no affected parent. The same tendency was found for urine albumin excretion. Beta-2-microglobulin excretion was highest among offspring whose mother had BEN. Urinary NAGA excretion was highest among offspring whose mother had BEN, and next highest among immigrants. Correlation among various markers was performed, with the CCR (Cockcroft and Gault) showing a significantly positive rank correlation with kidney length and cortex width.

Critical evaluation of environmental exposure agents suspected in the etiology of Balkan endemic nephropathy

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Endemic nephropathy (EN) results from prolonged exposure to one or more environmental toxicants acting alone or synergistically on genetically predisposed individuals/populations. Previous efforts have devoted considerably more attention to the clinical, toxicological, and epidemiological aspects of the disease rather than to exposure assessment. We report the results of field sampling and analysis studies to evaluate the potential for exposure to several classes of proposed agents and propose criteria for future testing of hypotheses on exposure to environmental agents. We believe that our field sampling and analysis contributes the following evidence to body of EN literature: 1.nitrogen compounds are unlikely agents, but their presence in water supplies may indicate a potential pathway for other agents; 2.PAHs can be eliminated from consideration, and while other coal-derived compounds could be involved, the primary evidence in support of the Pliocence lignite hypothesis is the spatial association between lignite deposits and EN endemic villages; 3.Ca and Mg deficiencies can be eliminated from consideration; 4.hypotheses related to exposure to heavy metals, and As and Se deficiency, could be neither confirmed nor rejected, but could be consider less likely since differences between EN and non-EN villages were not strong. We suggest six criteria that could be used to evaluate and compare different exposure agents: 1. Exposure – evidence that exposure occurs in the endemic areas.; 2.Follows spatial pattern – evidence that higher exposure may occur in endemic areas over non-endemic areas;3.Toxic levels – evidence that exposure levels for residents of endemic regions are high enough to cause health effects to potentially be biologically significant; 4.Nephrotoxic – evidence associating this agent with tubulointerstitial nephritis in human subjects or evidence of primary proximal renal tubular damage in experimental animals; 5. Carcinogenic – evidence associating this agent with upper urothelial cancers in human subjects or in experimental animals; 6.Absence of other arguments – absence of strong alternative explanations for evidence offered as supportive. Using these criteria, we conclude that several of the agents that have been proposed and remain under consideration can be eliminated or considered unlikely due to apparent inconsistencies between clinical or epidemiological evidence related to EN and toxicological or exposure evidence related to the agent. It appears that the toxicological evidence for two proposed agents, mycotoxins and aristolochic acid, is reasonably consistent with what is observed for EN, and while the evidence on exposure for both is consistent, it is insufficient to implicate either.

P-2.1

Health effects of toxic organic substances from coal: pandemic nephropathy

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Coal contains a myriad of organic compounds, some known to be toxic (e.g., polycyclic aromatic hydrocarbons, aromatic amines) and others that are potentially toxic (e.g. N-, S-, and O-containing heterocyclic compounds). Mobilization of toxic organic compounds from coal into the environment through combustion or leaching into water, and long-term human exposures to these compounds, may lead to disease occurrence. One example of a disease linked to coal-derived toxic organic compounds in water supplies is Balkan Endemic Nephropathy (BEN). BEN is a kidney disease with a high co-incidence of renal pelvic cancer (RPC) and occurs only in clusters of rural villages in Romania, Serbia, Bulgaria, Croatia, and Bosnia. The unusual geographic restriction of BEN is spatially correlated with the occurrence of Pliocene coal (lignite) deposits. The hypothesis being tested is that groundwater leaches toxic organic compounds from lignite located in the hills surrounding the endemic villages and transports these compounds to wells and springs that serve as water supplies. Exposure to these toxic, coal-derived organic compounds for more than 20 years may be one factor (combined with genetics and other factors) leading to BEN and RPC. Results of our field and laboratory studies have demonstrated that: (1) drinking water in BEN villages has higher concentrations and amounts of low- and high-molecular weight organic compounds than drinking water from control sites (nonendemic villages) and (2) organic compounds in drinking water from BEN villages are similar to compounds in laboratory water leachates of coal (lignites) from BEN areas. Toxicologic studies on human kidney and other types of cells have shown that organic compounds extracted from BEN area lignites, and organic compounds isolated from the well water in BEN villages, can stimulate excessive cell proliferation in culture, suggesting possible carcinogenic properties linked to RPC. In higher doses, the same extracts induce cell necrosis, effects that could explain the nephropathy characteristic of BEN. High rates of RPC are also found in the USA in states having low-rank coal deposits and rural populations that use groundwater for water supplies. Preliminary results show that wells in aquifers containing coal in WY and LA contain significantly higher concentrations of organic compounds compared to control sites. Some of the organic compounds identified in well water from WY and LA closely resemble those observed in wells in BEN villages. These results and other observations have led to the development of the concept of Pandemic Nephropathy, or BEN-like diseases worldwide, which appear to be linked to coal-derived toxic organic compounds in drinking water.

P-2.2

The Role of Lecithin cholesterol acyltransferase and organic substances from coal in the etiology of Balkan endemic nephropathy

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Balkan Endemic Nephropathy (BEN) occurs with a high prevalence in geographically restricted areas of the former Yugoslavia, Romania and Bulgaria. The etiology of BEN has been the subject of numerous studies, resulting in the publication of several hypotheses, including the impact of coal-derived nephrotoxic organic compounds in drinking water and the role of lipid metabolism (activity of lecithin cholesterol acyltranferase or LCAT activity) in the onset of BEN. Most researchers favor the idea of multiple factors operating in the etiology of BEN. In this study, we investigated a bi-factorial hypothesis for BEN by determining the impact of organic compounds isolated from drinking water in BEN villages on plasma LCAT activity. Water concentrates for the LCAT studies were prepared by tangential flow ultrafiltration (TFU) using 50 liters of clear water and yielding 100ml of final volume. The water was obtained from wells and springs in BEN villages (Serbia and Romania) and from control villages outside of BEN areas. TFU concentrates of well and spring water from BEN villages were much darker colored (more organic molecules) as compared with control sites. Plasma samples were mixed with the TFU concentrates at the raising step values of 10%. In this way, nine categories of diluted media were obtained, with the plasma volume ranging from 90% to 10%. Deionized water controls were mixed in the same fashion as the TFU concentrates. LCAT activity was measured by HPLC with fluorescent detector (change in 470/390 emission intensity) using an LCAT kit (Roar Biomedical). We found that TFU concentrates from drinking water wells in BEN villages in Serbia ($p<0.013$) and Romania ($p<0.003$) showed significantly higher LCAT inhibiting activity than TFU concentrates from drinking water wells in non-BEN villages in Serbia ($p<0.029$), Romania ($p<0.099$), and controls. Based on these results, we have concluded that the LCAT inhibiting activity of the organic compounds may trigger pathogenic mechanisms responsible for the development of BEN. It seems very likely that factor(s) operating in the etiology of BEN are of a ubiquitous nature and we believe that this pathological entity is not restricted to Balkan countries.

