UNIVERSITY OF MEDICINE AND PHARMACY
“VICTOR BABEȘ” TIMIȘOARA
FACULTY OF GENERAL MEDICINE
Department III - Functional Sciences

DRĂGHIA M.-I. LAVINIA-PAULA

UNIVERSITATEA
DE MEDICINĂ ȘI FARMACIE
VICTOR BABEȘ | TIMIȘOARA

DOCTORAL THESIS

THE ROLE OF ENVIRONMENTAL FACTORS INVOLVED IN THE MULTIFACTORIAL ETIOLOGY OF CHRONIC KIDNEY DISEASES

ABSTRACT

Scientific coordinator:
PROF. UNIV. DR. PĂUNESCU VIRGIL

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KEYWORDS: aristolochic acid, Balkan endemic nephropathy, chronic kidney disease, chromatographic methods, environmental factors, exposure pathways, GC-MS, HPLC, LC-MS

INTRODUCTION

Chronic kidney diseases (CKDs) represent a group of diseases characterized by a complex pathophysiological process that results in irreversible alteration of the structure and function of the nephron. This leads to the onset of the end-stage renal disease, in which long-term survival requires dialysis or kidney transplantation. However, a large proportion of CKD remain unexplained, this category is called chronic kidney diseases of unknown etiology (CKDu) or chronic kidney diseases of multifactorial origin (CKDmfo) and caused several global epidemics throughout the years.

The focus of this doctoral thesis is on one of the CKD group, Balkan endemic nephropathy (BEN), used as a disease model and the main area of study is related to the causes and environmental factors involved in the etiology of this multifactorial disease. BEN is an irreversible chronic kidney disease, often associated with upper urothelial tract cancer, having high incidence in rural areas within the Balkan Peninsula, along the Danube River, affecting sporadic populations in Romania, and for this reason this specific disease represents an ideal model to study CKD.

The etiology of this disease is a important matter, the social impact on the affected populations and the related medical costs are high. From a scientific perspective, BEN is a multidisciplinary study at the crossroads of medicine, environment sciences and chemistry. The precise identification of the primary and secondary causal factors and the exposure mechanisms to these factors will allow effective preventive measures to be undertaken.

Many hypotheses have been postulated in the past six decades regarding the multifactorial etiology of BEN, several environmental factors being mentioned: coal-derived organic compounds, plant toxins, ochratoxin, heavy metals, plant dervied nefrotoxine of the *Aristolochiaceae* and *Asarum* family, called aristolochic acids (AAs). Recent studies confirm the main cause of BEN as chronic exposure to AAS originating from Aristolochia spp. plants that grow among crop fields, farms and gardens cultivated by the locals in some endemic and non-endemic regions in the Balkans and thus contaminate food sources. However, the routes of exposure to these phytotoxins and their toxicological relevance to the environment are not yet fully understood.

PURPOSE AND OBJECTIVES OF THE RESEARCH

The motivation behind this doctoral thesis is to bring contributions to the causes of BEN, which is a public health problem targeting susceptible populations in well-defined rural areas in Romania, but also in other Balkan countries. The purpose of this thesis is to research BEN causes and the environmental factors involved in this disease.

The specific objectives of the research were :

1) Detection, monitoring and quantification of environmental factors associated with Balkan endemic nephropathy in endemic and non endemic areas; field investigations and environmental sampling (soil, soil organic matter and the crops) but also *Aristolochia clematitis*.
2) Preparation of samples for laboratory analysis of environmental samples using specific chromatography methods; analysis of the chemical composition of the soil in endemic and non-endemic areas.
3) Development and optimization of modern chromatographic methods based on gas chromatography, liquid chromatography and mass spectrometry in order to achieve aristolochic acids analysis from the environmental samples.
4) Identification of the exposure pathways to aristolochic acids which causes the contamination of susceptible populations in endemic areas and the involvement of other cofactors in the genesis of chronic kidney diseases.
5) Development of a prevention model in relation to nephrotoxic and carcinogenic etiological agents, sources of contamination for Balkan endemic nephropathy and other chronic kidney diseases.
6) Reducing the risk of Balkan endemic nephropathy by raising awareness of the impact on the environment of human health.

