This is to certify that the

thesis entitled

BALKAN ENDEMIC NEPHROPATHY.
RESULTS FROM A STUDY IN
VRATZA DISTRICT, BULGARIA

presented by

Plamen Stoianov Dimitrov

has been accepted towards fulfillment
of the requirements for

M.S. degree in Epidemiology

Major professor

Date April 25, 2001

BALKAN ENDEMIC NEPHROPATHY. RESULTS FROM A STUDY IN VRATZA DISTRICT, BULGARIA

By

Plamen Stoianov Dimitrov

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Epidemiology

2001

ABSTRACT

BALKAN ENDEMIC NEPHROPATHY.
RESULTS FROM A STUDY IN VRATZA DISTRICT, BULGARIA

By

Plamen Stoianov Dimitrov

Balkan Endemic Nephropathy (BEN), first described in 1956 in Vratza District, Bulgaria, may result from prolonged, chronic exposure to environmental toxicants, but the underlying etiologic factors remain elusive. There has been no recent characterization of the epidemiology of this disease. Our study conducted in May 2000 in Vratza District aimed to assess prevalence, incidence, age and gender distribution, survival time and life expectancy of BEN. The project also investigated a possible association of BEN with ochratoxin A. The results of the study suggest a decline of incidence of BEN in the endemic area over the period 1964-1987 - from 0.7 per 1000 to 0.3 per 1000. Reasons for the decline in incidence may be incomplete case identification, reduced efforts of screening, and diminished public health interest. The percentage of underreported cases rose from 6.0% for the period 1965-1969 to 18.9% in 1980-1984. Survival and life expectancy increased significantly over the same period, from 1.7 to 6.5 years; life expectancy increased from 59 to 68.7 years. Age at first diagnosis was raised from 53.9 to 56.3 years. Therefore the increase in survival was due to actual prolongation, not lead-time bias. Ochratoxin A could falsely be associated with BEN, due to behavior bias expressed by a modification of food storage in villages affected by BEN, but not by residents of non-BEN villages.

To Rady and Villy

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my advisor, Dr. Wilfried J.J. Karmaus, for his guidance, inspiration and valuable suggestions throughout my studies and during the writing of my thesis. His desire for his students to learn and excel in their field has been a continuous encouragement to me, as I was exploring the intricacies of Epidemiology.

I would also like to thank my committee members Drs. Dorothy R. Pathak and Evangelos A. Petropoulos who provided constructive insights during the conceptualization of the research project.

I am further grateful to Dr. Petropoulos, who as Program Director of the NIH-funded Environmental Health Project for Bulgaria, provided continuous moral, scientific and financial support during my graduate studies at Michigan State University.

I wish to express my gratitude to Dr. Aryeh D. Stein for his invaluable help in the design of the field research and in the collection of data in the Vratza District Hospital in Bulgaria.

The facilitation and support of Dr. Valeri Simeonov, Head, Division of Nephrology and Hemodialysis at the Vratza District Hospital, with the screening of the hospital records of Balkan Endemic Nephropathy patients, is very much appreciated.

The support of Dr. Varban S. Ganev, In-country Project Director, in various stages of my training under the project is gratefully acknowledged.

Other colleagues, who have provided valuable inputs to the research, are individually acknowledged on page 10.

This research was supported by Grant # 5-D43-TW00641 "Training and Research in Environmental Health – The Balkans" to the Institute of International Health at Michigan State University, by the Fogarty International Center, National Institutes of Health.

I would like to thank team members of Fisheaters project for their kindness and patience with me.

Finally, sincerest thanks to my Family members for their endless love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BEN - Balkan Endemic Nephropathy

OTA - Ochratoxin A

ppb - Parts per billion

OR - Odds Ratio

CI - Confidence Interval

INTRODUCTION

Balkan endemic nephropathy (BEN) is a noninflammatory chronic, familial, primarily tubulointerstitial bilateral kidney disease, usually without hypertension, that affects rural populations in several geographical regions in Bulgaria, Romania, Serbia, Bosnia-Herzegovina, and Croatia.

BEN (ICD-10 - N15.0) was first described in Vratza district, Bulgaria, by Tanchev in 1956. He surveyed the Vratza district in Northwestern Bulgaria and identified patients with this unique kidney disease. Cases of kidney disease with similar symptoms were subsequently reported in Yugoslavia in 1957, and in Romania in 1961. In 1964 the disease was recognized as a new nosological entity and was referred to as Balkan Endemic Nephropathy.

The disease has no acute onset, runs asymptomatically and progresses slowly for many years. The clinical manifestations of BEN are progressive renal insufficiency without hypertension, normochromic or slightly hypochromic anemia, salt loss, polyuria, polydipsia, nocturia. Laboratory indicators in urine samples include aseptic leukocyturia, low molecular proteinuria, creatinemia, and a minimal amount of urinary sediment. Several investigations have reported that beta2-microglobulin might be an early sign of BEN as it is the first protein to be excreted in increased amounts. The tubular structures of the kidneys are severely affected, as seen in ultrasound imaging, by the decrease in size of both kidneys, some to the size of a walnut with a weight of 30 g.

Clinical cases occur in various locations and possibly have a long "incubation" period before the clinical onset of the disease takes place. BEN

occurs predominantly in adults aged 40-60 years. Few children and individuals over 70 years of age have been found to develop the disease. The gender distribution of the disease shows a slight predominance in women, with a female/male sex ratio of about 1.5/1.

For over five decades, the occurrence of BEN has been characterized by its undefined etiology. Although various investigators have postulated that the disease may result from prolonged, chronic exposure to environmental toxicants, with possibly genetic and immune factors playing a role, no definitive risk factors have yet been identified.

Various potential risk factors were studied extensively, including ochratoxin A and other mycotoxins, polynuclear aromatic hydrocarbons, heavy metals, selenium deficiency, viral infection, and genetic susceptibility. However, the majority of the studies were not epidemiologically designed; they were limited to case series, or to single risk factors. As a result the cause of BEN is still unclear and evades the best efforts of researchers to date.

Initial studies describing the geographical distribution of the disease indicate that the disease is located only in Balkan countries and affects rural populations in a mosaic-like pattern. Thus far, BEN exists in some villages for decades, while in neighboring villages and towns (one to two miles away); no cases of BEN have been diagnosed. Similar to the mosaic distribution pattern throughout the villages, within these endemic villages unaffected households exist side by side with BEN households. Familial clustering of BEN was first described in 1957 and later reports have confirmed that observation. However,

no pedigree studies have been conducted. Over the years, the geographic distribution of BEN has remained stable. Villages afflicted in the past continue to be afflicted today. BEN predominantly occurs in rural areas, which are farmed. Urban BEN patients typically had resided in a rural endemic area during their childhood.

In Bulgaria the endemic area is situated in the Northwestern part of the country in the districts of Vratza and Montana, at the foot of the Balkan Mountains. During the 1970s, studies reported many cases of the disease in the Vratza district. Forty-one villages totaling 50,000 population were affected by BEN, 15 of them severely. However, the available reports did not provide adequate epidemiological data, such as incidence, prevalence, survival, life expectancy, age and gender distribution, etc. No epidemiological studies have been conducted in recent years in the Vratza district.

We decided to focus our investigation of BEN on the region, where the disease was first identified. Using modern epidemiological approach we conducted a field study in May 2000 in the Vratza district, during which we collected all available data from the BEN patient registry and from hospital records of current BEN patients, who are under dispensary observation. Both types of data were obtained from the Department of Nephrology and Haemodialysis at Vratza District Hospital. Additionally we collected samples of soil, well water, spring water, food and feed from BEN and non-BEN villages to investigate possible association of BEN with ochratoxin A (OTA), arsenic and polynuclear aromatic hydrocarbons (PAH). During the sampling of BEN

households, more BEN cases were identified by interviewing the inhabitants of the house.

Registry, hospital and field data collected during the field study were grouped and analyzed into four sections, addressing various epidemiological aspects of BEN. Figures 1 and 2 show the categorized data and different study populations described in chapters 1-4.

The first section of our study (Chapter 1) is an epidemiological analysis of the disease registry maintained in the Vratza District Hospital for the period 1964-1987. Using modern epidemiological methods we assessed the incidence, prevalence, age, gender, and geographical distribution of BEN. This section also analyzes time changes in the epidemiological characteristics of BEN. We were able to identify and analyze records for 1375 BEN cases for the period 1964-1987, distributed in 21 villages and the town of Vratza. This analysis provides an illustration of epidemiological characteristics of the region where the disease was first described. The data have not been analyzed previously, using modern epidemiological methods.

In the second section of the study (Chapter 2) we examined whether changes in incidence rates over the years are actual or biased. Figures 1 and 2 show the study population described in this chapter. Recently, a study conducted in Serbia suggested that the incidence of the BEN is decreasing in Serbian endemic areas, although no similar analyses have been published for other endemic areas. In the first section of our study we discuss a possible decline of incidence and prevalence of BEN in the Vratza District. Because changes in

Figure 1 – BEN cases - Categorized Data and Study Populations Used in the Four Sections of Analyses

