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High-performance liquid chromatographic determination of sphinganine and sphingosine in serum and urine of subjects from an endemic nephropathy area in Croatia

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Abstract

Endemic nephropathy (EN) is a chronic renal disease present as an endemic in Brodska Posavina, Croatia. The aim of the study was to assess the possible role of fumonisins, i.e., mycotoxins produced by *Fusarium moniliforme*, as causative agents for EN. Fumonisins inhibit ceramide synthase, the enzyme of de novo synthesis of sphingolipids, which leads to an increase in the sphinganine/sphingosine ratio. In the present study, a modified method has been used for the determination of the sphinganine/sphingosine ratio in human serum and urine of healthy subjects and EN patients from the endemic area. Free sphingoid bases, sphinganine and sphingosine, were obtained by base hydrolysis. Afterwards, precolumn *ortho*-phthaldialdehyde derivatisation, HPLC separation and quantification by fluorescence detection were performed. The results thus obtained pointed to a sphingolipid metabolism impairment, which may have been induced by fumonisins or fumonisin-like mycotoxins. As statistically significant differences were recorded in the subjects not yet affected with EN, an impairment in the metabolism of sphingolipids might be considered as an early indicator of EN. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Sphinganine; Sphingosine

1. Introduction

Endemic nephropathy (EN) is a chronic renal disease geographically restricted to a number of locations in Croatia, Bulgaria, Romania, Yugoslavia, and Bosnia and Herzegovina. In Croatia, the endemic area is Brodska Posavina. The disease was first described in the 1950s [1]; however, there is evidence it had been present before that time. Only rural populations are affected, and the disease shows a tendency of familial clustering; however, there is no correlation with other known genetic diseases. The villages known for their inhabitants to have been affected with EN in the past are still involved, while the inhabitants of non-endemic villages and towns, some of them in close neighborhood to the endemic villages, have been consistently spared. Only those

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living in an endemic village for more than 10 years are affected, irrespective of their sex, ethnic or religious affiliation [2]. EN patients have a higher prevalence of upper urinary tract tumors than the subjects from the endemic area not affected with EN, which is consistent with data on the populations from other countries with EN endemic areas [3,4].

EN is a progressive renal disease that ultimately leads to kidney damage. The disease develops without an acute phase and has slow progression. Kidney lesion follows the occurrence of nonspecific symptoms. (Clinical characteristics have been described in [2].) Tubular transport is reduced, and proteinuria ensues, usually very mild and accompanied by the presence of very low-molecular mass protein [5]. Morphopathologically, EN is relatively nonspecific. In the advanced stage of the disease, the kidneys become symmetrically diminished, weighing ≤50 g. Histological studies have revealed marked fibrotic changes of the interstitium, while the glomeruli are spared [2].

In spite of numerous investigations, the etiology of EN remains obscure. As the symptoms and pathology of EN are similar in all endemic areas, there is a strong belief that the cause of the disease must be of environmental origin. A number of hypotheses on the disease etiology have been proposed (e.g., streptococci and other bacteria; viruses; metals and nonmetals from the soil; drinking water and foods; genetic factors; etc.). Recently, the hypothesis on mycotoxins as the possible causes of EN has prevailed. According to this hypothesis, mycotoxins produced by molds from mildewy cereals and other foods (beans, meat, etc.) are the environmental factors that are most likely to play a role in the etiology of EN. One of the main 'suspect' mycotoxins is ochratoxin A [6], mainly produced by molds of the genera *Penicillium* and *Aspergillus*. The present study was focused on the concepts on the effect of fumonisins, i.e., mycotoxins produced by the mold *Fusarium moniliforme* and other related molds [7], on the metabolism of sphingolipids. Fumonisins include a group of related compounds structurally similar to sphingosine (Fig. 1), the basic component of all sphingolipids. Sphingolipids are a group of lipids found in the membrane of all eukaryotic cells. They have many biological functions, e.g., they play an important role in the maintenance of membrane structure [8]; they act upon growth factor receptors [9]; they serve as binding sites for some microorganisms, toxins and viruses [10,11]; and some of them play the role of second messengers. Fumonisins are specific inhibitors of de novo synthesis of sphingolipids [12], compounds having so-called long-chain bases in common. In mammals, sphinganine (Sa) and sphingosine (So) are most common bases. The primary target of fumonisine in animal cells is sphingosine (So) [sphinganine (Sa) *N*-acyltransferase], (ceramide synthase), the key enzyme in de novo biosynthesis of sphingolipids [12]. The inhibition of ceramide synthase leads to an increase in the cellular concentration of sphinganine, and occasionally although less pronounced, of sphingosine (So), resulting in an increased Sa/So ratio. As long-chain bases easily diffuse through the cellular membrane, the portion accumulated in the cells may leave them and migrate to the blood and urine. As these changes occur before other biochemi-