The thesis is divided into two major chapters: the general part and the special part. The general part consists of three main chapters. Chapter I includes the introduction of chronic kidney diseases of unknown etiology (CKDu) and information about causes, risk factors, and the classification of CKDu. In Chapter II the attention is directed to BEN and current data on clinical and pathological characteristics, etiology and a subchapter dedicated to the exposure pathways and the involvement of environmental factors in the etiology of BEN. Chapter III describes the main factor involved in the etiology of BEN, aristolochic acid (AA), its origin, use and toxicity, as well as extraction and analysis methods for these nephrotoxins.

The special part is divided into two large chapters, each representing an individual study with a well-defined purpose:

I . Study on soil composition from endemic and non-endemic areas and its contamination with aristolochic acid I

II . Study on the role of aristolochic acid I contamination of vegetable crops and the quantitative and qualitative analysis by using liquid chromatography and mass spectrometry methods

THE SPECIAL PART

I . STUDY ON SOIL COMPOSITION FROM ENDEMIC AND NON-ENDEMIC AREAS AND ITS CONTAMINATION WITH ARISTOLOCHIC ACID I (AAI)

This larger study on the chemical composition of the endemic or non-endemic soils and the further contamination with AAI and the involvement in BEN pathology, includes two separate objectives:

1. Identifying, by a simple and quick extraction and analysis, the organic composition of the first two layers of the soil (soil organic matter - SOM and soil itself) collected from endemic areas with active cases of BEN, as well as non-endemic areas, with or without the presence of *Aristolochia clematitis* in the sampling area. For this purpose, we used a solvent-based extraction method, with methanol, using microwaves followed by a gas chromatograph coupled with mass spectrometry (GC-MS) method.
2. To determine whether this soil contamination pathway is possible, taking as reference the soils in cultivated fields and gardens from endemic and non-endemic areas from several villages in Romania. At the same time, we want to gather scientific evidence for new hypotheses that contribute to the etiology of BEN, related to a new AAs exposure pathway through the surface layers of the soil, where dust and aerosols are formed, possibly contaminated with AAs particles from pollen or residues originating from *Aristolochia* plants.

For this study, chromatography methods were used in order to analyze the soil composition but also to separate and identify certain compounds, especially AAs from environmental samples (soils). Thus the instruments used were: gas chromatograph GC Scion 436-GC (Bruker Daltonik GmbH) coupled with an MS mass spectrometer with triple quadrupole Scion TQ, (Bruker Daltonik GmbH), high performance liquid chromatograph (HPLC Agilent 1100 Series) coupled with mass spectrometer (Bruker A maZon SL). The samples analyzed by HPLC-MS were extracted using 80% methanol and 20% aqueous solution of 10% formic acid.

Following HPLC and MS analysis, AAI could be detected and quantified at nanogram levels. Concentrations found in soil samples (both endemic and non-endemic areas) ranged from 140 to 606 ng/mL (**Figure 1**). Most of the samples, especially those from endemic areas, positively detected the presence of AAI. This may be explained by the fact that the rotting seeds or remains from *Aristolochia clematitis* plants, were taken together with the samples. However, there are exceptions, probably due to environmental and seasonal variations, in some soil samples that were collected under the same conditions, in the immediate vicinity of the *Aristolochia* plants, we could not detected AAI.