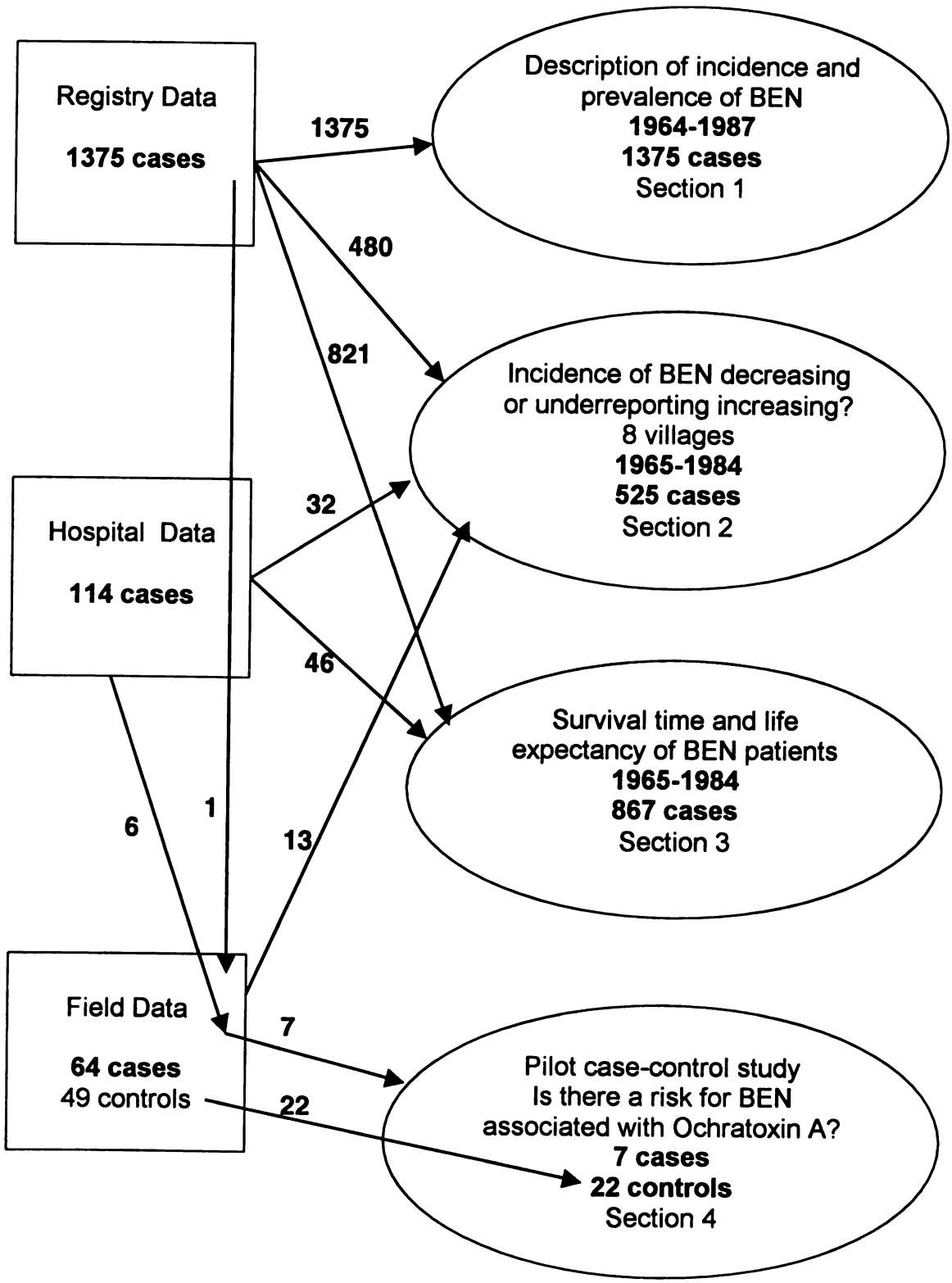
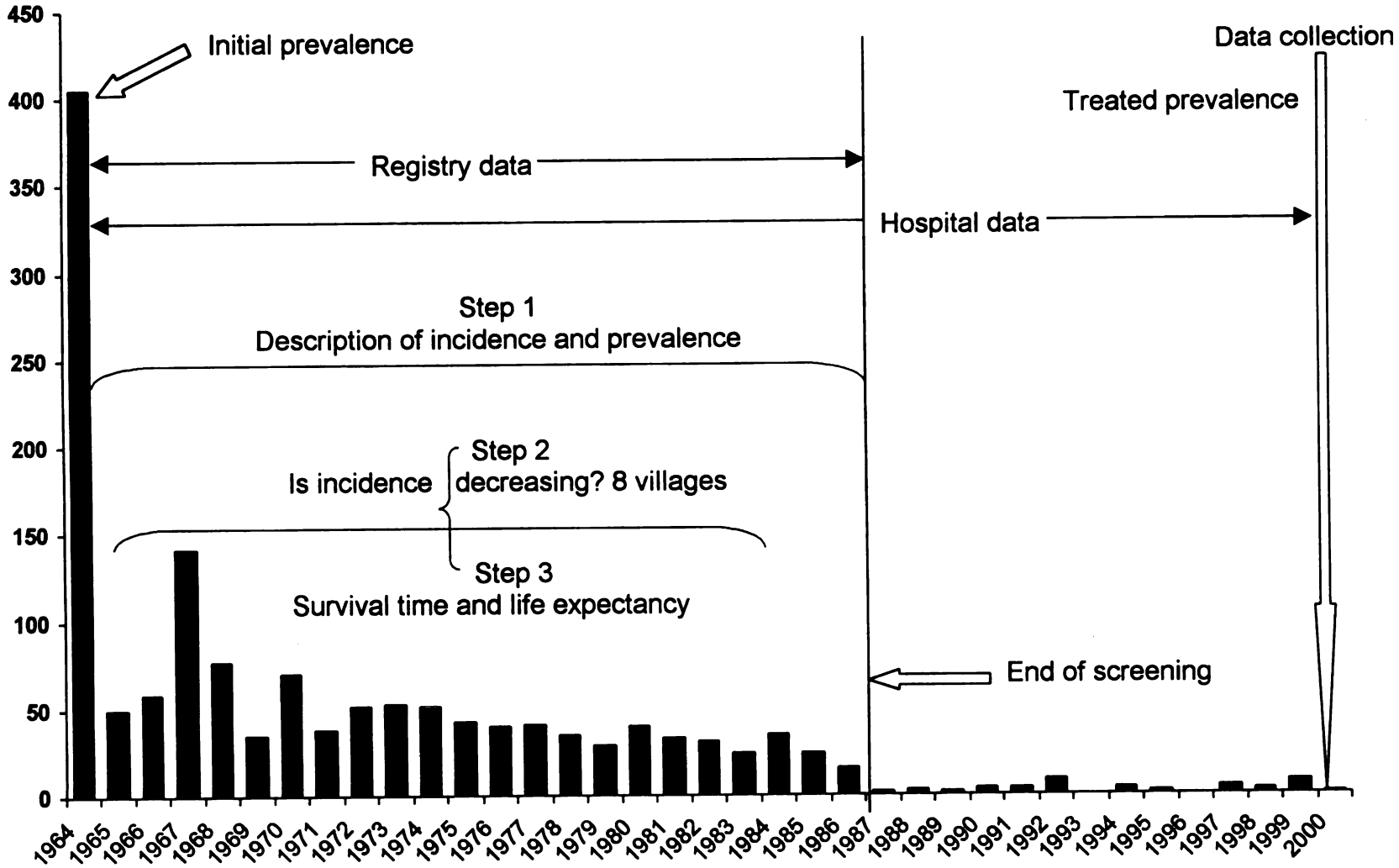


Figure 2 - Distribution of BEN cases by year of diagnosis, used in three steps of analyses



occurrence rates of BEN over time would influence the entire assessment of BEN, confirmation of the incidence data was required. We tested whether the reported decline is the result of reduced efforts to collect data and record BEN patients. Active screening for BEN was terminated in 1987; after this date it is possible that the interest in collecting data on BEN decreased. In this section we used three data sources based on data of disease registry, hospital records, and field study cases. We restricted our analyses to eight villages only, which were included in the field study. We attempted to confirm the completeness of the registry data and to assess any underreporting of BEN cases by comparing the registry data with other two data sources: hospital records and field study data. The registry included 1375 patients diagnosed during the period 1964-1987. The hospital records identified 114 current BEN patients, who were treated at least once in the Vratza District Hospital, and are currently under dispensary observation. The BEN patients identified in the hospital records were diagnosed over the period 1964 – 2000. During the field study we identified 64 BEN cases; these cases made up the third source for our analyses. Subsequently we restricted the data from registry and from hospital records to only those eight villages for the period 1965-1984. We excluded from our analyses data from 1964, because we did not know when those patients were recorded as BEN cases; therefore, they cannot be used for incidence analyses. We excluded data after 1984, as there were very few cases in the disease registry, which made the completeness of the data doubtful. We restricted our analyses to only one record per patient when the same individual was identified in more than in one data

source. By limiting the scope of the study to eight villages and to the period 1965 –1984, our data consisted of 480 BEN patients from the disease registry, 32 patients from hospital records, and 13 patients identified during the field study. If all past records of BEN data were complete, then all BEN patients in the hospital records, as well as all patients identified during the field study, if diagnosed before 1984, should be in the registry. Checking the completeness, we could analyze whether changes in epidemiological characteristics of BEN over time are actual, and the extend to which they might be affected by other factors.

The third section of the study deals with problems of survival and life expectancy of BEN patients (Chapter 3). The study population used in this section is presented in figures 1 and 2.

Because BEN is a chronic disease developing over many years the diagnosis is typically defined when the disease is in its later stages. That is why the problem of survival of BEN patients needs more studies and discussions. Analyses of the survival after onset of disease and of the survival after diagnosis could produce dissimilar results. We do not know the onset of the disease, and therefore only the year of diagnosis can be used to assess the survival of BEN patients. The year of diagnosis is a critical point of survival analyses and, if incorrect, could falsely affect the survival time. Records of the year of diagnoses depend on several factors: active screening, patients seeking medical care, good record keeping, and accurate diagnoses. Only a few studies report life expectancy and survival time for patients after diagnosis of BEN. Additionally, we found many of the data are contradictory. This was the reason, as a third step of

our study, to investigate the survival time and the life expectancy of BEN patients in the Vratza District, and to find out whether both have changed over time.

In this section we used data from the two sources: disease registry and hospital records. The inclusion criterion was that the year of diagnosis occurred within the period 1965-1984 for the reasons described previously. As a result of these restrictions, the third section of our analyses was based on 821 cases identified in the disease registry and 46 cases from the hospital records, all of which were diagnosed during the period 1965-1984.

In the fourth and final section of our study, we decided to investigate a possible association of ochratoxin A with BEN (Chapter 4). The etiology of the disease is still unknown, but some factors are more plausible than others as the cause of BEN, such as ochratoxin A. Ochratoxin A is a widespread contaminant in a variety of foods and animal feeds throughout the endemic areas. Even North Atlantic Treaty Organization has stressed this putative cause in an effort to protect their servicemen in Bosnia-Herzegovina from BEN. During the field study, samples of food and feed were collected and analyzed for OTA. We conducted a pilot case-control with the seven cases and 22 controls identified during the field study, for whom we had OTA measurements. These individuals were also identified in either the registry or hospital data (Figures 1 and 2).

In summary, the aim of the study is to analyze important epidemiological characteristics of BEN in the area, where the disease was first described, to determine how reliable the available data are, and to investigate one of the possible causative factors.

All four sections of the study were submitted separately for publication in various journals (1-4), and one of them (4) has already been accepted. Other authors participated in the preparation of these manuscripts. Herewith, it should be noted in particular, the contribution of Dr. Wilfried Karmaus to chapters 2,3 and 4, Dr. Evangelos Petropoulos to chapter 4, Dr. Aryeh Stein to chapter 1, Dr. Varban Ganev to chapters 2 and 3, Dr. Valeri Simeonov to chapters 1 and 3, and Dr. Mohamed Abouzied to chapter 4.

CHAPTER 1

BALKAN ENDEMIC NEPHROPATHY IN VRATZA, BULGARIA, 1964-1987: AN EPIDEMIOLOGIC ANALYSIS OF POPULATION-BASED DISEASE REGISTRY

Introduction

Balkan Endemic Nephropathy (BEN; ICD-10 - N15.0) is a non-inflammatory, slowly progressing, familial, chronic, primarily tubulo-interstitial bilateral kidney disease. First described in 1956 in Vratza, Bulgaria (5), it is localized to rural areas in several regions in Bulgaria, Romania, Bosnia-Herzegovina and Croatia (Figure 3). Although various investigators have postulated that the disease may result from prolonged, chronic exposure to environmental toxicants including ochratoxin and other mycotoxins (6-8), polynuclear aromatic hydrocarbons (9), heavy metals (10), selenium deficiency (11), herbs containing toxic compounds (12), viral infection (13), or a genetic susceptibility (14, 15), the underlying etiologic factors remain elusive (16).

In 1956, Tanchev surveyed the Vratza region in northwestern Bulgaria and identified prevalent cases of BEN (17). The affected villages were interspersed among villages in which, to the present day, no cases of BEN have been diagnosed. An incidence rate of 30-50 cases per year per 10,000 people in the endemic area in the 1970's has been cited (18). However, the available reports did not provide an adequate epidemiological profile of this disease. Recently, it has been suggested that the incidence of the disease is decreasing in Serbia (19), but no similar analyses have been published for other endemic areas.

Figure 3 – Map of the foci of Balkan Endemic Nephropathy in Bosnia and Herzegovina, Bulgaria, Croatia, Romania and Yugoslavia

Changes in the epidemiology of the BEN over time, if real, would suggest directions for focusing research into the etiology of the disease. We therefore describe the distribution of BEN in the region in which it was first identified, using population-based registries for the period 1964 through 1987 that have never, to our knowledge, been previously analyzed using epidemiological approaches.

Methods

Setting

Vratza is a rural district in the northwestern region of Bulgaria (Figure 3). The district consists of the district capital (Vratza, 1991 population 85174), and approximately 60 villages ranging in population from a few hundred to over 15000 people. The economy is based on farming. Since the 1960's the population of most villages has decreased, while the population of the town of Vratza has more than doubled and that of the village of Mezdra has increased to over 15000 inhabitants (Table 1). The population of one affected village (Karash) was systematically relocated in the early 1960's.

Sources of data

The sources of study population are presented in Figures 1 and 2.

Registration of BEN patients was established in 1964, following several years of research and surveillance activity in the district of which no records remain. All previously identified surviving BEN patients were logged into hand-written registries. A separate registry was maintained for each of 21 villages in which BEN cases had been identified and for patients living in the town of Vratza.