P-2.3

An environmental science approach to study the spatial distribution of endemic nephropathy

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Environmental exposure to toxic agents may adversely affect those who are vulnerable temporally (age, developmental stage), spatially (geographic location), or because of unique circumstances (nutritional status, socioeconomic status, genetics). The basic methodology of exposure assessment involves measuring exposure concentrations in the environment (e.g., water, air, soil, food), extrapolating this information to potential means of human contact (e.g., inhalation, ingestion, dermal contact), and measuring the concentration of parent compounds or their metabolites in biological samples (e.g., blood, urine). Such an approach assumes prior knowledge of the environmental agent where it causes a particular disease and has been detected in humans and an understanding of the media/route of exposure. In many instances, exemplified by EN, there are gaps in the knowledge base that make it difficult to develop strategies to protect humans. For example: (a) a disease may be present, but the causative toxin is unknown, (b) known toxic agents might be present in the environment, but their relationship (e.g., pathway and health effects) to the specific disease is unknown, or (c) a metabolite may be detected in humans indicating exposure to a toxic agent and the agent may have been identified in the environment, but the pathway by which exposure occurs is unknown. Understanding the sources, pathways, and fate of toxic agents in an endemic area both in terms of natural processes and human activities is essential for addressing these knowledge gaps and requires a sound application of principles of environmental sciences. In this paper, we consider environmental hypotheses offered to explain the etiology of EN, using an environmental science approach to exposure, which involves mechanisms and dynamics of environmental processes and agent behavior, together with biogeochemical signatures and indicators. Results show clear differences between the biogeochemistry (food, water, soil) of EN and non EN villages. Water quality has been clearly influenced by human activities and may have increased exposure to environmental toxins both by direct additions and indirectly through changes in the natural environment. These findings can be used to inform future investigations of exposure and approaches to the etiology of EN.

P-2.4

Endemic nephropathy remains a major renal disease in the Croatian endemic area

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Endemic nephropathy (EN) is a chronic renal disease of unknown etiology with a slow onset, progressive clinical course and frequent occurrence in farming households. In Croatia, EN occurs in the rural population in 14 villages located in the western part of Brodsko-posavska county near the city of Slavonski Brod. It has recently been suggested that EN is less common in this area than it was in earlier years. The aim of this study was to determine whether this hypothesis is correct. This is a retrospective study of patients beginning hemodialysis in the Center for Dialysis in the General Hospital in Slavonski Brod from 1992–2005. We then compared those results with the percentage of patients on hemodialysis in 1980. In last 14 years, 141 new patients began a program of chronic hemodialysis, 53 of whom had a diagnosis EN (37%). The highest number of new patients with EN occurred in the years 2002 (11 of a total of 21 patients starting dialysis) and 2005 (7 of a total of 20). The lowest number of new patients starting hemodialysis with EN occurred in the years 1992 (0 patients), 1994 (1 of a total of 4), and 2000 (1 with EN of a total of 4). In 1980, 39% of patients on dialysis had end-stage renal disease due to EN. EN still exists in the Croatian region with a prevalence similar to that in earlier years. This retrospective study provides strong proof that many new patients start hemodialysis because of end-stage renal disease caused by EN. We have also shown that the number of patients with end-stage renal diseases due to EN and the percentage of patients beginning hemodialysis because of EN remains above 30% of the total number of patients beginning dialysis for all reasons. Importantly, in the last 3 years, there has been an increase in the number of new patients beginning dialysis because of EN.

P-2.5

Erythropoietin requirement in endemic nephropathy

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Endemic nephropathy(EN) is an inflammatory, slowly progressive chronic tubulointerstitial bilateral desease with an endemic occurrence in some rural environments of Croatia, Bosnia and Hercegovina, Serbia, Bulgaria and Romania. Its etiology remains unknown despite many investigations into a possible relationship with environment, heritage, and immune mechanisms. The pathohistological findings in Chinese herbal nephropathy, now known as aristolochic acid nephropathy, are the same as those observed in EN. There are many similarities in clinical findings as well: damage occurs in the proximal tubule and patients exhibit very severe anemia in contrast to patients with other kidney diseases in the same stage of kidney damage. As of December 31, 2004, the Croatian Registry for Renal Replacement Therapy (CRRRT) had 2641 patients on hemodialysis, 199 of whom had EN as the primary cause of their end-stage renal disease. Among the 199 EN patients, 86 died (between 1999 and 2004), 14 went to Bosnia and Hercegovina; 99 are alive, of whom 83 are on hemodialysis, 11 were transplanted, and five are on CAPD. Dialysis centers caring for EN patients are located in: Slavonski Brod (EN patients constitute 31% of all patients), Vinkovci (26%), Sisak (6%), and Osijek(5%). Patients with EN have more severe anemia than do the other patients. In Slavonski Brod, EN patients on hemodialysis had an average hemoglobin level of 80.84 g/L while other patients on dialysis had an average hemoglobin of 91.88g/L. Approximately 96% of EN patients required rHuEPO, but only 64% of other dialysis patients needed rHuEPO. In April 2004, at the Center for Dialisis in Slavonski Brod, the average weekly dose of rHuEPO/erythropoietin was 4965 IU for EN patients and 3916 IU for other patients. Thus, the need for erythropoietin among EN patients is 21% higher than among others. In fact, EN patients need 62 IU of rHuEPO for g/Hb/L while other patients need 43 IU of rHuEPO/g/Hb/L, pointing to a 44% greater need per g/Hb/L than is observed for other patients

P-2.6

Characteristics of the population from a Croatian focus of endemic nephropathy

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The aim of this investigation was to establish clinical and laboratory characteristics of a population living in a Croatian region of endemic nephropathy (EN). This study included 738 individuals from an EN village (307 male, 431 female), and 120 subjects from a control village (K) (50 male, 70 female) from a total of 1081 subjects included in the perlustracija in 2005. No differences in the distribution of gender and age were observed between EN and control villages ($p<0.01$). Following administration of a detailed questionnaire and clinical examination, investigators obtained blood and urine samples from the subjects. The population was classified according to WHO criteria as diseased (D), suspect (S), at risk (R) and others (O). The following definitions were used: albuminuria, > 30 g/l; alpha-1-microalbuminuria (alpha-1) >20 g/l, hypertension, BP > 140/90 mmHg and/or antihypertensive drug therapy; and anemia (AN) as Hb < 120 g/l in M and <113 g/l in F. Renal disease (RD) was classified according to NKF classification.