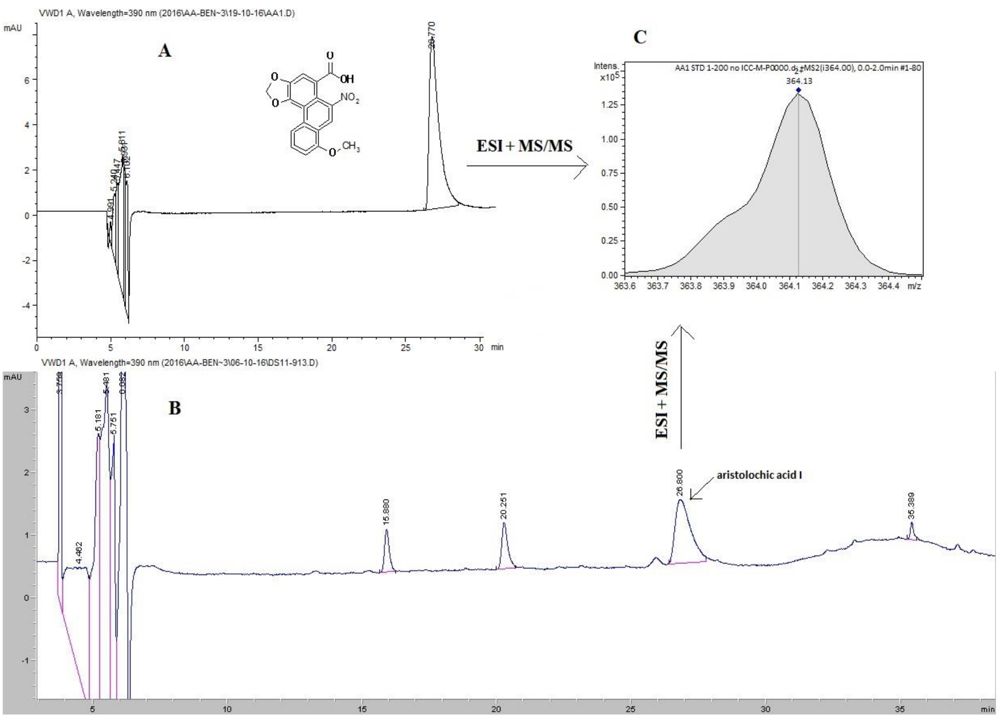

Figure 1 . HPLC and MS chromatography analysis of AAI
A . HPLC-UV analysis of AAI standard dilution
B . Chromatogram of a soil sample (collected together with *Aristolochia* seeds) from a garden in the endemic area of Romania
C . AAI ESI + MS / MS spectrum - Chromatogram of the extracted ion [AAI + Na] with m / z 364)

The analysis of organic compounds from soil samples was done after their separation from the chromatographic column, and their identification was based on chemical similarities between structures, obtaining a total of 103 compounds from different chemical classes, identified after employing the GC-MS analysis method. Among the identified compounds, in the largest proportion were aliphatic and aromatic hydrocarbons derivates , each peak representing a different compound. The compounds are presented in the chromatogram as the sum of the intensities of the mass spectra as a function of the retention time (**Figure 2**).

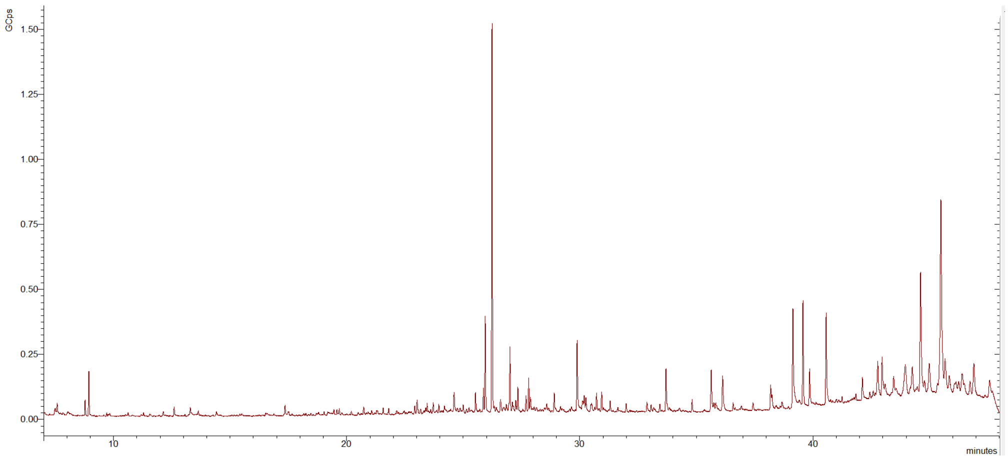

Figure 2. Chromatogram of a soil sample from an endemic area (Erghevita village, Mehedinți County)

This large study has shown that it is possible to contaminate the soil with AAI, a carcinogenic nephrotoxin primarily responsible for the incidence of BEN and other chronic kidney diseases. We were able to prove this using sensitive HPLC and MS methods. Both endemic and non-endemic areas were examined for the presence or absence of AAI, subsequently demonstrating that the degree of soil contamination with AAI was largely similar in both areas. A decisive factor in the detection of AAI in soils is the presence of *Aristolochia clematits* plants.