Table 1 - Population of Affected Villages and Cases of BEN Registered, 1964-1987, Vratza District, Bulgaria

	Population						Number of cases registered		
	1964	1970	1975	1980	1985	1991	Prevalent in 1964	Incident 1965 to 1975	Incident 1976 to 1987
Beli Izvor	1302	1910	1345	1250	1085	974	75	105	44
Bistretz (1)	1213	1322	1414	1506	1598	1653	50	69	34
Brusen	585	563	556	536	544	532	5	9	0
Darmantzi	709	661	642	593	517	415	4	7	3
Goliamo Babino	850	711	676	630	547	386	4	8	3
Goliamo Peshtene	1750	1351	1196	993	846	642	4	31	13
Gomo Peshtene	1275	1024	944	825	708	531	34	41	28
Hubavene	838	670	685	621	543	416	46	53	30
Kalen	434	429	342	254	265	201	21	15	5
Karash	417	101	61	56	37	32	11	1	1
Kravoder	1813	1813	1832	1640	1481	1052	6	22	6
Kunino	1480	155	1376	1527	1233	1184	0	7	2
Liliache	2609	1890	1997	1778	1943	1855	0	12	7
Mezdra	9885	11852	12701	14117	13949	15323	8	36	13
Pudria	1607	1271	1308	1159	1102	915	33	51	16
Radovene	868	670	647	576	569	418	13	17	10
Roman	2179	2872	3121	3174	4434	4772	17	43	34
Tishevitza (1)							17	19	5
Tsakonitza	592	437	306	257	217	179	43	37	12
Vesletz	645	438	329	300	207	185	0	9	7
Vlasatitza	809	701	646	582	501	381	0	9	7
All villages with registers	31870	30985	30751	32406	29642	30473	391	583	266
Vratza town	35826	51309	61134	65992	78669	85174	14	56	32

(1) Population count data were not available for Bistretz in 1970, 1975, 1985, or 1991, or for Tishevitza in any year. Bistretz population estimates for these years are interpolations from available data.

Information available included the individual's full name (first, patronymic, family) and age. Registries were updated each year until 1987. Each year's entry for a village consisted of the listing of all surviving BEN patients (prevalent and newly identified) living in the village at the time of the survey. New names were added as individuals were identified, and deaths and relocations were recorded. Some individuals were deleted from the registry in later years and not subsequently followed – there is no additional information in the registries, but it is our understanding that these individuals were later thought to have a disease other than BEN. No clinical details are available, either of these deleted cases or for those retained in the registry. Additional patients were identified in the summer of 2000 from the records of the Department of Nephrology at the District Hospital in Vratza. Most reside in the town of Vratza to have easy access to services (modified food, medical care) made available to registered BEN patients. Many undergo regular dialysis. For these patients we abstracted age, sex, year of diagnosis, and village of residence at the time of diagnosis.

Data abstraction and analysis

For each individual identified in a registry, we recorded the year of first registration, age at registration, and year of death. Sex was inferred from the individual's name; in Bulgaria this is unambiguous.

We considered cases entered into the registry in 1964 as prevalent, as no information is available concerning the year of first diagnosis. We considered cases registered in subsequent years to be incident in that year. Population

count data (without age and gender distribution) were available for 21 of the 22 villages with registries, and for the town of Vratza, for the years 1963-1967, 1970, 1975, 1976, 1980, 1985, and 1991. Population counts for years in which census data were not available were computed by linear interpolation. Prevalence in 1964, and incidence rates for each year from 1965 to 1987 were computed for each affected village, for all the affected villages of the district taken together, and for the town of Vratza. We also combined the years 1965-1975, and 1976-1987, to provide larger numbers of cases in each of two periods of approximately one decade, to compare trends in annual incidence rates and survival over time. For these periods we computed the average incidence rate by summing the new cases that occurred in the period, and then dividing by the total person-years at risk, calculated as the sum over the years 1964-1987 of the population counts for each year. Survival post registration was calculated as the difference between the recorded date of death and the year of registration, in complete years. Survival was considered censored at 1987.

For cases prevalent in the hospital records in 2000, we computed survival as the time (in complete years) between the year of diagnosis and 2000.

Results

Registries

A total of 1375 unique individuals were listed in the registries. Table 1 provides the population counts for selected years, and cases registered in 1964,

in 1965-1975, and in 1976-1987, for each village and for the town of Vratza. Of these, 405 (29.5%) were prevalent in 1964, 656 (47.7%) were first registered between 1965 and 1975, and 312 (22.7%) were registered between 1976 and 1987. Of the 1375 registered cases, 184 (13.4%) were subsequently considered as questionable diagnoses and deleted. The proportion of subsequently deleted cases decreased over time (19.8% of cases prevalent in 1964, 13.3 of cases incident in 1965-1975, and 4.8% of cases incident in 1976-1987).

The distribution of the cases by age and sex is provided in Table 2. The ratio of women to men was 1.5:1 among cases prevalent in 1964 and incident between 1965 and 1975, and it was closer to 1:1 among cases registered between 1976 and 1987. Age was unknown for 3 cases registered between 1965 and 1975, and for 32 cases registered between 1976 and 1987; among cases with known age, approximately 60% of cases were between 50 and 70 years of age, with little difference between men and women. Cases incident in 1976-1987 were older at registration than cases incident in the earlier period ($p<0.001$). In 1965-1975, 62% were 50 years and older; in 1976-1987, 79% were over 50 years old. Deleted cases did not differ from retained cases with respect to sex and age.

There was considerable variation in prevalence and incidence across the villages (Table 3). Overall prevalence in the affected villages was 6.0 per 1000 in 1964, and incidence rates fluctuated between 0.4 and 1.2 per 1000 from 1965 to 1987, with a clear decreasing trend over time. In the period 1965-1975, the average incidence rate was 0.7 per 1000 per year, and in the period 1976-1987 it

Table 2 - Registered Cases of Balkan Endemic Nephropathy in Vratza District, Bulgaria in 1964, 1965-1975, 1976-1987, and 2000, by sex and age.

	Population-based registers in 22 communities						Vratza District Hospital	
	Prevalent in 1964		Incident 1965-1975		Incident 1976-1987		Prevalent in 2000	
Age at first registration (years)	Male	Female	Male	Female	Male	Female	Male	Female
	n=148	n=257	n=277	n=379	n=150	n=162	n=33	n=81
	%	%	%	%	%	%	%	%
<30	1.4	1.6	0.4	2.1	0.0	0.0	0.0	1.2
30-39	9.5	10.1	6.9	7.7	2.2	0.7	6.1	23.5
40-49	20.3	21.0	24.9	26.9	17.7	20.1	24.3	22.2
50-59	35.1	31.5	30.3	26.9	35.3	34.7	45.5	28.4
60-69	25.7	28.0	27.4	25.9	29.4	32.6	18.2	14.8
70+	8.1	7.8	10.1	9.8	15.4	11.8	6.1	9.9

Age was unknown for 3 women registered 1965-1975 and 18 women registered 1976-1987, and for 14 men registered 1976-1987. Percentages in table were calculated after excluding patients with unknown ages.

Table 3 - Prevalence in 1964, and Incidence Rates (both per 1000 population) 1965-1987, for Cases of BEN, Vratza District, Bulgaria, by Village of Residence.

	Prevalence in 1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1965 to 1975	1975 to 1987
Beli Izvor	57.6	2.2	4.4	9.9	7.0	2.7	6.8	1.7	5.9	7.0	7.5	6.7	3.7	5.3	6.2	3.1	4.0	5.8	0.8	3.5	0.9	0.9	0.9	0.0	5.5	2.4
Bistretz	41.2	1.6	1.6	7.9	4.7	9.2	5.3	4.5	4.4	5.1	2.1	5.7	1.4	2.1	4.1	1.3	2.0	0.7	4.5	1.3	3.8	0.6	0.6	0.0	4.7	2.3
Brusen	8.5	1.8	3.6	1.8	0.0	3.6	1.8	1.8	0.0	0.0	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0
Darmantzi	5.6	0.0	1.5	0.0	3.0	1.5	3.0	0.0	0.0	1.5	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	1.9	1.9	0.0	0.0	1.0	0.4
Goliamo Babino	4.7	1.3	2.5	0.0	0.0	0.0	5.6	0.0	1.4	0.0	0.0	0.0	3.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.4
Goliamo Peshtene	2.3	1.2	3.8	5.3	2.7	0.7	0.7	1.5	2.3	0.0	0.8	2.5	0.0	1.8	0.9	2.9	2.0	1.0	1.1	2.2	0.0	1.2	0.0	0.0	2.0	0.9
Gorno Peshtene	26.7	2.4	5.1	7.9	1.8	0.9	2.0	2.0	3.0	3.1	6.3	4.2	2.2	3.3	3.4	4.7	3.6	3.7	1.3	2.6	4.1	2.8	1.5	1.5	3.5	2.5
Hubavene	54.9	10.0	3.9	28.5	4.2	2.9	7.5	3.0	0.0	2.9	8.8	1.5	7.5	4.6	6.2	3.2	8.1	5.0	1.7	3.5	7.2	1.8	0.0	0.0	6.8	3.9

Table 3 - (cont'd)

	Prevalent cases in 1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1965 to 1975	1975 to 1987
Kalen	48.4	0.0	2.0	12.7	2.2	6.8	2.3	2.4	0.0	0.0	5.6	0.0	3.0	3.2	0.0	0.0	3.9	3.9	0.0	0.0	3.8	0.0	0.0	0.0	3.2	1.1
Karash	26.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.8	0.0	0.0	0.0	0.0	0.0	0.0	17.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	1.0
Kravoder	3.3	0.5	1.0	2.5	2.6	0.0	0.0	2.2	1.1	0.0	0.5	1.1	1.1	0.0	1.2	0.6	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	1.1	0.3
Kunino	0.0	0.0	0.0	0.0	0.0	0.0	3.2	0.0	0.7	0.0	0.7	0.0	0.0	0.0	0.7	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.1
Liliache	0.0	0.8	0.4	0.0	0.0	0.0	1.6	0.0	0.5	0.0	2.0	0.5	0.0	0.0	0.0	0.5	0.6	0.6	0.0	0.5	0.5	0.0	1.0	0.0	0.5	0.3
Mezdra	0.8	0.1	0.4	0.4	0.6	0.4	0.3	0.3	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.0	0.2	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.3	0.1
Pudria	20.5	0.6	0.7	8.3	13.0	0.8	1.6	2.3	3.9	1.5	0.8	3.8	1.6	1.6	1.6	0.8	1.7	1.8	0.9	0.0	0.9	1.9	1.0	0.0	3.4	1.1
Radovene	15.0	1.2	2.5	9.1	1.4	0.0	3.0	0.0	1.5	1.5	3.1	0.0	1.6	1.6	3.3	0.0	0.0	3.5	1.7	0.0	1.8	0.0	3.6	0.0	2.2	1.3
Roman	7.8	2.1	0.7	5.2	2.8	0.0	0.7	0.3	0.3	1.3	0.7	0.6	1.6	1.6	0.0	1.3	1.3	0.6	1.4	0.5	1.2	0.5	0.0	0.0	1.3	1.0
Tsakonitza	72.6	7.6	3.9	20.3	12.7	4.4	4.6	4.9	5.2	8.4	9.0	3.3	0.0	7.0	3.6	0.0	3.9	8.0	8.3	0.0	4.4	9.2	4.7	0.0	7.9	2.7

Table 3 (cont'd)

	Prevalent cases in 1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1965 to 1975	1975 to 1987
Vesletz	0.0	2.1	4.2	0.0	0.0	0.0	6.8	0.0	0.0	5.4	2.9	0.0	6.2	3.1	0.0	0.0	0.0	0.0	0.0	4.1	8.9	0.0	4.9	0.0	1.9	1.6
Vlasatitza	0.0	1.4	2.7	0.0	0.0	0.0	4.3	0.0	0.0	3.0	1.5	0.0	3.2	1.6	0.0	0.0	0.0	0.0	0.0	1.9	3.9	0.0	2.1	0.0	1.2	0.9
All villages	12.3	1.2	1.5	4.2	2.4	1.1	1.8	1.0	1.4	1.3	1.5	1.2	1.0	1.0	1.0	0.8	1.0	0.8	0.7	0.6	0.9	0.5	0.3	0.0	1.7	0.8
Vratza	0.4	0.3	0.2	0.1	0.0	0.0	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1
All registers	6.0	0.7	0.7	1.9	1.0	0.4	0.7	0.4	0.6	0.5	0.6	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.1	0.1	0.0	0.7	0.3

Incidence rates could not be calculated for Tishevitza due to absence of population estimates

had decreased to 0.3 per 1000 per year (rate ratio 0.43; $p<0.001$). Incidence was much lower in Vratza town; among village residents, the period-specific rates were 1.7 and 0.8 per 1000 per year, respectively (rate ratio 0.47; $p<0.01$).