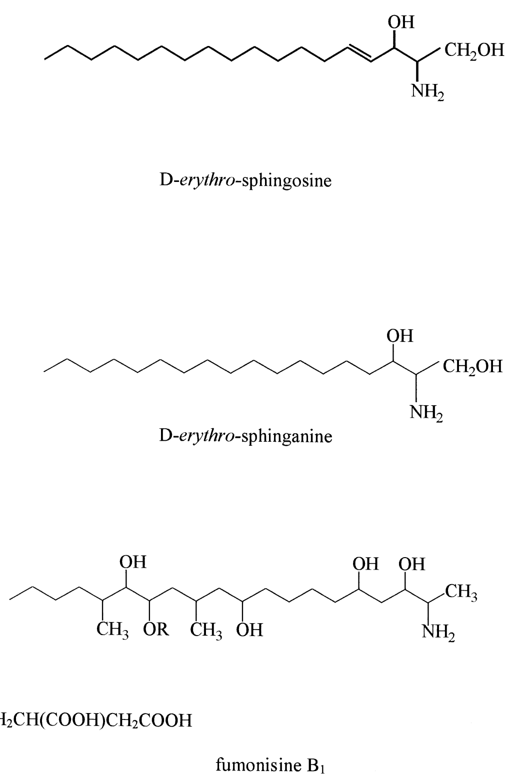


Fig. 1. The structures of sphingosine, sphinganine and fumonisine B_1.

cal indicators of cytotoxicity, the Sa/So ratio has been suggested as a potentially useful indicator of fumonisine exposure [13]. Direct measurement of fumonisins in biological fluids does not provide a good indicator of their intake into the body, because these mycotoxins are cleared from the body soon after ingestion [14].

This paper reports for the first time measurements of the Sa/So ratio in serum and urine of healthy subjects and EN patients from the endemic area. Generally, literature offers little data on sphingoid base contents in human serum [15,16] and urine [16,17]. There are more data about sphingoid bases in serum, urine and tissues of animals [18–20]. The determination of the Sa/So ratio, has generally been based on the original method published for determination of free sphingosine in liver tissue by HPLC [21]. In this work, the sphinganine/sphingosine ratio in human serum and urine was determined by HPLC according to the method of Riley et al. [13] with minor modifications. The method involved lipid extraction with chloroform/methanol, base hydrolysis to cleave acylglycerolipids and to hydrolyse lysosphingolipids and determination of the sphingoid bases by HPLC of their *o*-phthaldialdehyde (OPA) derivatives using fluorescence detection.

As there is a correlation between the body intake of fumonisins, and amount and ratio of sphingoid bases in body fluids [13,22], the aim of the present study was to determine the possible modifications in the concentrations of sphinganine and sphingosine as well as in the sphinganine/sphingosine ratio in the serum and urine of healthy subjects and EN patients from the EN endemic area in Croatia, i.e., whether fumonisins could be considered as a cause of EN.