The fact that we were able to identify AAI in the SOM samples may lead us to think of another hypothesis regaring contamination, a new route of exposure, the air pathway, through the dust generated by the upper layer of the soil. This exposure pathway through air, complements the other routes already listed in the general part: voluntary exposure (medicinal use) or involuntary exposure (food contamination with AAs). Each of these pathways can contribute in a partial, chronic or cumulative way to AAs exposure, the harmful effects produced over time are often irreversible.

Lipid fractions in the soil, especially aliphatic hydrocarbon derivatives were detected in the largest proportion in samples collected from endemic and non-endemic rural areas, as opposed to soil in urban areas, where a high concentration of polycyclic aromatic hydrocarbons was detected, a worrying fact for locals due to the fact that it accumulates in the soil and contributes to chronic exposure to these harmful substances.

Aristolone, a specific sesquiterpene, was identified in samples that contained seeds or remains of the *Aristolochia clematitis* plant, that have been collected together with soil samples. Therefore, we can deduce that the soil assimilated this compund, aristolone, due to the fact that *Aristolochia* plants grow near the cultivated gardens from where the samples were collected.

II. STUDY ON THE ROLE OF ARISTOLOCHIC ACID I (AAI) CONTAMINATION OF VEGETABLE CROPS AND THE QUANTITATIVE AND QUALITATIVE ANALYSIS BY USING LIQUID CHROMATOGRAPHY (LC) AND MASS SPECTROMETRY (MS) METHODS

In order to extend the scope of previous studies, referring to the occurrence and distribution of emerging contaminants in "soil - plant - environment" systems and which represents a public health problem, we proposed a sensitive liquid chromatography coupled with mass spectrometer with ion trap (LC-IT-MS) method for AAI detection and quantification in some of the most common cultivated plants, such as: green or spring onions (*Allium fistulosum*), lettuce (*Lactuca sativa*) and cucumbers (*Cucumis sativus*). We performed all the experiments in natural field conditions, the samples were collected from a non-endemic garden, an area where *Aristolochia* plants are present. This current study is a continuation of one of the investigations recently published by our research group. Moreover, we proposed the sample analysis by using a simple LC-MS method to analyze and quantify the AAI for the calculation of theoretical doses that the population is exposed to, by consuming the contaminated vegetables. In this regard, we developed a similar experimental approach for a better understanding of the causal relationship between AAI and BEN and how this environmental contaminant is translocated from different parts of vegetables, how AAI enters the onion bulb, the salad leaves and the cucumber fruits. We carefully chose these vegetables in order to cover several types of vegetable parts that have both under and above the ground, cultivated in an agricultural field or garden contaminated with AAI.

The instrumentation used to perform the chromatographic analysis experiments consisted of: an ultra-high performance liquid chromatography (UHPLC) Dionex UltiMate 3000 (Thermo Scientific) equipped with an autosampler, a pump and a diode array detector (DAD). The liquid chromatograph part is coupled to a mass spectrometer (MS) with an electrospray ionization source (ESI) and ion trap (IT) Amazon SL (Bruker).

A total of 141 samples were collected from two different locations, during field trips made between June and July 2017 from a non-endemic rural garden in Romania, where *Aristolochia clematitis* (which were flowering at that time) were present in some places, and an area where no cases of BEN have been reported. The locals use the gardens to grow vegetables for personal consumption, usually being the only source of food for the family. Although most publications mention that AAs are found exclusively in endemic areas, there are studies that present data that AAs are also found in non-endemic settlements, outside BEN areas, due to the fact that *A. clematitis* is considered a weed whose growth and spread cannot be controlled. Ever since the field trips in the previous study we noticed that *A. clematitis* is found in similar abundances in both areas: endemic and non-endemic villages.

The validated UHLPC–MS method was applied for AAI detection in samples: soil (n = 30), green onion - bulb and leaf (n = 24), lettuce - root, stem, leaf (n = 54) and cucumber - fruit (n = 6), collected from a cultivated garden, from a non-endemic area in Romania. In order to have a comparison term, we collected control samples (n = 27), from a non-endemic urban area where BEN or Aristolochia clamatitis were not reported. Chromatographic analysis confirms the presence of AAI in all samples collected from the garden possibly contaminated with Aristolochia clematitis . As expected, AAI was not detected in soil and vegetable samples collected from the urban garden (negative control). The MS / MS spectrum and retention times of the samples collected from the garden contaminated with Aristolochia in the presence of the plant were similar to those of AAI standards, and the concentrations were calculated.