Dates of death were noted for 83.2% of the 1191 cases retained in the registries. The distribution of times to death following registration is displayed in Figure 4. Among cases prevalent in 1964 median time to death was 3.0 years, among cases incident in 1965-1975 it was 2.0 years, and among cases incident in 1976-1987 it was 5.0 years (log-rank test, 2 d.f., $p<0.001$). Survival patterns did not differ notably between men and women.

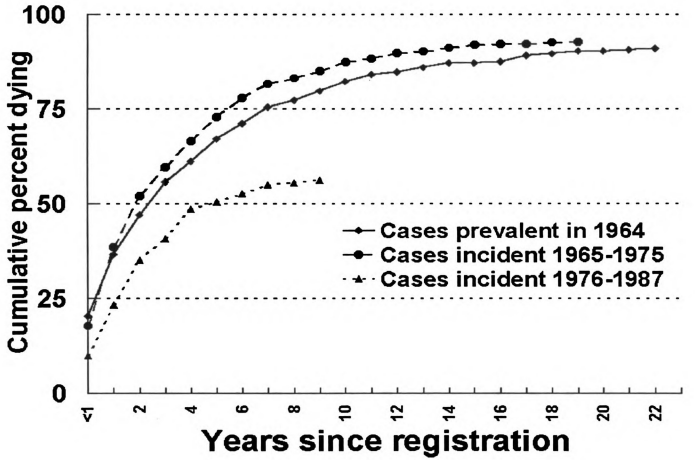
Prevalent Hospital Cases

The age-sex distribution of the 114 patients receiving care at the District Hospital is provided in Table 2. There were 2.5 times as many women as men. As a group, they were younger than the patients included in the registries. The median period since diagnosis was 15 years. Among 59 prevalent hospital cases diagnosed prior to 1987 and living in a village with a BEN registry at the time of diagnosis, only 8 could be matched to individuals listed in the respective registry.

Discussion

Our data suggest that the incidence of BEN declined by 50% between 1965-1975 and 1976-1987 in Vratza District, Bulgaria. If true, our findings

Figure 4 – Cumulative Density Function of Time to Death Following Registration as BEN Patient, Vratza District, Bulgaria



suggest that the epidemic of this disease, which is thought to have appeared in the late 1940's and was first described as a separate clinical entity in this region in 1956 (5), may have peaked sometime in the late 1960's and incidence has decreased since then.

Our data are consistent with those of Cukuranovic et al. (19), who studied a region of Yugoslavia during the period 1987-1997. There have been few other rigorous epidemiologic studies of BEN; most authors appear to cite incidence and prevalence data from the 1970's. Much of the focus in recent years has been the attempt to relate patterns of disease to selected environmental factors, usually at an ecological or geographical level.

Our study is limited by constraints of the data available to us. BEN is an elusive condition, with imprecise diagnostic criteria. Several other conditions, including hypertension and diabetes mellitus, also predispose to renal failure, so careful workup of suspected cases is required to establish the diagnosis. The registries contain no clinical data, therefore no retrospective confirmation of diagnoses is possible, either for cases deleted from the registries or those retained. However, all registries were maintained in one district hospital, which provided services to the whole region. It is therefore likely that diagnostic criteria remained consistent for this surveillance period.

It is possible that the reduced incidence that we observed resulted from reduced intensity of case finding during the more recent period. We lack any data to address this concern directly. We are concerned that we were unable to match in the registries a majority of the patients receiving care at the District Hospital in

2000 who should have been registered. This suggests that these individuals were not ascertained via village-based surveillance, and that the various ascertainment methods may not have been cross-referenced. It is also possible that the cases prevalent in 2000 represent a different form of the disease, given their very long survival and the marked preponderance of women in the prevalent series. Arguing against a deterioration of the surveillance system is our observation that the annual update was maintained consistently for cases who were identified in the villages, with no evidence of increased losses to follow-up. Thus follow-up of registered cases continued with the same rigor. The age of incident cases increased between 1965-1975 and 1976-1987, suggesting that cases were being identified at a later age, consistent with less aggressive case-finding and screening. However, median time to death following registration was longer among cases identified in the later period, arguing that these later cases do not appear to be weighted towards a later stage of the disease, as might be expected if active surveillance were scaled back.

The longer life expectancy for cases diagnosed in the periods 1976-1987, and the long median survival among cases prevalent in 2000 might also be due to the better treatment of BEN (20). A district renal dialysis unit was opened in 1972(17). It might represent a staging effect, as a result of earlier detection. It may also reflect changes in diagnosis, with a higher proportion of severe nephropathies being classified as diseases other than BEN (e.g., end-stage renal disease consequent to diabetes mellitus) in more recent years. It is important to note that any prevalent series will always be biased towards those with longer

survival, and hence true median survival is probably still lower than that observed for the prevalent cases, but we cannot establish the true distribution of survival times with certainty. In the absence of unambiguous diagnostic criteria it is unlikely that the reason for the changes in survival following diagnosis will be easily resolved. In this context, we note the report by Bukvic et al. (21), who found that mean survival in their patients exceeds 10 years, far greater than the 2-3 years reported in earlier descriptions of the disease (17).

Overall, the population in the villages (excluding Vratza) was stable (Table 1). However, it is important to note that the population of the eight villages that had a prevalence of 25 cases (or greater) per 1,000 inhabitants in 1964 (Table 2) lost about 36% of their population over the study period (from n=7,678 to 4,901), while that of Mezdra increased proportionally. This might indicate that a redistribution of the population of BEN-villages occurred (including current and potential future cases); this likely reduced the prevalence and incidence of the disease in the BEN-affected villages. In this regard, the systematic transfer of the population of Karash is noteworthy. The implications of these population transfers to previously BEN-free areas depends on the true underlying etiology. If the cause is related to a localized environmental exposure and indeed out-migration reduces risk, then we would expect incidence in the areas to which these people moved to remain low. If, however, the etiology is viral or genetic, then we might expect to have seen the emergence of BEN in areas previously free of the disease. Surveillance of the out-migrating population is required to address this question thoroughly.

We conclude that the incidence of BEN declined in the late 1970's and 1980's in the endemic region of Bulgaria, while survival of identified cases increased over the same period. Studies designed to identify etiologic factors need to take declining incidence into consideration in order to identify candidate factors and develop studies with adequate power to test hypotheses. There is also a need for additional epidemiological studies, including clinical examination of apparently healthy individuals using objective diagnostic criteria, to increase our confidence that the decrease in incidence is indeed occurring throughout the BEN endemic region.

CHAPTER 2

IS THE INCIDENCE OF BALKAN ENDEMIC NEPHROPATHY DECREASING?

Introduction

For over five decades, the occurrence of Balkan endemic nephropathy (BEN) has been characterized by insufficient evidence of its causes. BEN is a non-inflammatory, slowly progressing, familial, primarily tubulo-interstitial, bilateral renal disease, usually without hypertension, that affects rural populations in several regions in Bulgaria, Romania, Yugoslavia, Bosnia-Herzegovina, and Croatia (17, 22, 23). Despite investigations into the role of many environmental, genetic and immune factors, no definitive risk factors have yet been identified. Nevertheless, there are villages, which have been afflicted for decades, and others situated in the vicinity that have remained free of BEN (16, 17, 23).

In the '90's it was suggested that the incidence of BEN in Yugoslavia is decreasing (22, 23). Findings from a recent epidemiological study from Serbia supported this assumption (19). However, the authors also emphasize that in the '70's and '80's, the morbidity of BEN varied in regions and in time. Additionally, Radovanovic reported, that the onset of the disease has shifted towards older age groups (23).

We used data from the Vratza region in Bulgaria to investigate a potential time trend of the incidence of BEN.

Methods

The sources of study population are presented in Figures 1 and 2. The Vratza district hospital kept two registries of BEN patients from the region. One registry started in 1964 with coverage of all BEN patients identified during screening in the Vratza district that began in 1964. Data was collected until 1987, when the active screening measures stopped. The other registry includes current patients treated at least once in the hospital. In a recent investigation, a team of researchers went to various known BEN villages in the Vratza district and collected information on patients, current and historic, from households that were identified by the mayor, the health service, or neighbours. The abstracted medical information from these three sources (screening, current patients, and patients identified as part of a field study) was entered in electronical files. The three different data sets included information on the full name, the age at first diagnosis of BEN, year of diagnosis, and village. We identified patients who were listed in more than one of the three data sets. Only one record was kept for each patient when estimating the incidence. We restricted our analysis to the eight villages included in the field study (Bistretz, Beli Izvor, Goliamo Peshtene, Gorno Peshtene, Hubavene, Kravoder, Pudria, Vlasatitsa).

Population census data (without age and gender distribution) were available for all eight villages for the years 1963-1967, 1970, 1975, 1976, 1980, 1985, and 1991. Population estimations for years in which census data were not available were computed by linear interpolation. We estimated the crude incidence per year (number of cases / population size) for the eight affected villages of the

district taken together from 1965 to 1987. To investigate underreporting, we combined the years 1965-1969, 1970-1974, 1975-1979, and 1980-1984 to provide larger numbers of cases in each calendar period. After 1984, the number of cases from the incidence registry was too small to analyze the extent to which the different data sources contributed to the number of BEN patients.

Results

For the eight villages with a population of about 9,500, a total of 584 BEN patients were included during the period of 1965 to 1984 (Table 4). Of these, 480 cases were identified in the screening and confirmed as BEN, 28 were additional patients listed in the hospital, and 10 were additionally identified in the field study. In total, the population of the eight villages declined from about 11,000 to about 8,000 (Table 4).

Figure 5 shows the trend in the incidence of BEN over 20 years. After an initial peak between 1966 and 1969, the incidence remains quite stable, with about one case per 1,000 inhabitants.

In the period of 1965-69: 6.0% of the cases were not in the incidence registry (10 out of 166), from 1970-74: 2.2% were not in the registry (3 out of 138), from 1975-79: 5.9% (7 out of 119), and from 1980-84: 18.9% (18 out of 95). The age of the cases at the time they were registered changed over the four calendar periods. Between 1965-69, 67.5% of the cases registered were below 60 years of age when the disease was diagnosed; in 1970-74 the proportion was 68.4%. Then it started to decline—from 1975-79 the proportion was 59.5% and was

Table 4 - Population, Number of BEN Cases and Incidence Rate of BEN in 8 Villages in the Vratza District in Bulgaria

	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976
population	11082	10893	10622	10435	10249	10062	9924	9785	9647	9508	9370	9202
registry cases	17	24	63	32	20	32	22	26	29	26	31	20
hospital cases	1	3	2	1 (1 reg)	0	1	2	0	0	0	0	2
field study cases	0	1	0	2	2 (1 reg)	0	0	0	0	0	2	1
total	18	28	65	34	21	33	24	26	29	26	33	23
incidence rate	1.62	2.57	6.12	3.26	2.05	3.28	2.42	2.66	3.01	2.73	3.52	2.50

Table 4 (cont'd)

	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	total
population	9046	8889	8733	8576	8423	8270	8116	7963	7810	7612	7413	211630
registry cases	22	25	14	20	16	13	13	15	8	4	1	493
hospital cases	1	2 (1 reg)	0	6 (1 reg)	2	4	0	5 (1 reg)	5	3	1	41
field study cases	0	0	0	0	1	1	0	3(1reg 1 hosp)	0	0	1	14
total	23	26	14	25	19	18	13	20	13	7	3	541
incidence rate	2.54	2.92	1.60	2.92	2.26	2.18	1.60	2.51	1.66	0.92	0.40	2.56

Legend reg - case already included in the disease registry
hosp - case already included as hospital case