2. Experimental

2.1. Chemicals

Standards of sphingoid bases (C_{18} D-sphingosine and C_{18}-DL-*erythro*-dihydrosphingosine), *ortho*-phthaldialdehyde (OPA), and 2-mercaptoethanol were purchased from Sigma–Aldrich Chemie (Steinheim, Germany); $CHCl_3$ (p.a.) and CH_3OH (HPLC gradient grade) were purchased from J.T. Baker, Mallinckrodt Baker (Deventer, The Netherlands); CH_3OH (Pa), used for extraction, was purchased from Riedel de Hähn (Seelze, Germany); NH_4OH, KOH and HCl were purchased from Kemika (Zagreb, Croatia); and C20 sphinganine was a gift from Professor A.H. Merrill (Emory University, Atlanta, GA, USA).

2.2. Samples

Sphingolipid analysis was performed on 89 serum samples and 30 urine samples obtained from men and women living in the EN area (Kaniza, Croatia), with the following diagnoses:

- affected (A): an individual with proteinuria, anemia and elevated serum creatinine, with no signs of another renal disease;
- suspected (S): (a) an individual with two of the three laboratory findings positive, with no signs of another renal disease; (b) an individual with one of the three laboratory findings positive and positive family history;
- at risk (R): a healthy individual from a family with present or past EN history; and
- healthy (H): an individual with no positive laboratory findings and history.

2.3. Determination of Sa and So in serum and urine

Sphingolipids were extracted from serum and urine, whereafter base hydrolysis was performed, according to the method of Riley et al. [13] with minor modifications.

2.3.1. Extraction procedures

To 0.5 ml of serum sample, 2.25 ml of a mixture containing $CHCl_3$:CH_3OH (1:2, v/v) and 0.150 ml of 2 N NH_4OH solution are added, and the content is thoroughly mixed on a vortex. Then the mixture is incubated at 37°C for 1 h in a closed screw tube and cooled down to room temperature; then, 1.5 ml $CHCl_3$ and 3 ml alkaline water (0.1 ml 2 N NH_4OH+250 ml distilled deionized water, pH 8.0–10.0, prepared daily) are added and centrifuged for 20 min at 3000 rpm for phase separation. Upon centrifugation, the upper aqueous phase is discarded,

and 3 ml alkaline water are added to the lower chloroform phase; the content is mixed and centrifuged. Rinsing of the chloroform phase with alkaline water is repeated once, whereafter the aqueous phase is removed, and the chloroform phase is evaporated to dryness. Thus prepared, the sample can be stored overnight in nitrogen at +4°C or proceed with base hydrolysis.

2.3.2. Base hydrolysis

The purpose of the base hydrolysis is to cleave acylglycerolipids and hydrolyse lysosphingolipids (free sphingoid bases modified at hydroxyl group on carbon 1 of sphinganine or sphingosine) to release free sphinganine and sphingosine. The phospholipids (phosphatidylethanolamine and phosphatidylserine) that may react with OPA reagent for sphingosine base derivation are successfully eliminated by the base treatment [21]. This procedure does not release sphingoid bases from complex sphingolipids.

A dry extract is dissolved in 1 ml of 0.1 *M* methanol KOH:$CHCl_3$ (4:1, v/v) mixture and placed for 1 min in ultrasonic bath, whereafter it is incubated at 37°C for 1 h. The mixture is cooled down, and 1 ml $CHCl_3$ and 1 ml alkaline water are added, gently mixed and centrifuged for 20 min at 3000 rpm; then the upper aqueous phase is discarded, and the lower chloroform phase is washed with 2 ml alkaline water, centrifuged again for 10 min at 3000 rpm, whereafter the upper aqueous phase is removed, and the chloroform phase is evaporated to dryness, and stored at −20°C until analysis.

The starting serum volume for base hydrolysis was 0.5 ml, while the starting urine volume for sphingolipid extraction was 10 ml because of low levels of sphinganine in male urine. In this case, the sample was frozen and lyophilised in an evaporator with a refrigerating unit, and 0.5 ml distilled water were added before extraction.