A comparision of the results obtained in soil samples, green onions and lettuce is represented in the graph in **Figure 3**, the concentrations varying between 600 - 1500 ng / mL (361.45 - 903.61 ng / g). The samples are divided into two categories depending on the collection area and whether or not the presence of the Aristolochia clematitis plant was detected at the time of sample collection. The highest amount of AAI was found in soil samples that had no direct correlation with the presence of *Aristolochia* plants. A remarkable amount was observed in the underground parts of the plants, in the green onion bulb and in the lettuce root, values that attest the path of absorption from the soil in the cultivated plants. In this case, the presence of *Aristolochia* plants increases the concentration of AAI in the samples, the green onion bulb reaching an average concentration of 441.3 ng / g. On the other hand, the amount of AAI found in the aerial parts of vegetables (leaves or stems) was two thirds lower than the concentrations calculated in the underground parts. The lowest amount of AAI was found in cucumber fruit, with concentrations between 120.5 and 187.5 ng / g. One explanation may be that the fruits grow at a later stage, grow above the ground or stand on the ground, and even if the cucumbers were located near the *Aristolochia* plants, they probably did not have enough time for the AAI to accumulate in the fruit tissue.

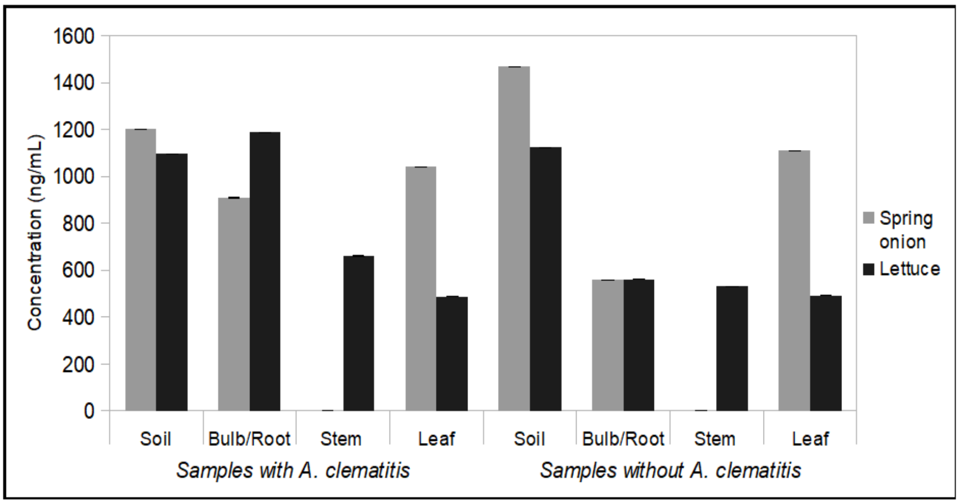

Figure 3 . Graphical representation of average AAI concentrations in vegetables (green onions versus lettuce) in parallel with soil samples corresponding to nearby areas

Our results demonstrate for the first time that concentrations of AAI can also be found in non-endemic soils, as long as *Aristolochia* plants grow in that area. Assuming that a villager is exposed to the maximum amount of AAI by consuming all these vegetables throughout the period in which they grow (three months a year), it means that he is exposed to almost 10 mg of AAI annually. If we take into account that BEN develops between 40 and 60 years, it means that the cumulative exposure of AAI is around 400 mg, corresponds to the period of time in which BEN develops.

The presence of AAI in parts of plants grown above or below ground is attributed to a specific pathway that explains how AAI circulates in the environment. AAI concentrations decrease from soil to bulb (or root) to leaves and fruits; the highest concentrations are in the underground parts, more precisely in the green onion bulb. AAI from leaves and stem can be attributed to volatilization and air deposition by air, or transport through the whole plant starting from the root (in salad) or bulb (in green onions) and further to the aerial parts, such as leaves (in green onions and salad) and fruit (in cucumber), while AAI from the root can be associated with a passive transport from the soil or by binding humic acids from the soil, thus making available AAI for other plant roots.

FINAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

This doctoral thesis aimed to study the environmental factors involved in the multifactorial etiopathology of a chronic kidney disease manifested in the Balkan Peninsula, a disease called Balkan endemic nephropathy. In the context of this disease, the factors involved in its appearance were studied, the most recent and intensively studied being aristolochic acids, phytotoxins present in plants of the Aristolochiaceae family , and this species grows mainly in the Balkans, for Romania the species Aristolochiaclematitis is characteristic. The studies within this thesis complement the current studies carried out by other research groups with the common goal of elucidating the cause and preventing this fatal disease.

The thesis includes several studies of analysis and identification of aristolochic acid I, the predominant compound in *Aristolochia* plants, with nephrotoxic and carcinogenic effect. Contamination of the environment with this phytotoxin is in itself a complex study, given that so far have been identified in the soil, in cultivated plants consumed by locals, in water from wells, so the study hypotheses start from these experimental data. The fact that the compound can contaminate the soil surrounding the Aristolochia plants and then can diffuse into the soil and thus end up contaminating the plants that grow near it. This fact led to the analysis of samples collected from endemic and non-endemic areas because there are certain rural areas where Aristolochia plants grow, but where there are no cases reported by Balkan endemic nephropathy. In general, aristolochic acids prefer a soil with a composition similar to that in which grown cultivated plants consumed by locals. Several research groups have studied how this compound contaminates: soil in endemic areas, crops grown in endemic gardens, such as onions, tomatoes, cucumbers, lettuce; but also those cultivated in endemic fields, such as: corn, wheat, the waters of the wells.

These multiple contamination pathways occur in the chain: Aristolochia plants soil - crop plants - water and it is necessary to understand them because they could guide the causes of the disease and explain the unknown causes, both for Balkan endemic nephropathy and other chronic kidney disease. similar, which have among the triggers contaminants in the environment. Usually, environmental studies are carried out over a longer period due to the great variability of the triggering or potentially responsible factors: Aristolochia plant diversity , crop plant properties, particularities of the region of origin of the plants, soil type in which the plants grow, seasonal differences, period collection, analysis techniques.

The main conclusions highlighted by the work done in this doctoral thesis are:

1) By using sensitive analysis methods, we demonstrated that the soil and the soil surface layer - soil organic matter (SOM) are largely contaminated with AAI at nanogram levels, in areas where *Aristolochia clematitis* exists.

2) Due to the fact that *Aristolochia* plants grow on endemic and non-endemic soils, the AAI compound was found in both types of soils, the level of contamination was found similar.

3) In the soil samples collected in September 2016, we found a much higher AAI concentration than in the samples of other seasons, probably this fact is due to a higher abundance of *Aristolochia clematitis* plants in the mature stage, when the fruits and the seeds have an increased concentration of AAI.

4) The GC-MS method has been successfully applied in order to analyze the chemical composition of the soil collected from endemic and non-endemic areas, thus identifying more than 100 organic compounds.

5) Our results demonstrate for the first time the presence of AAI in non-endemic soils, as long as the presence of *Aristolochia* plants is observed in that area.

6) We managed to develop and validate a new ultra-performance liquid chromatography coupled with ion trap mass spectrometer (UHPLC-MS-IT) method, capable of detecting and quantifying AAI at the nanogram levels in soils and vegetables.

7) All AAI-positively identified samples were collected from the same location, a non-endemic cultivable garden with or without the direct presence of the plant *Aristolochia clematitis*, which did not particularly influence the concentrations of AAI.

8) We demonstrated that AAI concentrations vary amongs different plants parts, depending if they grow above- or under- ground, and this fact is attributed to a specific pathway that explains how AAI circulates in the environment: from soil to the direction of root - leaf - fruit, therefore the highest concentration of AAI was identified in the bulb of the green onions.

Balkan endemic nephropathy together with its main trigger, aristolochic acid I, is a matter of concern for the public health of some populations. The likelihood of this phytotoxin contaminating vegetables, the way it is taken up, then transported and bioaccumulated in the tissues of the edible vegetables, is a first step in taking preventive measures and avoiding the direct contamination of other foods. Frequent soil analysis are also needed in order to identify toxic organic compounds in the soil, such as polycyclic aromatic hydrocarbons, which have implications for public health. To conclude, a multidisciplinary approach is needed to cover the complexity of all the hypotheses developed so far: solving the mystery of the factors responsible for triggering Balkan endemic nephropathy, together with other chronic kidney disease of unknown etiology induced by various environmental factors.