The frequency of the WHO categories in this group was: D = 1.4% (M, 1.73% vs. F, 1.34%, respectively; $p=0.929$), S = 5.6% (M, 7.79% vs. F, 4.83%; $p=0.137$). If we analyze only the portion of population who resided > 20 years in an EN village, then the incidence of D is 1.8% ($p> 0.05$) and of S is 6.8%. In the subgroup of persons living in EN villages < 10 years, there were none in the D group and only 2% were in the S category. In the EN villages, we found an increased MA frequency compared with the control village (20.0% vs. 9.8% M, respectively; $p=0.09$; 16.6% vs. 9.37% F, respectively; $p=0.0001$), as well as an increased frequency of alpha-1 (21.73% vs. 13.72% M, respectively; $p = 0.205$; F 9.48% vs. 4.54%, respectively; $p=0.172$). No similar differences were observed between subgroup O and the control village ($p> 0.05$). The prevalence of hypertension was slightly lower in EN villages than in the control village (51.75% vs. 58.9%; M 54.07% vs. 57.1%; F 51.97% vs. 58.46%, respectively; $p>0.05$). There were no differences in the frequency of RD stages >2 (by NKF classifications) or in anemia between EN and control villages ($p>0.05$ for both categories). The duration of residence in EN villages was significantly correlated to MA ($r=0.264$; $p<0.00001$), alpha-1 ($r=0.368$; $p<0.00001$) and serum creatinine ($r=0.284$; $p<0.00001$), but was not significant for those living < 20 years in EN area and in control village residents ($p>0.05$). A significant correlation was observed between years living in an EN area and the presence of MA, alpha-1 and serum creatinine in persons in WHO categories R and O ($p<0.001$). The incidence of D and S in this group has remained the same as before. The duration of residence in the endemic area affects EN prevalence. The two village populations differed in MA, alpha-1, and hypertension frequency but not in anemia and renal disease stage >2. The known significant association between early renal impairment in persons classified as R and O living in EN villages, indicates that new diagnoses of S and D will be registered in the next several years regardless of whether the causative agent is still present or active.

P-2.7

Endemic (Balkan) nephropathy remains a significant medical problem in the Bosnian endemic region

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Balkan Endemic Nephropathy (BEN) is a chronic renal disease of unknown etiology. It is a slow, progressive bilateral tubulointerstitial disease with an endemic occurrence in rural regions of Bosnia and Hercegovina, Croatia, Serbia, Bulgaria and Romania. In Bosnia and Hercegovina, BEN occurs in a rural area near the river Sava in Posavska County near the cities of Odžak and Orašje. We initiated a study to determine the role of BEN in public health and the severity of the medical problem caused by BEN. This was a retrospective study of the history of illness of patients starting hemodialysis in the Center for Dialysis in Odžak from year 2000 to 2005. In those 6 years from 2000–2005, 116 new patients started hemodialysis in the Center for Dialysis in Odžak. Of these patients, 67 had a diagnosis of BEN (57.8%) and 49 had other diagnoses. The number of patients with BEN starting dialysis was similar during the study years, with the lowest number in the year 2000 (6 BEN of 14 total) and 2003 (6 BEN of 16 total) and the highest numbers in year 2001 (15 BEN of 25 total) and in 2002 (10 BEN of 19 total). In 2004, 22 new patients entered the chronic dialysis program, 10 of whom had BEN; in 2005, there were 20 new patients, 9 of whom had BEN. On January 1, 2006, the Center for Dialysis in Odžak had 89 patients on chronic hemodialysis program, 56 of whom had a diagnosis of BEN (62.9%). Among these patients, seven had urothelial cancer (12.5%) and underwent surgery and all had BEN. BEN is still a major cause of end-stage renal disease in the Bosnian endemic region and in Posavska county. It remains the most frequent diagnosis among patients in the Center for Dialysis in Odžak. The consistent number of new patients starting dialysis with a diagnosis of BEN suggests that BEN shows no sign of disappearing as a disease. BEN is also associated with a high prevalence of urothelial cancer. Therefore BEN is still a great medical problem in the Bosnian endemic region.

P-2.8

Endemic nephropathy as a cause of end-stage renal disease in Croatian and Bosnian endemic areas: Do the focuses differ?

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Our aim is to compare two focuses of endemic nephorpathy (EN) – in Croatia and in Bosnia and Hercegovina – with respect to clinical parameters at the at the start of chronic hemodialysis. The rationale for this study was to compare the length of time from disease diagnosis to dialysis, the laboratory findings at the beginning of dialysis, and the age of patients starting dialysis because of EN. This was a retrospective study of patients starting dialysis because of EN in the Center for Dialysis in Slavonski Brod (SB), Croatia and the Center for Dialysis in Odžak, Bosnia and Hercegovina during the period 2000–2005. We evaluated the following clinical data: erythrocytes, hemoglobin, creatinine, age of patient, predialysis period from the first diagnosis of illness, and number of urothelial tumors. In the 6-year period, there were 36 new EN patients on dialysis in SB and 67 new EN patients on dialysis in Odžak. The table below shows the different clinical parameters evaluated:

	Slavonski Brod	**Odžak**
Mean age at dialysis	67.5 7.8	63.28 12.6
Predialysis period (yrs)	9 1.5	5.9 2.2
Erythrocytes (x 10^{12}/L)	2.7 0.5	2.5 0.3
Hemoglobin (g/L)	82.6 22.1	81.7 24.2
Creatinine (μmol/L)	870 325	855 544
Urothelial cancer	3 patients (8.3%)	7 patients (10.4%)

We compared EN focuses in two different countries: Croatia and Bosnia and Hercegovina according to clinical parameters at the beginning of end-stage renal disease and at the start of dialysis. The data show that the focuses are statistically different in clinical factors. In Croatia, the predialysis period is longer and the mean age at the start of dialysis is older than in Bosnia and Hercegovina, while in Bosnia, urothelial cancer is more frequent than in Croatia (10.4% vs 8.3%, respectively). The mean levels of creatinine, hemoglobin and erythrocytes at the beginning of dialysis do not differ statistically, suggesting that the criteria for starting dialysis are the same. The greater incidence of urothelial cancers and the shorter predialysis period for endemic nephropathy patients in Bosnia may be due to some variabilities of EN in this focus as compared with Croatia and indicates a need for further study.

P-2.9

Microalbuminuria as a potential marker of susceptibility to Balkan endemic nephropathy

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The aim of this study is to evaluate the urinary albumin excretion rate (UAER) in inhabitants from a Balkan endemic nephropathy (BEN) region and to evaluate whether microalbuminuria could be a marker of early tubular damage in individuals at risk of developing endemic nephropathy. This cross-sectional study was conducted with 34 healthy non-proteinuric subjects from a known BEN region (test group) and the obtained data were compared with the known prevalence of microalbuminuria outside the endemic region (control group of 40,548 sex- and age-matched individuals from the general population of the city of Groningen, The Netherlands). In the control group, UAER was determined in the morning, whereas in the test group, a random urine sample was obtained and analyzed by a commercial immunoturbidimetry assay (Dade Behring). Microalbuminuria was defined as UAER between 20–200 mg/L. Statistical analyses were performed using MedCalc for Windows, version 8.1.0.0 (MedCalc Software, Mariakerke, Belgium). To eliminate the confounding influence of age-dependent microalbuminuria and hypertension, one sample T test and Kruskal-Wallis test were used, respectively. Blood pressure was categorized as: normal, mild (diastolic >90 and <110) and severe (diastolic >110). Hypertensive persons were further categorized as treated or untreated and the patients were assigned to categories 1, 2 and 3. Signed rank sum test and Fisher's exact test were used for the comparison of UAER between the groups. Microalbuminuria was found in 26.5% of the subjects in the test group, in contrast to 7.2% with microalbuminuria in the controls ($p=0.0001$). The median level was 11.6 in the test group, with P5–P95 between 2.62–73.6, and 6.1 in the control, with P5–P95 between 2.3–28.7 ($p<0.0001$). Among the experimental group, 35.3% had hypertension compared to 11.2% in the control ($p<0.0001$). Both treated and untreated hypertensives were present in the test group, while all the hypertensives in the control group were on treatment. There was no significant relationship between microalbuminuria and the degree of hypertension ($p=0.49$) nor between microalbuminuria and any of the hypertension categories (normal, treated or untreated) ($p=0.50$). These results persisted after excluding five individuals with untreated hypertension. Microalbuminuria may be a useful marker of early tubular injury in individuals who live in endemic regions and who are at risk of developing BEN.