2.4. Chromatographic analysis

2.4.1. Instruments

The following equipment manufactured by Perkin-Elmer (Norwalk, CT, USA) was used for the procedure of high-performance liquid chromatography (HPLC): isocratic pump with a single ram (Series 10, liquid chromatograph); fluorescent detector (model LC 240), (λ_{ex}=334 nm, λ_{em}=440 nm); interface (series 900); column oven (series 200); and chromatography software (Turbochrom 4.1.2). For HPLC, the following equipment was used: reversed-phase analytical column (Radial-Pak™ cartridge, Nova-Pak™ C_{18}, 10×0.8 cm, 4 μm); column module (RCM 8×10) and holder with precolumn filter (Guard-Pak assembly, Nova-Pak C_{18}, 4 μm), purchased from Waters (Milford, MS, USA); manual injector (model 7125) and injector loop of 50 μl, Rheodyne (Cotati, CA, USA); and syringe for loop filling with the sample of 500 μl, from Hamilton Supelco (Bellefonte, USA). The instruments for solvent filtration for HPLC (Millipore, Bedford, USA) were used.

2.4.2. Derivatization

Before HPLC analysis, a dry sample extract was derivatised with OPA reagent. OPA reagent was prepared according to Riley et al. [13]: 5 mg reagent and 5 μl 2-mercaptoethanol were dissolved in 0.1 ml ethanol and made up to 10 ml with a borate buffer (pH adjusted to 10.5 by 1 *M* KOH). Thus prepared reagent remains stable for 7 days if stored in the dark at +4°C.

2.4.3. Sample preparation

The sample for HPLC was prepared by the addition of 250 μl mobile phase to a dry extract (obtained by base hydrolysis) and mixed for 1 min on a vortex. Then 50 μl OPA reagent were added and the sample was mixed on a vortex for 30 s, filtered by centrifugation through a filter with 0.45-μm pore size (1 min), and left for 1 h at room temperature before injection. The injected sample volume was 50 μl.

2.4.4. Standard mixture

The standard mixture for HPLC was prepared by mixing 5 μl of 10 μ*M* solutions of C_{18} D-sphingosine, C_{18} DL-*erythro*-dihydrosphingosine and C:20 sphinganine each, adding 485 μl mobile phase, then adding 100 μl OPA reagent. The mixture was well mixed and left for 20–30 min at room temperature. Then the mixture was filtered by centrifugation in cuvettes with a filter of 0.45-μm pore size for 1 min, and injected.

2.4.5. Chromatography conditions

A mixture of CH_3OH:H_2O (9:1, v/v), filtered by use of nitrocellulose filter paper, 0.45-μm pore size, and degassed by 3- to 5-min helium passage, was used as a mobile phase. The flow-rate was 2 ml/min. The column oven temperature was 30°C. The identity of individual sphingosid bases in the sample was determined by comparing the sample chromatogram with the standard mixture chromatogram. Sphinganine and sphingosine were quantified by measuring the peak area using the Turbochrom 4.1.2. chromatography software.

2.5. Statistical analysis

Statistical analysis of the results was performed by use of the Statistica program, StatSoftInc, ver. 5. The following tests were used: (1) Kolmogorov–Smirnov test (test for normality); (2) *t*-test (testing a difference between the means of two independent samples); (3) ANOVA (analysis of variance); (4) post-hoc Scheffe test; and (5) Mann–Whitney *U*-Wilcoxon rank sum test [23].