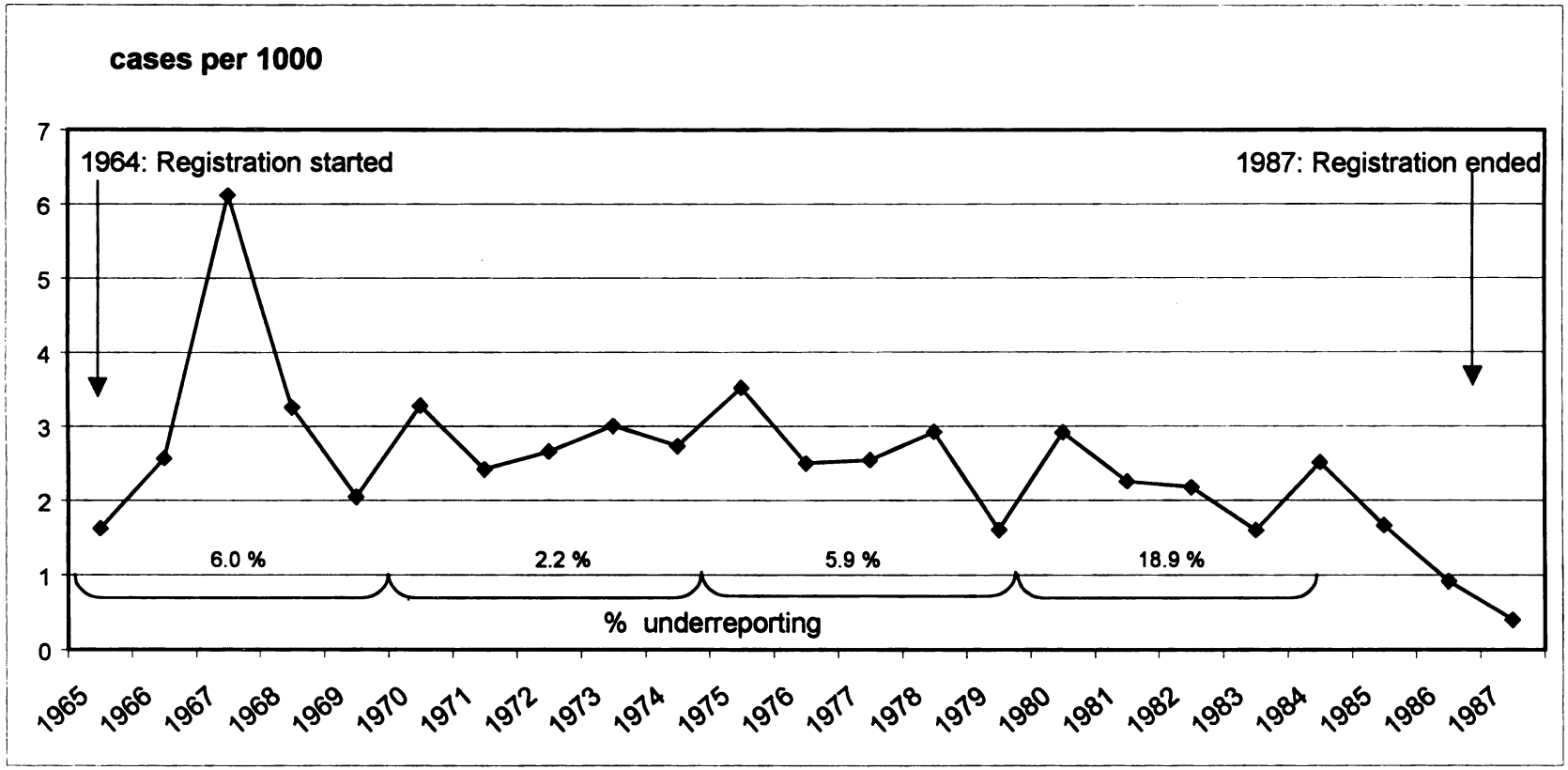
57.6% for the period 1980-84. Of the additional cases found in the lists of hospital-treated patients and in the field study, nearly all (36 out of 38) were younger than 60 years.

Discussion

The data suggest a reduction in the incidence of BEN in eight villages of the Vratza region in Bulgaria. This is in agreement with studies from other countries (19, 22, 23), where BEN is prevalent. However, a more rigorous investigation raises doubts about this conclusion. First, the case identification was less complete in the years after 1979. Of all cases before 1979, less then 6% identified were not recorded in the incidence registry; after 1979 the proportion rose to 19% (Figure 5). Thus, a declining trend may have resulted from declining interest in active screening efforts.

Second, age at diagnosis increased for the registered cases, but not for cases additionally identified. Thus, the age at onset of the disease did not change, but the age of case-identification moved to an older age, probably because cases were not identified at an early stage, but only after they sought medical treatment. These two points indicate that the efforts to screen or to register all cases with BEN were reduced. Our additional data from the hospital list and from the field study are also limited. As BEN has a very distinct characteristic, we assume that it is unlikely that the residents have reported the disease without reason (no false positives). However, it is likely that we missed cases that would

Figure 5 - Incidence of Balkan Endemic Nephropathy from 1965 to 1987 – eight villages in the Vratza district in Bulgaria



have been identified with a more rigorous screening, in particular at an early stage.

Third, the disease poses a mysterious threat for the people living in these villages. Migration seems to be a reaction to keep away from the disease. In one village, Karash, with about 400 people, nearly all inhabitants were relocated to another village near Sofia. Only 32 still resided there in 1991. Therefore, we assume that migration might also have contributed to the decline in the registered cases. Actually, we found four cases of BEN in the lists of hospital-treated patients who were born in so called non-BEN villages and currently resided in Vratza. As a consequence, BEN appears in villages that were previously BEN-free. Thus, the paradigm of scattered BEN villages among non-BEN villages would be challenged.

In summary, our data suggest that after an initial peak, the incidence of Balkan Endemic Nephropathy was stable from 1970 to 1984, with about 1-2 cases per 1,000 residents. We attributed the decrease from 1985 to 1987 to some decline in the interest in record-keeping and active screening. In conclusion, we would recommend a rigorous monitoring of BEN in the afflicted countries before concluding that the incidence of BEN has decreased. It is feasible to employ an active screening with ultrasound in order to detect BEN patients in early stages of the disease.

CHAPTER 3

SURVIVAL TIME AND LIFE EXPECTANCY OF BEN PATIENTS IN VRATZA DISTRICT, BULGARIA. ANALYSIS OF DISEASE REGISTRY AND HOSPITAL RECORDS

Introduction

In 1956 Balkan Endemic Nephropathy (BEN; ICD-10 - N15.0) was first described in Vratza District, Bulgaria by Tanchev. He surveyed the Vratza region in Northwestern Bulgaria and identified patients with a unique kidney disease (5). Similar diseases were described in Yugoslavia in 1957 by Danilovich (24, 25) and in Romania in 1961 by Fortza and Negoescu (26). In 1964 the disease was recognized as a new nosological entity and was named as Balkan Endemic Nephropathy. Clinically BEN manifests as a non-inflammatory, slowly progressing, familial, tubulo-interstitial, bilateral kidney disease, usually without hypertension.

First studies aimed to describe clinical characteristics of BEN and to determine spatial patterns of the disease. It was shown that the disease could be found only in Balkan countries and affects rural populations in a mosaic-like pattern. There are villages, which have been afflicted for decades, and others situated in the vicinity that have remained free of BEN (16, 17, 23). Despite investigations over five decades into the role of many possible causative factors, no definitive risk factors have yet been identified (7-11, 13, 14, 16, 27-36).

Only a limited number of studies report on prevalence and incidence, and we identified only four studies on survival and life expectancy. Additionally, the data cover different calendar periods and are contradictory (4, 17, 21, 22, 37). For these reasons, we investigated the survival time and life expectancy of BEN patients in Vratza District in two time periods, 1965-74 and 1975-84.

Materials and Methods

The sources of study population are presented in Figures 1 and 2. We followed registered patients, prospectively collected from 1964 to 2000. We obtained two different data sources from the Vratza District Hospital, Bulgaria in May 2000. One was a disease registry, which started in 1964 with coverage of all BEN patients identified during the screening program for BEN between 1964-1987. All identified BEN patients in Vratza district were recorded into hand-written registries, with separate patient rosters for each village in which a BEN case had been identified. The available information included full name, age, gender, year of diagnosis, year of death, and for unconfirmed BEN cases, the year of clearance from the records. Each year's record for a village consisted of the list of all living BEN patients (prevalent and newly identified) in the village that year. New BEN cases were added into the registry, as individuals were identified. Deaths and migration of BEN patients were recorded. Individuals with unconfirmed diagnoses were discarded in later years and not all were followed. The registry was updated each year until 1987, when active screening efforts most likely stopped, and no further records were kept.

The second source was patients treated at least once at the Vratza District Hospital (treated prevalence). They were identified in May 2000 from the records of the Department of Nephrology at the Vratza District Hospital. For these patients, we abstracted full name, age, gender, and year of diagnosis. Many of them were currently undergoing regular haemodialysis.

The abstracted medical information from these two sources (registry data, list of current patients) was entered in electronic files. We kept only one record of those patients who were part of the disease registry or were currently being treated in the hospital.

For patients from both data sources, we restricted our analyses to those diagnosed in the period 1965-1984. The year 1964 was the starting point of the registry, and the data included all cases prevalent in that year. Thus, before 1964 the onset of disease was often unknown and therefore, we could not include these patients. Information about new patients was incomplete after 1984. Information on survival was complete within this time-window from the patient registry of the BEN villages and from the list of treated patients.

We calculated the survival time as the period between first diagnosis of the disease and the year of death. For purposes of survival analyses, we right-censored the survival for all patients who were alive at the end of the observation period (2000) or when they reached the age of 85 (38).

In order to investigate whether the survival time after diagnosis has changed, we analyzed two separate groups, one group diagnosed between 1965-1974, the other group diagnosed between 1975-1984.

All statistical analyses were performed using SAS 8.0 (39). We applied the Cox proportional hazards model using the 'proportional hazards regression model' (PHREG) (40, 41). The PHREG procedure estimates survival probabilities and the median time of survival. We controlled statistically for gender, and age at diagnosis. To account for the occurrence of ties, exact maximum likelihood estimates were calculated. The estimated survival probabilities are plotted against years of observation for the different groups separately.

Results

For the period 1964-2000, data for 1481 BEN patient were collected, 880 first diagnosed in the period 1965-1984. In our analyses we excluded 13 patients due to missing age data (Table 5). For the period 1965-1974, we obtained data for 544 confirmed BEN patients. For the period 1975-1984, we extracted information for 323 confirmed BEN patients.