P-2.10

Prevalence, treatment and control of hypertension in a Croatian focus of EN – comparison with Epidemiology of hypertension in Croatia (EHUH) study results

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Hypertension is not a characteristic of endemic nephropathy (EN) and occurs only at the advanced stage of renal disease (RD). This study compares the prevalence, treatment and control of hypertension in a current EN focus with the general EHUH study results. Of the 1081 inhabitants included in the perlustracija (approximately 10% of population), we enrolled in our study 738 subjects from EN villages (307 men, 431 women) and 120 subjects from a control village (Klakar; 50 men; 70 women) who underwent complete clinical and laboratory examinations. There were no gender and age differences between the two populations ($p<0.01$). The EN population was classified according to WHO criteria as diseased (D), suspected of having EN (S), at risk (R), and others (O). Blood pressure (BP) was measured by mercury sphygmomanometer with the subject in the seated position, following a 10-minute rest, on two occasions, and the mean value was calculated. Hypertension was defined as BP >140/90 mmHg and/or administration of antihypertensives. The two studies showed no difference in hypertension prevalence between EN villages (our study) and other rural parts of Croatia (EHUH results), with the prevalence being 51.7% vs 49.17%, respectively; $p>0.05$, both in males (M) (53.0% vs. 47.12%) and in females (F) (50.5% vs. 50.6%). Hypertension prevalence was the lowest in the O category (M: 48.4%, F: 46.3%) and the highest in the suspected EN category (M: 83.3%, F: 94.1%). However, people in the S groupwere also the oldest ($p= 0.0004$) and contained the highest percentage of obese subjects (35.2% in the S group vs. 16.6% in the O group; $p=0.046$). When we analyzed only subjects with GFR > 60 ml/min, we found hypertension prevalence to be lower in both genders (M: 47.7%, F: 46.6%), and, again, there were no differences when compared to the control village, Klakar (M: 55.3%, F: 56.3%; $p>0.05$). There were no differences between the endemic area and the general population in the EHUH study (53.8% vs. 59.6%, respectively). More F than M were treated (63.9% vs 40%; $p=0.001$) in the endemic region, but the percentage of women treated in the endemic area was lower than treated women in the EHUH (63.9% vs 81.6%, respectively; $p=0.0001$). Compared to the EHUH study, we found no differences in the control of all hypertensive patients (15.7% vs. 15.5 %; $p>0.05$) or treated hypertensive patients (24.4% vs 20.8%; $p>0.05$). Unlike the EHUH study, control of hypertension among the EN population was worse in F than in M (14.5% vs.17.4%; $p>0.05$) and in treated HT (23.0% vs. 32.7%; $p>0.05$). The prevalence of hypertension in EN villages did not significantly differ from that found in the EHUH study results. The higher-than-expected prevalence of hypertension among persons in the WHO »S« category is likely due, in addition to their being of older age, to the fact that that group contains the greatest number of obese individuals. The increased incidence of obesity leads to an earlier onset of hypertension in EN and also to poorer BP control. The observed poorer control among women in the EN focus as compared to those participating in EHUH was likely due to the lower number of treated hypertensive females and the higher number of obese females in the EN endemic region as compared with the general population.

P-2.11

The role of cytology in the diagnosis of endemic nephropathy

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Endemic nephropathy (EN) was first described in 1957. It is a chronic tubulointerstitial kidney disease in persons living in specific areas of southeast Europe. The etiology is the most important unanswered problem in EN. A high incidence of upper urothelial tumors has been described in endemic nephropathy regions and the etiology of these tumors is still unknown. The geographic correlation between EN and upper urothelial tumors suggests the possibility that the same etiologic factor is operative in both diseases. Several factors have been investigated. Studies have demonstrated a genetic predisposition to EN as well as some related environmental factors. The similarity of renal morphological changes and clinical features of Chinese herb nephropathy and of EN suggest the possibility of a common etiologic agent, namely, aristolochic acid. The upper urothelial tumors are a common feature of both nephropathies. The aim of this study is to determine the correlation between number of cells, detection of different cellular elements and quantity of DNA in urine sediment, in conjunction with an effort to detect aristolochic acid-DNA adducts in exfoliated urothelial cells. The second morning urine was collected from EN patients treated at our hospital (selective samples) and from healthy subjects older than 25 years (non-selective samples). Samples were limited to patients with 10 μg DNA or more in their urine sediment. Two samples from each patient were prepared in a cytocentrifuge at 2000 rpm for 5 min. The samples were air-dried and stained according to Papenheim (May-Grunwald-Giemsa). We conclude that the quantity of DNA in urine sediment depends upon the number of cells but the quality depends on the type of cells.

P-2.12.

Serum paraoxonase activities in endemic nephropathy patients on long-term hemodialysis

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The aim of this study is to determine whether paraoxonase activity, paraoxonase phenotype and lipid status are altered in endemic nephropathy (EN) patients on long-term hemodialysis treatment as compared to healthy population. EN patients (n=31) and control subjects (n=145) were residents of the area of Slavonski Brod, Croatia. Paraoxon was used as a substrate for measuring basal and sodium chloride-stimulated (NaCl-stimulated) paraoxonase activity, and phenylacetate for measuring arylesterase activity. The double substrate method was used to assign phenotypes. Cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-cholesterol) were determined by methods routinely used in medical-biochemical laboratories. Results are expressed as median values and tested by Mann-Whitney rank sum test. The values $p<0.05$ were considered significant. Basal and NaCl-stimulated paraoxonase activity as well as arylesterase activity were significantly lower in the patient group compared to controls; 68% ($p<0.001$), 71% ($p<0.001$), and 58% ($p<0.001$), respectively. The distribution of paraoxonase phenotypes in the patient group and controls was as follows: AA, 45% and 39%; AB, 37% and 48%; BB 185 and 13%, respectively. Cholesterol and HDL-cholesterol were significantly decreased (87%, $p<0.001$ and 71% $p<0.001$, respectively), whereas triglycerides were significantly increased (195 %, $p<0.001$) in the patient group compared to control median value. We conclude that endemic nephropathy patients on long-term hemodialysis have decreased paraoxonase /arylesterase activity, which may indicate a greater risk of premature atherogenesis.