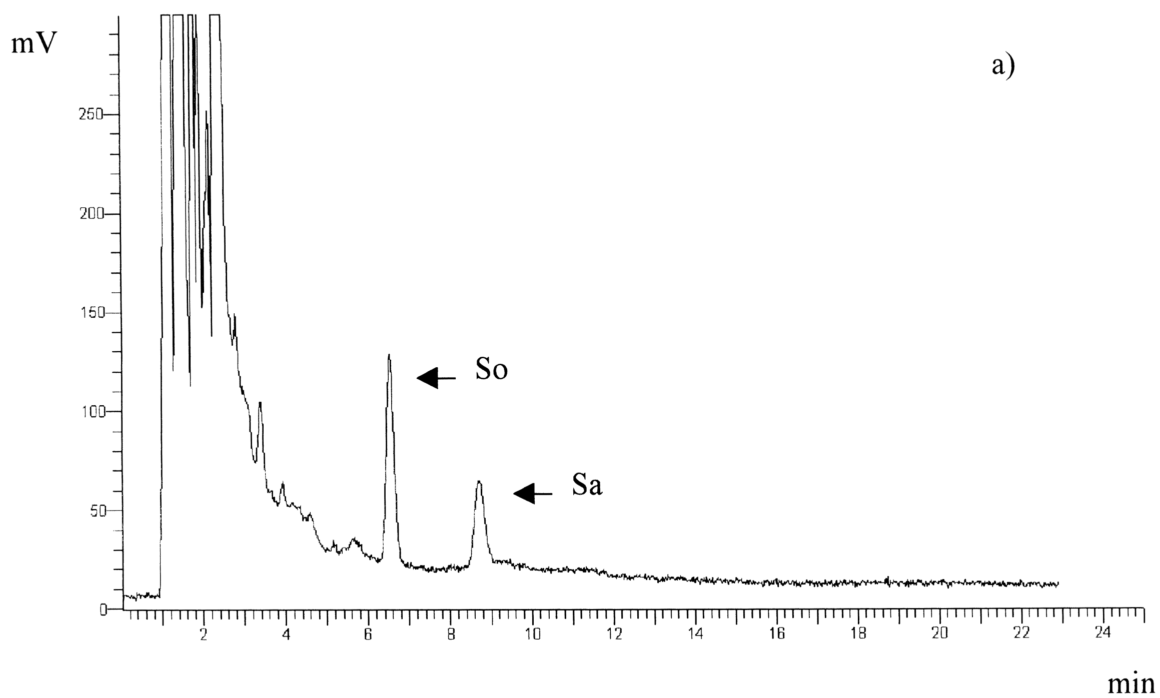
3. Results and discussion

Some animal and human diseases have been related to mycotoxins produced by the molds found as contaminants on cereals, especially corn. The *Fusarium moniliforme* mold is the most widely spread corn contaminant all over the world. This mold produces a group of mycotoxins, fumonisins, which are structurally similar to sphingosine, the basic component of sphingolipids. As fumonisins lead to the accumulation of free Sa, a precursor of So, by inhibition of de novo biosynthesis of sphingolipids, it has been proposed that an increased Sa/So ratio might be used as an early biomarker of fumonisine exposure [13]. Studies have revealed the Sa/So ratio to considerably increase prior to any other serum and renal biochemical marker in pigs given feed containing 5 ppm fumonisin [24]. The sensitivity of Sa/So ratio as a biomarker for fumonisins was studied in various animal species (rats, pigs, horses, rabbits, poultry) [12,13,25,26]. Determination of Sa/So ratio in the serum, lungs, liver and kidneys following fumonisine exposure showed the kidneys to be the most sensitive organ [13].

The aim of the present study was to investigate whether the concentrations of Sa and So, and Sa/So ratio were modified in the people living in the EN endemic area, including both unaffected individuals and EN patients. Serum and urine concentrations of Sa and So, and Sa/So ratio were determined (Fig. 2). The values obtained were compared with those recorded in healthy subjects from a nonendemic area (control group, S. Ribar, unpublished results). Stimulated by literature data pointing to a correlation between dietary intake of fumonisins, and the amount and ratio of sphingoid bases in body fluids [13,15,17,19,27], we embarked upon this study to investigate the possible association of EN with fumonisins or fumonisin-like mycotoxins. The results obtained in the study pointed to statistically significant differences (ANOVA, $P<0.05$) in serum concentrations of So in men from the group of healthy individuals (H), and of Sa in the groups of subjects at risk (R) and suspected individuals (S) (Table 1). In the women from the endemic area, statistically significant differences were observed in the concentrations of Sa and So in the groups of subjects at risk (R) and suspected individuals (S) (Table 2). There was no statistically significant difference in the serum Sa/So ratio in either men or women from the endemic area as compared with the control group of subjects (Tables 1 and 2).