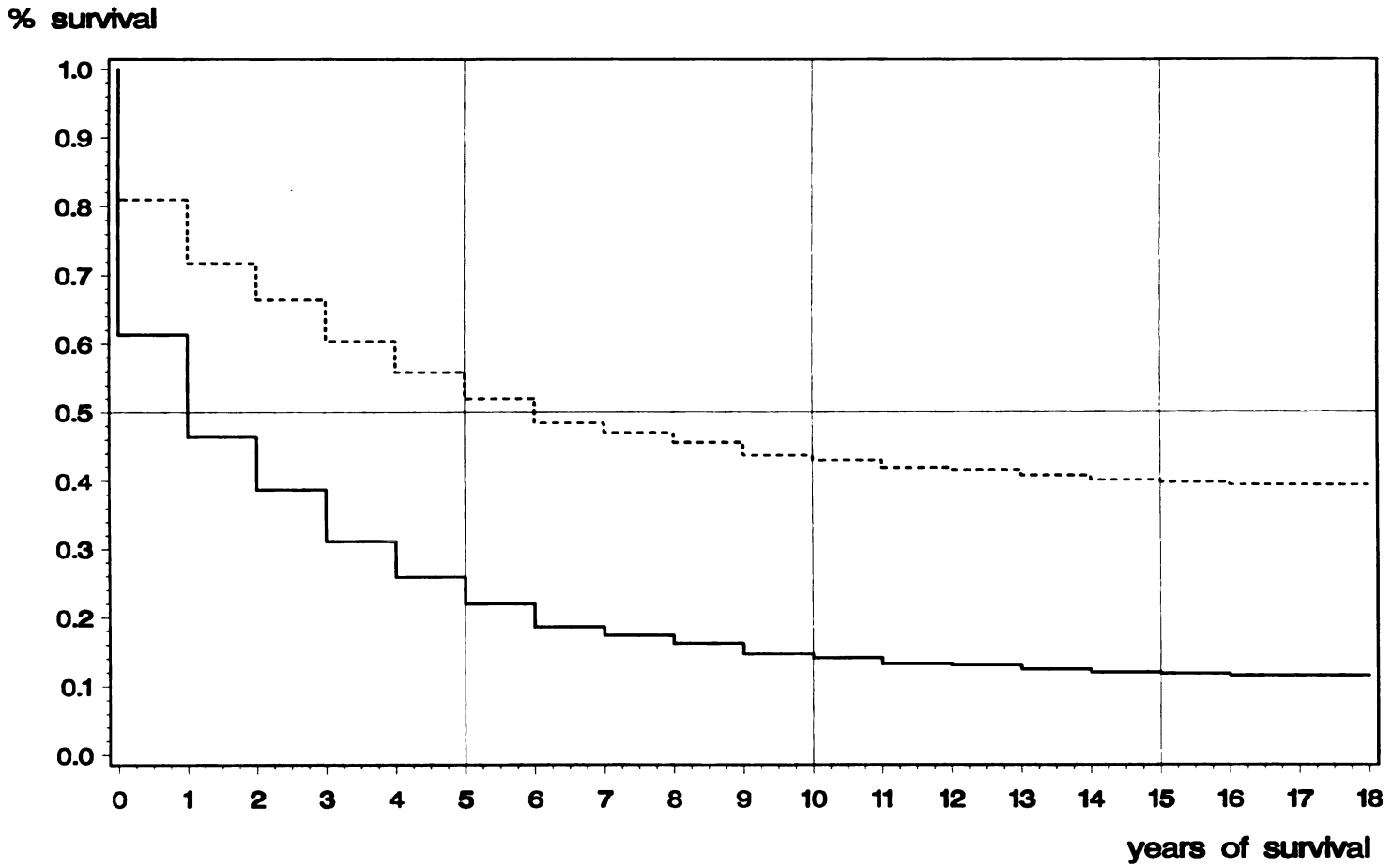
The survival probabilities of the BEN patients diagnosed between 1965-1974 and 1975-1984 are shown in Figure 6. BEN patients diagnosed in the period 1975-1984 have a longer survival after diagnosis.

Table 6 gives the survival probabilities in the two calendar periods. For 1965-1974, 46% survived two years after diagnosis. For the period 1975-1984, we established that 51% survived 6 years after diagnosis. The survival analyses indicated that men had a significantly shorter survival time (Chi-square – 9.82, p=0.0017). Separate analyses for men and women indicate that 57% of the male BEN patients diagnosed between 1965-1974 survived only one year, 41% survived two years. Among women diagnosed in the same period, 50% survived

Table 5 - Description of Population in Vratza District, Bulgaria, Used for Survival Analyses for the Period 1965-1984

	1965-1974			1975-1984		
Age	Total	Men	Women	Total	Men	Women
< 50	192	80	112	57	27	30
50 - 59	146	70	76	110	51	59
60 - 69	143	67	76	111	48	63
>70	63	26	37	45	25	20
Total	544	243	301	323	151	172

Figure 6 - Survival Curves of Confirmed BEN Patients in the Intervals 1965-1974 and 1975-1984



Legend: ——— diagnosed between 1965-74, n=544 - - - - - diagnosed between 1975-84, n=323

Table 6 - Survival Time and Survival Function for Confirmed BEN Patient for the Period 1965-1974 and for the Period 1975-1984

1965-1974						1975-1984					
BEN-all		BEN-men		BEN-women		BEN-all		BEN-men		BEN-women	
Survival time	Survival function	Survival time	Survival function	Survival time	Survival function	Survival time	Survival function	Survival time	Survival function	Survival time	Survival function
0	1.00	0	1.00	0	1.00	0	1.00	0	1.00	0	1.00
0	0.82	0	0.80	0	0.83	0	0.91	0	0.90	0	0.92
1	0.61	1	0.57	1	0.64	1	0.80	1	0.78	1	0.82
2	0.46	2	0.41	2	0.50	2	0.71	2	0.68	2	0.74
3	0.38	3	0.33	3	0.42	3	0.66	3	0.62	3	0.69
4	0.31	4	0.26	4	0.35	4	0.60	4	0.56	4	0.63
5	0.25	5	0.21	5	0.29	5	0.55	5	0.51	5	0.59
6	0.21	6	0.17	6	0.25	6	0.51	6	0.47	6	0.55
7	0.18	7	0.14	7	0.22	7	0.48	7	0.43	7	0.52
8	0.17	8	0.13	8	0.20	8	0.46	8	0.42	8	0.50
9	0.16	9	0.12	9	0.19	9	0.45	9	0.40	9	0.49
10	0.14	10	0.11	10	0.17	10	0.43	10	0.38	10	0.47
11	0.14	11	0.10	11	0.17	11	0.42	11	0.38	11	0.46
12	0.13	12	0.09	12	0.16	12	0.41	12	0.36	12	0.45
13	0.13	13	0.09	13	0.16	13	0.41	13	0.36	13	0.45
14	0.12	14	0.09	14	0.15	14	0.40	14	0.35	14	0.44
15	0.12	15	0.08	15	0.14	15	0.40	15	0.35	15	0.44
16	0.11	16	0.08	16	0.14	16	0.39	16	0.34	16	0.43
18	0.11	18	0.08	18	0.14	18	0.39	18	0.34	18	0.43
19	0.11	19	0.08	19	0.14	19	0.39	19	0.34	19	0.43

two years, and 42% survived three years. For the period 1975-1984 among male BEN patients, 51% survived five years, and 47% survived six years, whereas 50 % of the female patients survived eight years and 49% survived nine years after diagnosis.

Median age at first diagnosis, median survival time, and median life expectancy for confirmed BEN patients based on survival analyses are presented in Table 7. Age at first diagnosis changed slightly between two periods. For the period 1965-1974, the age at first diagnosis was 53.9 (men 54.7, women 53.2), whereas for the period 1975-1984, age at first diagnosis was 56.3 years (men 56.9, women 54.9). Overall, comparing the two calendar periods of first diagnosis, median survival time after diagnosis was augmented from 1.7 years to 6.5 years. For women, the median survival time increased from 2 to 8.1 years, and for men, from 1.8 to 5.2 years. The data show a significantly increased life expectancy from 59 years for the period 1965-1974, to 68.7 years for the period 1975-1984 (Table 7). There are only minor differences in the increase of life expectancy between men and women, respectively from 58.7 to 68.2 and from 59.5 to 69.2.

Discussion

We found an increase in the survival of BEN patients from about 2 to 6.5 years between the calendar periods of 1965-1974 and 1975-1984. The year of first diagnosis did not change in these two calendar periods. The life expectancy in BEN cases increased from 59 years to 68.7 years.

Whenever an increase in survival time after diagnosis is identified, a major

Table 7 - Median Age at Diagnosis, Median Survival Time and Median Life Expectancy, Based on Survival Analyses, Among Confirmed BEN Cases for the Period 1965-1984

Population	Years	Age at Diagnosis	Survival Years	Life Expectancy
Total	1965-1974	53.9	1.7	59.0
	1975-1984	56.3	6.5	68.7
Men	1965-1974	54.7	1.8	58.7
	1975-1984	56.9	5.2	68.2
Women	1965-1974	53.2	2.0	59.5
	1975-1984	54.9	8.1	69.2

concern is lead-time bias. The bias describes that the supposed prolongation is only due to a shift of the diagnosis to a younger age without effective prolongation of life expectancy. Our data, however, indicate an actual prolongation, since the age at diagnosis did not change and the age at time of death occurred later.

Survival of BEN depends on a correct diagnosis. The diagnosis must differentiate BEN from other kidney diseases. The clinical characteristics of BEN include: progressive renal insufficiency without hypertension, normochromic or slightly hypochromic anemia, salt loss, polyuria, polydipsia, or nocturia (42-44). Laboratory markers in urine samples include aseptic leukocyturia, low molecular proteinuria, creatinaemia (45). Usually, urinary sediment is minimal. Several investigations have reported that beta2-microglobulin might be the first protein excreted in increased amounts and is an early sign of BEN (46-48). Hypertension and urinary tract infections are rare, and renal edema is absent. Ultrasound pictures of the kidney display a decrease of the size of both kidneys, even in some cases to the size of a walnut. The disease has well-established features, which also were applied for diagnostic in our study. The main criteria of diagnosis are family history and anamnesis. The disease has a family clustering. Previous diagnosis in another family member is used as one criterion. Another condition is the geographical setting. There are villages afflicted with BEN, and other villages in very close proximity with the same water supply, similar food habits and supply, which are totally free of BEN. If a patient has some signs of kidney disease and the patient is from a BEN village, the diagnosis of BEN is more

likely. We have no indication that these major criteria have changed in the calendar period from 1965 to 1984.

We included data from both registries that were first established in the '60s and from rosters of patients treated in the Vratza district hospital. Inclusion criterion for both data sources was that the patients were first diagnosed either between 1965-74 or between 1975-84. Thus, we avoided a distortion of the estimated survival time due to a loss of follow-up of patients from the registry data or from the selective survival of current patients. Additionally, to avoid problems with reporting at higher ages, we censored all observations at the age of 85.

Only a few studies describe the survival time of BEN patients. Tanchev and Dorosiev (17) reported that 60.2% of BEN patients died within 6 months after the appearance of the first clinical symptoms (diagnosis), 23.3% of patients died within 1 year after diagnosis, and only 4.9% of BEN patients survived two years. Another study by Tanchev et al. showed that more than half of the patients with BEN died within the first 5 years of the onset of disease (37). Ceovic et al (22) found a gradually increasing survival time of BEN patients in Yugoslavia. Patients diagnosed in the period 1957-1960 survived a median time of 0.6 years after diagnosis. For the period 1967-1970, the survival time increased to 1.9 years, for the period 1977-1980 to 3.7 years, and for the period 1987-1990, the survival time reached 5.6 years. A recent study of Bukvic (21) demonstrated a much longer mean survival time of 16.4 years for BEN patients who were diagnosed after 1971.

Compared to the findings of Bukvic and coworkers (21), we detected a shorter

survival time for the recent period (about 6 years, compared to 16.4 years). One explanation for this difference is that Bukvic et al. used the mean survival. This mean value is biased, as the survival is not normally distributed. Additionally, their 50% value in their graphical presentation (13-14 years) is larger than our median survival time. We do not presume that this difference can be explained by a stricter diagnostic in the Vratza hospital, which would have excluded, however, more suspected BEN cases who experienced a better survival period, as shown by Bukvic (21). Bukvic et al. included 67 confirmed cases. Our data is based on 544 confirmed cases from 1965-75 and 323 confirmed cases from 1975-84. The estimated survival times in the other investigation might also differ as the separate investigations included populations with dissimilar occupational or lifestyle characteristics.

There is not sufficient evidence in the literature for a time trend of an increasing survival after BEN. With the exception of Ceovic (22), all other studies covered a similar time period (1965-1985). Nevertheless, our data support the assumption of an increase in the survival period over two decades.

Other investigators have shown that the onset or diagnosis of the disease had shifted to an older age (22, 23). Thus, we expected for the period 1965-1974, that the first diagnosis occurred at a younger age. However, we did not detect a change in the age of first diagnosis. We assume that because there was no change in the age at first diagnosis, there was a substantial increase in the life expectancy of BEN patients.

For the same period, the mean life expectancy for the Bulgarian population has

not changed as much (Table 8). Available data showed a life expectancy for the period 1960-1976 of 70.7 years, and for the period 1974-1986, of 71.2 years (49). Obviously, the increase of the life expectancy of BEN patients was higher than in the total population.

The median life expectancy of BEN patients increased by approximately 10 years for the period 1975-1984 (Table 7). This prolongation might also be attributed to better treatment in the period 1975-1984.