P-2.13

Endemic nephropathy: should the diagnostic criteria be revised?

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A basic problem in establishing the diagnosis of endemic nephropathy (EN) concerns the lack of specific symptoms and laboratory tests. WHO diagnostic criteria, established in 1965, rely on proteinuria, azotemia and anemia in addition to dwelling in endemic areas >10 years and absence of renal disease of known etiology. There is considerable controversy regarding the meaning of individual diagnostic components, resulting in inconsistency in the interpretation of results from different studies. In those studies, the diagnosis of EN often relied on the analysis of a single specimen of blood and/or urine. The situation is further complicated by inadequately defined control groups. **Aim:** To explore the significance of individual diagnostic components of EN, we compared the prevalence of anemia, proteinuria and azotemia in the endemic area and in an age-matched control group. We also analyzed the impact of a single measurement vs. more than 25% positive findings in groups from an endemic area. **Methods:** The unselected population included 355 inhabitants from an endemic area and 109 from a nonendemic village, aged 16–84 years. Proteinuria, azotemia and anemia were determined using standard laboratory tests. Using the WHO criteria, we recognized the following groups: »diseased,« »suspect,« »at risk« and »others« (in endemic village), and the »control« group – a population from a nonendemic village. **Results:** Proteinuria was observed in all study groups ranging from 28% to 100% in the control and the affected group, respectively. Anemia ranged from 41% to 100% and azotemia from 11% to 100 %. Following the criterion of >25%, we found proteinuria in 100% of the »diseased,« 55% of »suspect«, 3% of subjects »at risk,« 10% of »others,« and 6% of control subjects. Azotemia ranged from 100% to 0%, and anemia up to 15% (in »others«). Using age matching criteria in unaffected subjects aged >45 years, azotemia and anemia were found in 9% to 37%, and 40% to 51%, respectively.**Conclusion:** Proteinuria, azotemia and anemia are frequent in both populations examined. Therefore we suggest that the criterion of >25% of positive findings for the diagnosis of EN be used. Our results showed that there is a need to establish »persistent» proteinuria, anemia, or azotemia by multiple analyses using the above criterion as a minimal percentage of pathological laboratory tests. The frequent finding of anemia raises doubt about the reliability of this laboratory parameter as a criterion for the diagnosis of EN, or a suspicion of its presence.

5. UROTHELIAL CANCER IN ENDEMIC (BALKAN) NEPHROPATHY

INVITED LECTURES

Nikolić J (Serbia)

Epidemiology of upper urothelial cancer worldwide and in endemic regions

Krušlin B (Croatia)

Pathomorphological characteristics and classification of upper urothelial cancers

Slade N (Croatia)

Defining molecular pathways in endemic nephropathy –associated upper urothelial carcinoma

Pleština S (Croatia)

Diagnosis and treatment of patients with upper urothelial cancers

Bukvić D (Serbia), Janković S, Arsenović A, Marić I, Djukanović Lj

Urinary tract tumors in endemic region

Bašić-Jukić N (Croatia), Kes P, Bubić-Filipi L, Reiner Ž, Pasini J, Hudolin T

Renal transplantation in patients with endemic (Balkan) nephropathy

POSTERS

P-3.1

Mišić M, Vukelić M, Jakovina K, Medverec Z, Jakovina T, Stanić G, Jelaković B

Characteristics of urinary tract carcinomas in patients from endemic neprhopathy focus and non-endemic nephropathy region in Slavonski Brod county in 6-year period

P-3.2

Belicza M, Dubravić A, Leničeк T, Pavić I, Tomić K, Jakovina K, Vukelić M, Jakovina T, Mišić M, Krušlin B

Comparison of occurence of upper urinary tract carcinomas in Brodsko-posavska endemic nephropathy region and in pathoanatomical registry of Sestre milosrdnice University hospital in Zagreb

P-3.3

Kovač-Peić A-M, Miletić-Medved M, Kos J, Pećin I, Barešić M, Leko N, Bistrović D, Fuštar-Preradović Lj, Dika Ž, Brdar B, Jelaković B

Carcinoma frequency in a Croatian endemic focus

P-3.4

Medverec Z, Lučić Đ, Martinović M, Dittrich D, Perković A

Endemic nephropathy – the urologists' point of view

P-3.5

Mokos I, Pasini J, Štern-Padovan R, Mrsić S, and Ries S

Renal allograft preserving surgery in the patient with endemic nephropathy and urothelial carcinoma in transplanted kidney

P-3.6

Garneata L, Mircescu G, Capusa C

Endemic (Balkan) nephropathy – urinary tract tumors association: a case presentation

P-3.7.

Škegro D, Sabljar-Matovinović M, Miletić-Medved M, Prkačin I, Knotek M, Škegro I, Čeović S*

Renal tumors in population exposed to endemic nephropathy: an ultrasound follow-up study

Epidemiology of upper urothelial cancer worldwide and in endemic regions

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Upper urothelial tumors (UUT) develop in patients at all stages of EN, from preclinical to terminal stage of the disease. Data were obtained from 2110 patients (995 males, X= 58,78 years, 1115 females, X = 62,94 years) who underwent surgery because of histologically proven UUT in Serbia from 1955–1998 (94.4% TCC, 4.25% squamous cell carcinomas and 0.81 adenocarcinomas).

We concentrated on one subregion approximately 100 km wide, which contained 42/91 (46%) of proven and 128/285 (44.9%) potential EN focuses in Serbia. Village settlements (851 patients) were classified into ten groups according to UUT incidence. The average age of patients was found to be inversely proportional to the incidence in a settlement. The increase in the average age is constant in the first 6 groups of villages, suggesting that the disease is dose dependent and implying a corresponding concentration of etiologically acting agents in the area.

A lower concentration of etiologic agent in the area could result in a lower incidence in a village and higher age of patients due to the longer latent period and slower development of nephropathy. A new term »sporadic EN« is introduced and is characterized by a low incidence of disease in a settlement, accompanying UUT and equal incidence in both sexes. This term would be used to describe the older age groups (6th, 7th and 8th decade) with slowly progressive renal insufficiency, which are not family related. Characteristics of 145 patients suffering from sporadic EN from 33 settlements (with three or more affected persons) not included in the list of confirmed or potential endemic places will be presented. Of these, 28/33 are border, and 5/33 are located in the immediate vicinity of the known endemic places. Our UUT patients do not fulfill the criteria for the »classic« EN diagnosis.

We will present the different incidences of UUT in the hyperendemic, endemic, and hypoendemic areas and their trends over the period from 1955–1998. The data led us conclude that sporadic EN cases are the key to understanding the borders of EN regions, revealing at the same time the epidemic nature of the disease. The older age of affected patients in the specific areas are probably the result of a longer latent period due to a smaller ingested dose of the etiologic agent in the past.