Urine concentrations of Sa and So, recorded in men and women from the endemic area were also compared with the respective values found in the control group of subjects from a nonendemic area. Statistically significant differences were observed for urinary Sa concentration in men from the groups of healthy (H), suspected (S) and affected (A) individuals (Table 3). The Sa/So ratio differ significantly in the groups of healthy (H), suspected (S) and affected (A) men (Table 3). There was no statistically significant difference in urinary Sa and So concentrations between the women from the endemic area and those from a nonendemic area (Table 4). Urinary Sa/So ratio was found to differ statistically significantly only in the women from the groups of subjects at risk (R) and suspected individuals (S) (Table 4).

The results of the study showed the concentrations



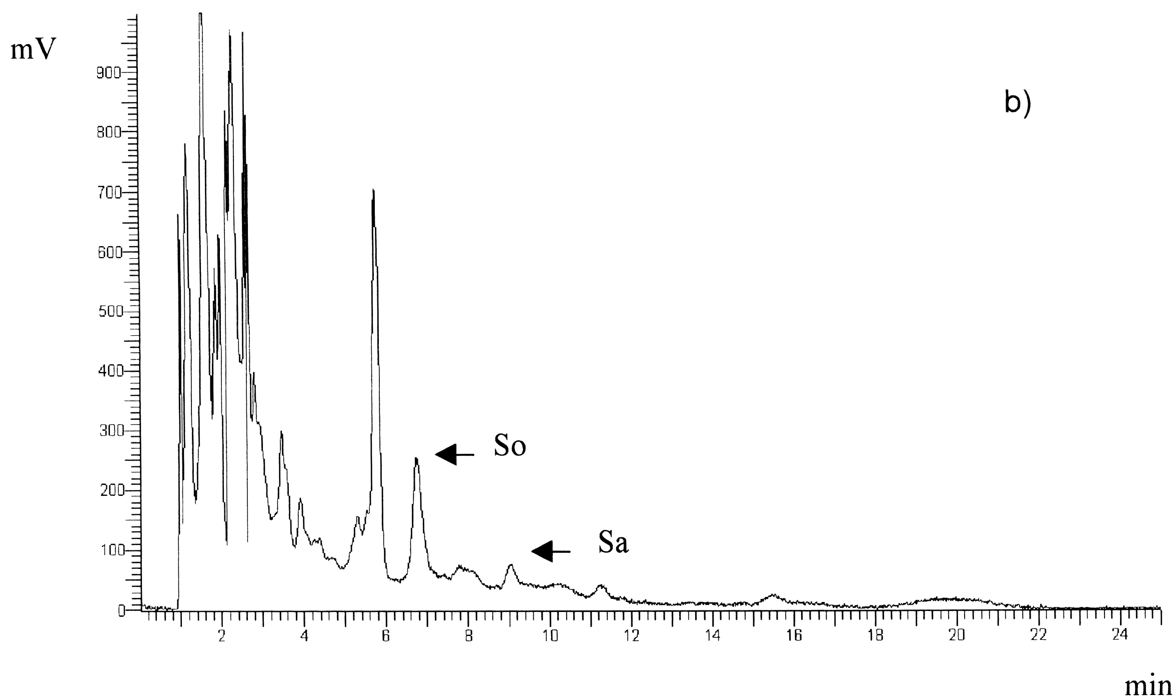


Fig. 2. (a) HPLC chromatogram of free sphingoid bases in serum. (b) HPLC chromatogram of free sphingoid bases in urine. So, sphingosine; Sa, sphinganine.