An additional explanation of the prolongation is a change in the severity of the disease. Improvement of living conditions and behavior changes also might have contributed to increased survival time. People from BEN regions became aware of the possibility of contracting the disease, and by changing their lifestyle, perhaps they avoided suspected etiological factors.

In summary, our data showed an increase of survival and life expectancy in BEN patients from 1965-74 to 1975-84. The survival time after diagnosis detected in our patients was longer than the survival times in three earlier publications from Yugoslavia and Bulgaria and shorter than the survival periods reported in a recent publication from Yugoslavia. The increase in survival time and life expectancy of BEN patients needs further explanation. We could gain a better understanding if other studies that addressed the survival issue would have included age at first diagnosis. If the patients in the recent study from Yugoslavia were younger at first diagnosis (earlier detection) then patients could have had a much longer survival period without an increase in life expectancy. Thus, a second requirement is to consider life expectancy in order to exclude that a

Table 8 - Life Expectancy in Bulgarian Population

Interval	All	Male	Female
1935-1939	51.75	50.98	52.56
1956-1957	65.89	64.17	67.65
1960-1963	69.59	67.82	71.35
1969-1971	71.11	68.58	73.86
1974-1976	71.31	68.68	73.91
1978-1980	71.14	68.35	73.55
1984-1986	71.19	68.17	74.44
1989-1991	71.22	68.02	74.66
1991-1993	71.10	67.70	74.70
1993-1995	70.60	67.10	74.90
1994-1996	70.58	67.12	74.62
1995-1998	70.50	67.10	74.30

longer survival time resulted from a lead-time bias (shift of first diagnosis into younger ages without increasing life expectancy). It is critical to gain a better understanding of the natural history of BEN and whether early diagnosis and treatment could affect life expectancy.

CHAPTER 4

OCHRATOXIN A CONTAMINATION AND BEHAVIOUR BIAS IN INDIVIDUALS WITH AND WITHOUT BALKAN ENDEMIC NEPHROPATHY? RESULTS OF A PILOT STUDY

Introduction

For over five decades, the occurrence of Balkan endemic nephropathy (BEN) has been characterized by insufficient evidence of its causes. BEN is a non-inflammatory, slowly progressing, familial, primarily tubulo-interstitial, bilateral renal disease, usually without hypertension, that affects rural populations in several regions in Bulgaria, Romania, Yugoslavia, Bosnia, and Croatia (17, 22, 23). Despite investigations into the role of many environmental, genetic and immune factors, no definitive risk factors have yet been identified. Nevertheless, there are villages which have been afflicted for decades and others situated in the vicinity that have remained free of BEN (16, 17, 23). Even though there is a clear-cut pattern regarding villages, there are BEN-free households in BEN-afflicted villages (17). The majority of the studies, however, were not designed to address risk properly. Some investigations only included case series; other studies focused only on single risks factors (8, 14, 33). For people living in these areas, the disease is still a mystery. To protect themselves, a variety of lay coping responses exist, from avoidance of marriage with inhabitants of BEN villages to changes in food storage and consumption. The latter response may

be related to the mycotoxin hypothesis, which assumes that BEN is caused by Ochratoxin A, ingested in small amounts over long periods by the individuals in the endemic regions. Ochratoxin A is a widespread contaminant in a variety of foods and animal feeds in the endemic areas. NATO has also stressed this putative cause in an effort to protect their servicemen in Bosnia-Herzegovina from BEN (50).

Subjects and Methods

Study population

The sources of the study population are presented in Figure 1.

We conducted a pilot case-control study to assess the risk of ochratoxin A in the Vratza district in Bulgaria. One team identified cases from the Vratza district hospital records. Another team of researchers went to various known BEN and non-BEN villages in the same district and collected food, feed, and other types of samples from BEN and non-BEN households. BEN villages comprise both BEN and non-BEN households. We combined information from both teams and identified eight BEN cases, which were included in both data sets, and 49 controls from both BEN and non-BEN villages. For the current work, we will focus on bean contamination with ochratoxin A based on data of seven BEN cases and 22 controls.

Exposure data and Analysis

Beans were a food that could be sampled from the majority of all households. We were able to sample beans from seven of the eight households with a BEN

patient and from 22 of 49 households of the control group. Participating samplers were trained in advance in the sampling method and protocol. All samples were taken and labelled by samplers wearing polyethylene disposable gloves. Samples were collected in wired, heavy-duty polyethylene bags that were immediately sealed with the wire at the sampling spot and kept at 4^0C for 1-4 days until processed for analysis. To ensure that the samples accurately represented the whole storage stock of beans, sub-samples of 30-50 g were taken from 10 different places of the stock and mixed well. The mixture was subsequently ground in an electric grinder in the laboratory. Ten grams from each ground sample were then taken, extracted and assayed for Ochratoxin A by Veratox Quantitive Ochratoxin Test, based on a competitive direct ELISA assay, following the instructions of the assay kit manufacturer (Neogen Corporation, Lansing, Michigan, U.S.A.).

We included only control households whose inhabitants were 50 years or older, as the age of diagnosis of the patients from the seven BEN households was over 50. Odds-ratios (OR) and their 95% confidence interval (95%CI) of ochratoxin A exposure in BEN cases, compared to controls, were estimated by logistic regressions. We controlled for age and number of years the individual resided in the household from which the bean sample was collected. All statistical analyses were done using SAS (39). In order to investigate the effect controls from different sites might have, we stratified for controls from BEN and non-BEN villages.

Results

We were able to include seven BEN cases and 22 controls with measurements of ochratoxin A in beans (Table 9). The median age in the cases was 67.5 years and 70 in the controls. The median duration of the participants' residence in their current house was 40 years for BEN cases and 53 for controls. Overall, there is a 3.67-fold higher odds of exposure in cases (Table 9). In BEN cases, the median ochratoxin A concentration in beans was 9.6 ppb (min. to max.2.4-24 ppb). When comparing with controls only from BEN-villages, we see a 10.88-fold increased risk with a marginal significance (p=0.07). When controls are taken from non-BEN villages only, there is essentially no association left (OR=1.43, p=0.74). The median ochratoxin A concentration was 3.6 ppb in beans from control households in BEN villages and 7.3 ppb in beans from control households in non-BEN villages.

Discussion

The results of our pilot study suggest that ochratoxin A could falsely be associated with BEN. We assume that some of the residents in BEN villages might have changed their behaviour on how to store foodstuffs, e.g. beans, as a measure to prevent falling ill. However, in non-BEN villages, this approach is missing and thus, comparing cases and controls from these villages did not identify an ochratoxin A-related risk.

The increased odds ratio for ochratoxin A contamination in BEN cases compared with controls from BEN-villages probably represents a behaviour bias (in food

Table 9 - Odds Ratios (OR) and Their 95% Confidence Intervals (CI) for Ochratoxin A Exposure in BEN Cases and Controls, Controlling for Age and the Number of Years Lived in the House.

Ochratoxin A concentration in beans	All BEN cases and controls				All BEN cases and controls from BEN villages				All BEN cases and controls from non-BEN villages			
	case	control	OR	95%CI	case	control	OR	95%CI	case	control	OR	95%CI
< 6 ppb	2	12	3.67	0.53-25.45	2	8	10.88	0.82-144.4	2	4	1.43	0.17-12.01
≥ 6 ppb	5	10			5	2			5	8		

Legend

ppb – parts per billion

storage), not a causal effect (51). Our pilot study shows that, in order to identify risk factors for BEN, we have to pay attention to changes in lifestyle and migration that possibly result from fear of contracting the disease. These findings suggest that in order to avoid effects of behaviour bias, potentially resulting from the mystery of the disease, it is necessary to recruit controls from BEN and non-BEN villages.

DISCUSSION

The first part of our analyses demonstrates that there is a decrease of incidence of Balkan Endemic Nephropathy in Vratza district, Bulgaria (Chapter 1). The incidence declined from 1.7 per 1000 for the period 1965-1975 to 0.8 per 1000 for the period 1976-1987 among village residents. Including data from the town of Vratza town in the analyses, the overall decline is from 0.7 per 1000 to 0.3 per 1000. In the town of Vratza only a small number of cases were recorded, but the town's population is more than twice that of all villages included in the study together.

However, further analysis of the same data questions whether the incidence of BEN is decreasing or is the result of two other phenomena: underreporting of cases and migration (Chapter 2). The percentages of underreported cases over time were as follows: 6.0% for 1965-69, 2.2% for 1970-74, 5.9% for 1975-79, and 18.9% during the period 1980-84. This increase in underreporting can be explained by the decline in case identification resulting from reduced screening efforts. As a result, some cases were identified only after they sought medical treatment.

Migration may also have contributed to the decline in the registered cases, as people move away to avoid the disease in their neighborhood, hence, a redistribution of the population likely reduced the incidence of the disease in the BEN-affected villages.

In the third section (Chapter 3) we found that survival time was in fact

increasing after diagnosis: from 1.7 years in period 1965-1974 to 6.5 years in period 1975-1984. For the same time periods the age at diagnosis changed only slightly from 53.9 to 56.3 years, and the life expectancy increased considerably from 59.0 years to 68.7 years. Therefore we can presume from our data that a lead-time bias is not suspected to produce an increased survival time after diagnosis, since the identified prolongation of survival was not due to a shift of the diagnosis to a younger age without effective prolongation of life expectancy. When studying survival time the inclusion in the analyses the age of first diagnosis is critical to exclude the possibility that a longer survival time resulted from a lead-time bias. The prolongation of survival might be explained by better medical treatment or by changes in disease severity. Improvement of living conditions and behavioral changes in the latter years also may play a role. People from the affected villages being aware of contracting BEN, by changing their lifestyle unintentionally perhaps avoided suspected etiological factors. The increase in survival time and life expectancy of BEN patients needs further explanation. It is important to investigate whether the actual prolongation of survival and life expectancy is due either to better treatment, or to changing of the natural history of BEN.

In section four we demonstrated that ochratoxin A may not necessarily be associated with BEN. The overall odds of exposure is 3.67 fold higher when comparing cases with all controls. However, a comparison of BEN cases with controls recruited from BEN villages gives a 10.88 fold increased risk, in contrast to a 1.43 fold increased risk when controls are taken from non-BEN villages.

Thus, the increased odds ratio for ochratoxin A contamination in BEN cases might represent behavior bias of people living in BEN villages. These findings suggest that to avoid effects of behavior bias, it is necessary to recruit controls from BEN and non-BEN villages.

Relevance

There are no recent BEN studies in the Vratza district and to our knowledge the data we collected have not been previously analyzed using epidemiological methodology. Our results add new information on incidence, prevalence, survival time, and life expectancy of BEN. In terms of possible etiological factors of BEN, the conducted pilot case-control study demonstrated that OTA is unlikely to be a cause of BEN. Therefore our study provides a more comprehensive epidemiological profile of BEN, and we present our results as a basis for future research.

Limitations

The weakness of the study is potentially the lack of completeness of some data. We have identified that a reduced efforts in case diagnosis and a reduction in (or lack of) screening may have contributed to insufficient data recording. Case identification in latter years was less complete. Therefore our results may not represent all BEN cases in the area. We have not been able to identify registry data for the last decade (1990-2000). Consequently, it was not possible to extend

the study and nor to define how the natural history of disease may have changed in the last decade.

Future steps

The epidemiology of Balkan Endemic Nephropathy requires further studis to better define the disease and to establish an appropriate public health intervention. Together with all clinical features of the disease, the most unique characteristic is the shrinkage of kidneys. An active ultrasound screening program in affected areas is needed in order to detect BEN in early stages of the disease. Identifying cases at the earliest disease stages will provide a better understanding of the onset and development of BEN. A rigorous screening program will provide the data to determine whether incidence of BEN is in fact decreasing or stable. By identifying patients in the early stages of disease through screening, the intervention of early treatment will enhance their chances for survival.

In future survival studies, survival and treatment should be monitored simultaneously to determine whether longer survival is due to better treatment, changes in living conditions or to changes in the characteristics of BEN.

Further studies are necessary to gain a better understanding of whether a shift in survival time is due either to treatment, or is the result of changes in behavior and living conditions.