Pathomorphological characteristics and classification of UTC

Krušlin B

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A new WHO classification was published in 2004 to standardize nomenclature and criteria for grading and invasion of transitional/urothelial cell tumors. The changes included a recommendation to use the term urothelial neoplasms instead of the designation »transitional.« The aim of this reclassification was to avoid the use of »cancer« for neoplasms that very rarely invade, recur, and/or cause death of the patient. Therefore, a new category of »tumor of low malignant potential« (LMP) was introduced into the group of noninvasive urothelial neoplasms, In addition, the three-tier grading of papillary noninvasive tumors was replaced by a low- and high-grade category. Criteria for the grades are cited in the classification but are somewhat indeterminate and difficult to apply. Here, the papillary urothelial neoplasm of LMP is a papillary urothelial tumor resembling exophytic urothelial papilloma, but with increased cellular proliferation exceeding the thickness of normal urothelium. Noninvasive papillary neoplasm, low grade, is defined as a neoplasm of urothelium lining papillary fronds, which shows an orderly appearance but has easily recognizable variations in architecture and cytological features. Noninvasive papillary urothelial carcinoma, high grade, is a neoplasm of the urothelium lining papillary fronds, which shows a predominant pattern of disorder with moderate to marked architectural and cytological atypia. Regarding invasive tumors, it is well known that identification of foci of lamina propria invasion is occasionally quite difficult. The criteria for invasion are: isolated cells or small nests, larger cells and cell nuclei, and marked cytoplasmic eosinophilia relative to the surface urothelium. The problems of the identification of lamina propria invasion are still not solved and therefore the use of additional histochemical and immunohistochemical methods is recommended in difficult cases. On the basis of data from the literature and our own experience, we recommend the use of WHO 1973 simultaneously with the new one during the transitional period.

Defining molecular pathways in endemic nephropathy-associated upper tract urothelial carcinoma

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Mutations of p53 have been found in nearly all tumor types and are estimated to contribute to more than 50% of all cancers. Inactivation of the p53 gene is predominantly due to missense mutations (90% of cases) in the central effector domain of the protein. Mutations lead to the synthesis of highly stable, inactive proteins that accumulate in the nucleus of cancer cells. Among 393 codons of the human p53 gene, 222 are targets of 698 different types of mutations. Alterations of codons 175, 248, 273 and 282 constitute 19% of all mutations and are considered general hot-spot mutations. The vast majority of urothelial cancers are transitional cell carcinomas of the bladder, which arise from urothelium, the epithelial lining of the urinary tract. Currently, 93 mutations of the p53 gene are recorded for urothelial carcinomas (IARC p53 database). The mutational spectrum shows hotspots in codons 175, 220, 248, 280 and 282. Codon 220 is relatively specific for urothelial carcinomas and is the only mutational hotspot in urothelial tumors associated with an adenine change. Of note, A:T T:A mutations are very rare in sporadic bladder cancers. The major causative agent in the development of urothelial carcinoma in patients with Chinese herb nephropathy is thought to be aristolochic acid (AA). Aristolochic acid preferentially binds to purines in the p53 gene and is associated with the predicted A T mutations, generating a carcinogen fingerprint. Aristolochic acid also causes A:T T:A mutations in codon 61 of the c-H-ras gene in rats.

Importantly, Endemic (Balkan) Nephropathy (EN) is associated with a 100- to 200-fold greater risk (compared to persons in nonendemic near-by towns) of urothelial carcinoma, albeit of an unusual high location (proximal ureter or renal pelvis), supporting a role for exogenous carcinogen(s). These carcinomas overexpress mutant p53. To gain insight into the causative agent of EN-associated carcinoma, it is important to determine the mutational spectrum of urothelial tumors of EN patients and compare it with that from sporadic urothelial tumors of the general population. We have initiated mutational analysis of the p53 gene in DNA obtained from confirmed upper urothelial cancers removed from patients residing in endemic villages in Croatia. Mutational analysis was performed on tumor samples that stain strongly with antibody directed against p53. The new Roche p53 sequencing AmpliChip powered by Affymetrix was used to sequence exons 2–11. Mutations at A:T pairs was found to account for >50% of mutations sequenced to date, supporting the hypothesis that exposure to aristolochic acid is responsible for the urothelial cancer associated with EN.

Diagnosis and treatment of patients with upper urothelial cancers

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Upper urinary tract tumors are uncommon, a situation that makes the diagnostic and staging procedures problematic and less accurate than for the transitional cell carcinomas of the bladder. Painless gross hematuria is the presenting symptom in 75% to 95% of all patients with renal pelvis and ureter tumors. Urinary cytology is a very important part of the workup for an upper tract tumor, though it is less useful than in bladder cancer because the sensitivity is only 10%–40% in detecting low-grade lesions. However, the sensitivity may be as high as 80% with high-grade tumors. Intravenous urography has been the mainstay of radiographic evaluation of upper urinary tract tumors, but helical CT scan has recently become a more preferred imaging method in most centers; MRI urography may also be useful. Retrograde pyelography is an option in patients with poor renal function or in those with hypersensitivity to intravenous contrast agents. In some patients, flexible fiber endoscopes allow direct viewing as well as the possibility of obtaining tissue for pathologic confirmation. At the moment, the most appropriate treatment and standard therapy is radical excision of the kidney and ipsilateral ureter, whenever possible. The success rate of surgical procedures and specific and overall survival is strongly influenced by the pathologic stage of the disease at the time of resection. Most patients have superficial disease, with a generally favorable prognosis. However, patients with tumors that invade beyond the muscularis propria have a significantly worse prognosis. When the disease has spread beyond the muscularis, cure rates are low despite aggressive surgery, with 5-year survival rates varying between 0 and 34%. Poor outcome may be additionally predicted by the overexpression of p53 and a higher Ki-67 labeling index, although this finding has not been supported by all studies. Radiation and chemotherapy, often used in combination, may prove to be important in survival prolongation, but their place has yet to be established in carefully planned studies.

Urinary tract tumors in an endemic region

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A high incidence of upper urothelial tumors was described in endemic nephropathy regions since the earliest investigations into the disease. A field investigation conducted in 1982 in the Kolubara region discovered upper urothelial tumors only in the Lazarevac and Lajkovac municipalities, most frequently in the three villages most affected by endemic nephropathy: Petka, Šopić and Cvetovac. Our recent retrospective study involved all patients with urinary tract tumors treated at the Institute of Endemic Nephropathy from 1974 – 2005. These data confirmed that the incidence of the upper urothelial tumors and the incidence of endemic nephropathy in Kolubara region are not decreasing. Urothelial tumors affected females more frequently than males (1.4:1) aged 50–80 years, most frequently in persons in the seventh decade of life. Agriculture was the main or additional occupation of patients with tumors.

The etiology of the upper urothelial tumors is still unknown. Our study, conducted from 1992–1994 among 73 patients, found that factors influencing tumor development included: smoking, history of endemic nephropathy in second- and third-degree relatives, presence of other malignant tumors in first degree relatives; agriculture as occupation, urinary tract infection and some types of food. Our studies found that about 40% of the tumors were localized in the renal pelvis and ureters; simultaneous appearance of tumors in the pelvis and ureter (18%) was less frequent, while bladder tumors were documented in 18% of patients with the upper urothelial tumors. The tumors appeared to be unilateral more frequently than bilateral (82% vs. 18%). Although endemic nephropathy often precedes the appearance of the upper urothelial tumors, our investigations in the Lazarevac endemic area also found patients whose upper urothelial tumors appeared before clinical manifestation of endemic nephropathy. In addition, upper urothelial tumors were seen in 29.8% of patients with endemic nephropathy maintained by hemodialysis in Lazarevac.

Renal transplantation in endemic (Balkan) nephropathy

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Balkan endemic nephropathy (BEN) is a chronic tubulointerstitial disease prevalent in Croatia, Romania, Bulgaria, Bosnia and Herzegovina, and Serbia. In addition to the renal disease, an increased incidence of upper urothelial carcinomas (UUC) has been observed in the foci of BEN. Carcinoma may occur alone or in combination with BEN. Immunosuppression is associated with an increased risk for development of several malignancies and there are no data in the literature about the outcome of patients with BEN after transplantation. This study consisted of a retrospective evaluation of the database and a review of the charts and pathology reports of 601 renal transplant recipients treated at our institution. From January 1995 to December 2004, nine patients with BEN underwent kidney transplantation. One-year graft survival was 100%. One man who was transplanted in 1997 died 2 years after transplantation with a functioning graft; cause of death was disseminated cancer of the pelvis of his own kidney. A female patient developed UCC two years after transplantation. They were both treated with a bolus of methylprednisolone before transplantation because of 4 HLA mismatches. Other patients have had an uneventful post-transplantation course with excellent graft function. Thus, 22.2% of patients with BEN developed UUC, compared with a prevalence of 0.67 % urinary tract tumors in transplanted patients with other causes of ESRD. Patients with BEN are at increased risk for development of UCC after transplantation and therefore regular screening for early detection of malignancy is mandatory. Longer follow-up and results from other transplant centers are needed to further investigate the relationship between BEN and UCC after renal transplantation. Bilateral nephroureterectomy might be necessary to achieve long-term benefits after renal transplantation.

P-3.1

Characteristics of urinary tract carcinomas in patients from endemic nephropathy and non-endemic areas in Slavonski Brod county in a 6-year period

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The literature documents that endemic nephropathy is a disease associated with an increased frequency of urothelial carcinomas. The aim of our study was to compare the pathomorphological characteristics of urinary tract tumors in patients from areas of endemic nephropathy with those of patients living in nonendemic areas of Slavonski Brod county. We therefore conducted a retrospective study to investigate: differences in age and sex of the patients, anatomical locations of tumor, pathohistological types, nuclear grading, staging, capability of recurrence, and multicentricity of the urinary tract tumors. The medical data bases from the Departments of Pathology, Forensic Medicine and Clinical Cytology, General Hospital Dr.J.Bencevic, Slavonski Brod were accessed and we evaluated all available tissue samples from surgical and small-biopsy specimens of patients undergoing surgery in the Department of Urology during 2000–2005. Medical data were divided in two groups depending on the place of birth and current residence of the surgical patients. During the period from 2000–2005, 33,341 biopsies were analyzed by the Department of Pathology in Slavonski Brod, of which 1502 were urinary tract biopsies. Further analyses of the medical data will be done.

P-3.2

Comparison of upper urinary tract carcinomas in Brodsko-posavska endemic nephropathy region with those in the pathoanatomical registry of Sestre milosrdnice University hospital in Zagreb

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Balkan endemic nephropathy (BEN) is a chronic tubulointerstitial nephropathy with an incompletely known etiology. It is associated with an increased frequency of urothelial carcinomas, especially those of the upper urinary tract. The aim of this study was to compare the occurrence of upper urinary tract carcinomas between Brodsko-Posavska Region (BPR) and Zagreb (ZG) in two six-year periods, 20 years apart. We used histopathological databases from two Pathology Departments (Slavonski Brod and Zagreb) for two observed periods. In the first period (1980–85), 122 urologic cancers were reported in Department of Pathology in Slavonski Brod, and 1095 in Department of Pathology in Zagreb, while in the second period (2000–2005), there were 588 cancers in Slavonski Brod, and 3017 in Zagreb. Primary tumors of the kidney represented 27.9% (34 cases; M:F =17:17) of all urologic tumors in the BPR, and 9.9% (108 cases; M:F =73:35) in ZG in the first period; in the second period these tumors represented 26.4%(155 cases; M:F=64:91) in BPR, and 17.4% (526 cases; M:F=332:194) in ZG. Among all kidney tumors in BPR, urothelial carcinomas of pyelon and ureter represented 79.4% (27 cases; M:F= 13:14), and 14.8% (16 cases; M:F= 12:4) in ZG in the first, and 65.8% (102 cases; M:F=64:91) in BPR, and 14.3% (75 cases; M:F=41:34) in ZG in second period. There were 6 cases (17.6%; M:F =3:3) of renal cell carcinoma (RCC) in BPR and 85 cases (78.7%; M:F= 59:26) in ZG in the first period and 48 cases (31%; M:F=27:21) in BPR and 447 cases (85%) M:F=291:156) in ZG in second period. In the second period, carcinoma of the pyelon and ureter was diagnosed at the average age of 67.2 years in males and 75.3 years in females in SB, and 64.3 years in males and 72.6 years in females in ZG. Conclusion: The occurrence of urothelial carcinomas of the pyelon and ureter is significantly higher in the Brodsko-Posavska Region, likely as a consequence of BEN. Women are affected more frequently and at an older average age than men. The association of BEN and urothelial cancer should be further analyzed.

P-3.3

Carcinoma frequency in a Croatian endemic focus

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The aim of this study was to establish the frequency of carcinoma (Ca) in the personal and family history of inhabitants of endemic (EN) villages and in the control village of Klakar. The analysis included data on 780 subjects (333 men, 447 women): 9 had disease, 55 had suspected disease, 165 were at risk, 408 others; 121 were residents of Klakar. The data were collected by completion of a detailed questionnaire during the 2005 perlustracija. There were no differences between subjects with various types of carcinoma with regard to age and smoking habit. A diagnosis of carcinoma was made in 30 subjects (3.8%) and those subjects were older than the rest of the population ($p=0.0007$). Personal medical history revealed no differences in the frequency of all types of Ca between EN villages and Klakar ($p=0.10$). However, no subject from Klakar had urinary tract Ca (UTC), whereas in EN villages a diagnosis of pyelon Ca was made in one patient and of urinary bladder Ca in two subjects, thus UTC represented 10.7% of total carcinomas in the EN area. Regarding family history, there were no differences in the frequency of all types of Ca between EN villages and Klakar (46.9% vs. 39.1%, $p=0.94$). However, we observed a higher frequency of UTC in the EN villages (9.2% vs 3.4% $p=0.022$). The difference in the upper UTC frequency did not reach statistical significance (5% vs 3.4%, $p>0.05$), which is very likely due to the low number of subjects. An increased frequency of UTC was observed in EN villages, but there was no increase in other types of Ca. These results support the correlation between residence in the endemic area and UTC, which is consistent with the hypothesis that a toxic agent is the cause of EN.