CONCLUSIONS

1. The data suggest a decline of incidence of BEN in all villages in the endemic area of Bulgaria over the period 1964-1987. Overall incidence rate among village residents declined from 1.7 per 1000 in 1965-1975 to 0.8 per 1000 in 1976-1987. By including the town of Vratza in the analyses, incidence decreases from 0.7 per 1000 to 0.3 per 1000. Although Vratza has only a small number of cases, its population is more than twice that of all villages together.

2. Reasons for the decline in incidence may be incomplete case identification, reduced efforts of screening, and diminished public health interest in BEN. The percentage of underreported cases rose from 6.0% for the period 1965-1969 to 18.9% in 1980-1984.

3. Survival and life expectancy increased significantly over the same period, from 1.7 to 6.5 years; as well as life expectancy increased from 59 to 68.7 years. Age at first diagnosis was raised slightly (from 53.9 to 56.3 years), therefore the increase in survival was due to actual prolongation, not lead-time bias.

4. Ochratoxin A could falsely be associated with BEN, due to behavior bias expressed by a modification of the behavior of food storage in villages affected by BEN. The overall odds of exposure to ochratoxin A in BEN cases compared with controls (OR=3.67) decreases when controls are recruited from non-BEN villages (OR=1.43). In contrast, when controls are from BEN villages we detected a much higher risk of exposure (OR=10.88).

BIBLIOGRAPHY

1. Dimitrov PS, Ganev VS, Karmaus WJ, Abouzied MM, Petropoulos EA. Ochartoxin A Contamination and Behaviour Bias in Individuals with and without Balkan Endemic Nephropathy? Results of a Pilot Study. Occup Environ Med 2001 February;Submitted for publication.

2. Dimitrov PS, Simeonov VA, Stein AD. Balkan Endemic Nephropathy in Vratza, Bulgaria, 1964-1987: an epidemiologic analysis of population-based disease registers. Eur J Epidemiol 2001 January;Submitted for publication.

3. Dimitrov PS, Karmaus WJ. Survival time and life expectancy of Balkan Endemic Nephropathy patients in Vratza district, Bulgaria. Analysis of disease registry and hospital records. Nephron 2001 March;Submitted for publication.

4. Dimitrov PS, Simeonov VA, Ganev VS, Karmaus WJJ. Is incience of Balkan Endemic Nephropathy Decreasing? Pathol Biol 2001 March;Accepted for publication.

5. Tanchev Y, Evstatiev Z, Dorossiev D, Pencheva J, Zvetkov G. Studies on the nephritides in the District of Vratza. Savremenna Medicina 1956;7:14-29.

6. Krogh P. Role of ochratoxin in disease causation. Food Chem Toxicol 1992;30:213-24.

7. Krogh P, Hald B, Plestina R, Ceovic S. Balkan (endemic) nephropathy and foodborn ochratoxin A: preliminary results of a survey of foodstuffs. Acta Pathol Microbiol Scand [B] 1977;85:238-40.

8. Vrabcheva T, Usleber E, Dietrich R, Martlbauer E. Co-occurrence of ochratoxin A and citrinin in cereals from Bulgarian villages with a history of Balkan endemic nephropathy. J Agric Food Chem 2000;48:2483-8.

9. Tatu CA, Orem WH, Finkelman RB, Feder GL. The etiology of Balkan endemic nephropathy: still more questions than answers. Environ Health Perspect 1998;106:689-700.

10. Wedeen RP. Environmental renal disease: lead, cadmium and Balkan endemic nephropathy. Kidney Int Suppl 1991;34:S4-8.

11. Maksimovic ZJ. Selenium deficiency and Balkan endemic nephropathy. Kidney Int Suppl 1991;34:S12-4.

12. Cosyns JP, Jadoul M, Squifflet JP, De Plaen JF, Ferluga D, van Ypersele de Strihou C. Chinese herbs nephropathy: a clue to Balkan endemic nephropathy? Kidney Int 1994;45:1680-8.

13. Uzelac-Keserovic B, Vasic D, Ikonomovski J, Bojanic N, Apostolov K. Isolation of a coronavirus from urinary tract tumours of endemic Balkan nephropathy patients [letter]. Nephron 2000;86:93-4.

14. Toncheva D, Dimitrov T. Genetic predisposition to Balkan endemic nephropathy. Nephron 1996;72:564-9.

15. Toncheva D, Dimitrov T, Stojanova S. Etiology of Balkan endemic nephropathy: a multifactorial disease? Eur J Epidemiol 1998;14:389-94.

16. Ganev V, Petropoulos E. Balkan Endemic Nephropathy and its Putative Environmental and Genetic Causes: A Review. In: Lekkas T, ed. 6th International Conference of Environmental Science and Technology. Pythagorion, Samos, Greece: University of Aegen, 1999:11-21.

17. Tanchev Y, Dorossiev D. The first clinical description of Balkan endemic nephropathy (1956) and its validity 35 years later. In: Castenagro M, Plestina R, Dirheimer G, Chernozemsky IN, Bartasch H, eds. Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. Lyon: International Agency for Research on Cancer, 1991:21-8.

18. Chernozemsky IN. Balkan endemic nephropathy and the associated tumours of the urinary system: A summary of epidemiological features in Bulgaria. In: Castenagro M, Plestina R, Dirheimer G, Chernozemsky IN, Bartasch H, eds. Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. Lyon: International Agency for Research on Cancer, 1991:3-4.

19. Cukuranovic R, Petrovic B, Cukuranovic Z, Stefanovic V. Balkan endemic nephropathy: a decreasing incidence of the disease. Pathol Biol (Paris) 2000;48:558-61.

20. Stefanovic V, Radenkovic S, Cukuranovic R, Kostic S. Balkan endemic nephropathy. Slowed progression of kidney disease by avoidance of etiological factors [letter]. Nephron 1999;83:85-6.

21. Bukvic D, Jankovic S, Dukanovic L, Marinkovic J. Survival of balkan endemic nephropathy patients [In Process Citation]. Nephron 2000;86:463-6.

22. Ceovic S, Plestina R, Miletic-Medved M. Epidemiological aspects of Balkan endemic nephropathy in a tipical focus in Yugoslavia. In: Castenagro M, Plestina R, Dirheimer G, Chernozemsky IN, Bartasch H, eds. Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. Lyon: International Agency for Research on Cancer, 1991:5-10.

23. Radovanovic Z. Epidemiological characteristics of Balkan endemic nephropathy in Eastern regions in Yugoslavia. In: Castenagro M, Plestina R, Dirheimer G, Chernozemsky IN, Bartasch H, eds. Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. Lyon: International Agency for Research on Cancer, 1991:11-20.

24. Danilovic V, Djurisic M, Mokranjac M, Stojimirovic B, Zivojinovic J, Stojakovic P. Chronic nephritis caused by poisoning with lead via the digestive tract (flour). Presse Med 1957;65:2039-2040.

25. Danilovic V, Djurisic M, Mokranjac M, Stojimirovic B, Zivojinovic J, Stojakovic P. Porodicna oboljenia bubrega u selu Sopic izvazvana hronicnom intoksikacijom olovom. Srpski Arh Tcelok Lek 1957;85:1115-1125.

26. Fortza N, Negoescu M. Nefrita cronica azotemia endo-epidemica. Stud Cercet Med 1961;1:217-221.

27. Petkova-Bocharova T, Castenagro M, Michelon J, Maru V. Ocharatoxin A and other mycotoxins in cerelias from an area of Balkan endemic nephropathy and urinary tract tumors in Bulgaria. In: M. Castenagro RP, G.Dirheimer, I.Chernozemsky, H.Bartsch, ed. Mycotoxins, Endemic Nephropathy and Urinary Tract Tumors. Lyon: International Agency for Research on Cancer, 1991:83-87.

28. Petkova-Bocharova T, Castenagro M. Ochratoxin A in human blood in relation to Balkan endemic nephropathy and urinary tract tumours in Bulgaria. In: M.Castenagro RP, G.Dirheomer, I.Chernozemsky, H.Bartsch, ed. Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. Lyon: International Agency for Research on Cancer, 1991:135-137.

29. Fuchs R, Hult K. Ochratoxin A in blood and its pharmacokinetic properties. Food Chem Toxicol 1992;30:201-4.

30. Stoev SD. The role of ochratoxin A as a possible cause of Balkan endemic nephropathy and its risk evaluation. Vet Hum Toxicol 1998;40:352-60.

31. Mihailovic M, Lindberg P, Jovanovic I, Antic D. Selenium status of patients with Balkan endemic nephropathy. Biol Trace Elem Res 1992;33:71-7.

32. Feder GL, Radovanovic Z, Finkelman RB. Relationship between weathered coal deposits and the etiology of Balkan endemic nephropathy. Kidney Int Suppl 1991;0:S9-S11.

33. Orem WH, Feder GL, Finkelman RB. A possible link between Balkan endemic nephropathy and the leaching of toxic organic compounds from Pilocene lignite by groundwater: preliminary investigation. International Journal of Coal Geology 1999;40:237-252.

34. Tatu CA, Orem WH, Feder GL, et al. Additional support for the role of the pilocene lignite derived organic compounds in the etiology of Balkan endemic nephropathy. J Med Biochem 2000;4:95-101.

35. Mitic-Zlatkovic M, Cukuranovic R, Lecic N, Stefanovic V. Urinary creatinine excretion in children from families with Balkan endemic nephropathy: evidence for genetic predisposition to the disease. Pathol Biol (Paris) 2000;48:554-7.

36. Ceovic S, Hrabar A, Radonic M. An etiological approach to Balkan endemic nephropathy based on the investigation of two genetically different populations. Nephron 1985;40:175-9.

37. Tanchev I, Naidenov D, Chepel N, Nankov M. [Possibility for studying the life expectancy of patients with Balkan endemic nephropathy by dispensary observation]. Vutr Boles 1983;22:57-63.

38. Lee ET. Statistical Methods for Survival Data Analysis. Belmont, California: Lifetime Learning Publications, 1980.

39. SAS Institute. SAS/STAT Software Inc.,. Cary, NC: SAS Institute Inc., 2000.

40. SAS Institute Inc. SAS Technical Report P-229. Cary, NC: SAS Institute Inc., 1992.

41. Cox DR. Regression models and life-tables. J R Stat Soc 1972;Series B:187-220.

42. Stefanovic V, Polenakovic MH. Balkan nephropathy. Kidney disease beyond the Balkans? [editorial]. Am J Nephrol 1991;11:1-11.

43. Polenakovic M, Stefanovic V. Balkan nephropathy. In: Davidson A, Cameron J, Grunfeld J, Kerr D, Ritz E, Winearl C, eds. Oxford texbook of clinical nephrology. Oxford - New York - Tokyo: Oxford University Press, 1998:1203-1210.

44. Radonic M, Radosevic Z. Clinical features of Balkan endemic nephropathy. Food Chem Toxicol 1992;30:189-92.

45. Plestina R. Some features of Balkan endemic nephropathy. Food Chem Toxicol 1992;30:177-81.

46. Hrabar A, Aleraj B, Ceovic S, Cvoriscec D, Vacca C, Hall PW. A fifteen year cohort based evaluation of beta 2-microglobulin as an early sign of Balkan endemic nephropathy. Kidney Int Suppl 1991;34:S41-3.

47. Hrabar A, Aleraj B, Ceovic S, Cvoriscec D, Vacca C, Hall PW. Beta 2-microglobulin studies in endemic Balkan nephropathy. Kidney Int Suppl 1991;34:S38-40.

48. Stefanovic V, Mitic-Zlatkovic M, Cukuranovic R, Miljkovic P, Pavlovic NM, Vlahovic P. Beta 2-microglobulin in patients with Balkan nephropathy and in healthy members of their families. Kidney Int Suppl 1991;34:S21-6.

49. National Center of Health Information B. Statistical Information. Mean life expectancy in Bulgarian population., 2000.

50. Atkins JL. Balkan Nephropathy. [Online] Available: http://www.wramc.amedd.army.mil/departments/medicine/nephrology/education/lectures/balkan/sld002.htm, 1998.

51. Weinberg CR, Baird DD, Wilcox AJ. Sources of bias in studies of time to pregnancy. Stat Med 1994;13:671-81.