P-3.4

Endemic nephropaty – the urologists' point of view

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In the last 11 years, 481 patients underwent surgery because of a diagnosis of urinary-tract cancer: 81 from the EN area Brodska Posavina and 325 from other locations. The frequency of upper-urinary-tract tumor vs. bladder tumor is statistically significantly higher in patients from the EN area whereas patients from the other locations resembled those at the national level. One hundred patients from the EN endemic area Bosanska Posavina had surgery, 38 of whom had upper-urinary-tract tumor.

When classified by type, there was a higher prevalence of transitional cell (papillary) cancer then of infiltrative cancer. The prevalence of upper-urinary-tract cancer in patients from the EN area is 50 times higher than seen in patients from other locations. Analyzing patients from EN area, serum creatinine levels above 132.6 μmol/L were found in 60% of patients with cancer of pyelon and/or urether and in 40.5% of patients with bladder cancer. Hemoglobin levels below 120 g/L were recorded in 100% of the cases and proteinuria was observed in 91% of the cases. In contrast, among all patients from areas located outside of EN areas, azotemia was present in 35.1% and anemia in only 13%; proteinuria was detected in 92% of the cases. According to our results, renal impairment and anemia are more pronounced in patients with upper urinary cancers from the EN area compared to patients from other regions.

P-3.5

Renal allograft-preserving surgery in a patient with endemic nephropathy and urothelial carcinoma in the transplanted kidney

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Our aim was to describe the diagnostic procedure, preoperative cytopathologic and cytogenetic tumor analyses, and conservative surgical treatment of urothelial allograft carcinoma in an immunocompromised, transplanted patient. A 52-year-old male patient from the endemic area of east Croatia developed chronic renal failure after endemic nephropathy and began hemodialysis in 1995. The patient received a renal transplant in 1998 from a female cadaveric donor who had lived outside the endemic region. Three years later, a low grade pTa urothelial carcinoma was diagnosed in the left contracted kidney. In 2004, ultrasound and MRI revealed an expansive process in the renal allograft. Percutaneous biopsy of the tumor under ultrasound guidance, preoperative cytopathologic and FISH analysis (UroVysion and CEP X/Y DNA Probe Kit, Abbott Diagnostics) for aneuploidy on chromosome 3, 7, 17 or losses of the 9p21 locus were performed. Cytopathologic analysis detected well-differentiated urothelial tumor cells. UroVysion was normal, without evidence of aneuploidy on chromosome 3, 7, 17 or loss of the 9p21 locus. FISH with CEP X/Y DNA Probe Kit showed the presence of X and Y chromosomes within the biopsied tumor. A right-side nephroureterectomy, followed by resection of the upper quarter of allograft (with negative surgical margins on frozen section), was performed. Histologic analysis showed low-grade urothelial carcinoma with *muscularis propria* invasion of the involved calyx. Postoperative graft function was stable; the creatinine clearance decreased from 82 to 56 mL/min at 18 months after surgery, without evidence of tumor recurrence on urinary cytology, cystoscopy, or MRI. In the absence of data about recurrences and progression of upper urothelial tumors after tumor excision in immunosupressed transplanted patients, further studies should include long-term follow-up in similar cases to determine the oncologic risk of graft-sparing procedures.

P-3.6

Endemic (Balkan) Nephropathy – urinary tract tumor association: A case presentation

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A 56-year-old Caucasian woman, born in Erghevita, Mehedinti County, Romania, who spent only her first 17 years of life in this region, with a family history of kidney disease, was admitted to our hospital. The patient was found to have chronic kidney disease (CKD, eGFR=42mL/min/m^2), signs of interstitial nephritis (TIN), symmetrically shrunken kidneys and severe normochromic normocytic hyporegenerative anemia, discrepant with eGFR. The differential diagnosis included myeloma kidney and medullary cystic diseases. The association of TIN with severe anemia suggests toxic impairment: lead-induced nephropathy, other toxic TIN (analgesic nephropathy, Chinese herb disease, 5-ASA nephropathy) and endemic Balkan nephropathy (EBN), the latter strongly supported by the clinical and biological picture, as well as by her residence in the endemic region during childhood and her family history. A relatively slow decline of renal function and a parallel increase in severity of anemia were noted during the annual monitoring. After 4 years of follow-up, intermittent total macroscopic painless hematuria without clots was noted. Ultrasonography revealed a right renal pelvic tumor, confirmed by CT scan. Right nephrectomy was performed. Microscopic examination of the tumor identified stage III renal pelvis transitional carcinoma (AJCC).

The diagnosis of EBN is based on epidemiologic data, the clinico-biological picture (TIN, severe anemia, shrunken kidneys, absence of UTI, slowly progressive CKD), histologic aspect of the kidney obtained at nephrectomy and on the association of urinary tract tumour (UTT). The association of EBN with UTT is proved by the increased incidence of UTT in families of patients with EBN and by the recently reported increased frequency of urinary bladder tumors. This case has certain particularities: the relatively high eGFR for the patient's age at first presentation and diagnosis, the short period spent in the endemic area and the early appearance of the pelvic tumor.

P-3.7

Renal tumors in population exposed to endemic nephropathy: an ultrasound follow-up study

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The first clinical sign of endemic nephropathy (EN) is tubular functional abnormalities which occur long before overt renal failure supervenes. This peculiar disease of unknown origin is also characterized by extreme reduction of renal dimensions, often asymmetric, and increased incidence of urothelial cancers. Both changes can be visualized by ultrasound (US) directly or indirectly (signs of obstruction). Ultrasonographic examination of the kidneys and abdominal organs was performed with a 3.5 MHz abdominal probe three times in 15 years (1990, 1996, and 2005). The unselected population examined included 388, 299 and 269 inhabitants of the endemic area, aged 16 – 84 years. In 1990, solid expansive lesions in kidney parenchyma were found in 7 persons (1.8 %). In 2 persons the lesions had US characteristics of hypernephroma (0.5 %), while in 5 persons (1.3 %) hyperechoic, sharply delineated nodules in the parenchyma up to 17 mm in diameter were found. Those US findings are typical for angiomyolipoma. In 1996, when the control study was performed, these renal lesions with benign characteristics increased up to 34 mm in diameter. The most recent study, performed in 2005, again revealed 7 persons (2.6%) with solid lesions in kidney parenchyma. One had US characteristics of hypernephroma (with enlarged local lymph nodes), and six individuals (2.2%) had hyperechoic nodules typical of angiomyolipoma (multiple nodes in two persons). The largest lesion increased further up to 51 mm in diameter. This first ultrasound follow-up study on an unselected population in an EN affected area revealed that the incidence of some pathologic findings significantly differed from what has been reported in the literature. The number of expansive lesions in the renal parenchyma, especially angiomyolipomas, indicates that the as-yet unidentified etiological factor responsible for EN causes a significant incidence of renal tumors in addition to uroepithelial tumors. The results of this study suggest that further systematic ultrasound examination is mandatory in the population living in an endemic area.

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