Table 1
The concentrations of free sphingosine (So), sphinganine (So) and Sa/So ratio in serum samples of men from the endemic nephropathy area in Croatia in comparison with healthy men from the non-endemic area[a]

Group of subjects	Sphingosine (pmol/ml) $\bar{X}\pm$SD (range)	Sphinganine (pmol/ml) $\bar{X}\pm$SD (range)	Sa/So $\bar{X}\pm$SD (range)
Healthy ($n=27$)	22.20±10.70* (4.00–52.00)	4.70±2.50 (1.00–12.00)	0.24±0.15 (0.09–0.81)
At risk ($n=12$)	16.70±9.30 (4.00–39.00)	5.70±2.10* (1.00–9.00)	0.36±0.11 (0.15–0.53)
Suspected ($n=7$)	14.10±7.20 (4.00–24.00)	5.10±3.80* (1.00–12.00)	0.33±0.17 (0.09–0.55)
Affected ($n=1$)	29.00	3.00	0.10
Control ($n=20$)	9.80±4.00 (4.00–19.00)	2.80±2.00 (1.00–8.00)	0.29±0.12 (0.14–0.57)

[a] SD, standard deviation; $\bar{X}$, mean.
*Statistically significant difference ($P<0.05$).

of Sa and So, and the Sa/So ratio to be increased in the serum and urine of subjects living in the EN endemic area. The elevated levels of Sa and So and changed Sa/So ratio point to a sphingolipid metabolism impairment. In the light of literature data pointing to Sa/So ratio as an early indicator of fumonisin intake [13,28], our study subjects could be presumed to have been exposed to fumonisins. However, such a presumption is hampered by the lack of information on food contamination with fumonisins. If not induced by fumonisins, the changes observed may have been caused by some other mycotoxin or environmental factor. As statistically significant differences were mostly recorded in the subjects from the families with present or past history of EN (at risk, R), or in those with two of three laboratory findings positive and with no signs of another renal disease, and in the subjects with one positive laboratory finding and positive family history (suspected, S), a sphingolipid metabolism im-

Table 2
The concentrations of free sphingosine (So), sphinganine (So) and Sa/So ratio in serum samples of women from the endemic nephropathy area in Croatia in comparison with healthy women from the non-endemic area[a]

Group of subjects	Sphingosine (pmol/ml) $\bar{X}\pm$SD (range)	Sphinganine (pmol/ml) $\bar{X}\pm$SD (range)	Sa/So $\bar{X}\pm$SD (range)
Healthy ($n=21$)	19.60±10.70 (2.00–46.00)	5.50±3.70 (2.00–18.00)	0.40±0.52 (0.11–1.10)
At risk ($n=11$)	25.90±17.50* (10.00–74.00)	6.40±4.50* (1.00–17.00)	0.25±0.13 (0.11–0.47)
Suspected ($n=9$)	20.70±6.70* (13.00–34.00)	6.10±4.50* (3.00–16.00)	0.30±0.21 (0.17–0.84)
Affected ($n=1$)	9.00	6.00	0.67
Control ($n=20$)	8.60±3.00 (2.00–15.00)	2.50±1.00 (1.00–5.00)	0.31±0.13 (0.13–0.60)

[a] SD, standard deviation; $\bar{X}$=mean.
*Statistically significant difference ($P<0.05$).

Table 3
The concentrations of free sphingosine (So), sphinganine (So) and Sa/So ratio in urine samples of men from the endemic nephropathy area in Croatia in comparison with healthy men from the non-endemic area[a]

Group of subjects	Sphingosine (pmol/ml) $\bar{X}$±SD (range)	Sphinganine (pmol/ml) $\bar{X}$±SD (range)	Sa/So $\bar{X}$±SD (range)
Healthy ($n=5$)	0.58±0.02 (0.57–0.60)	1.29±0.07* (1.23–1.36)	2.22±0.06* (2.16–2.26)
At risk ($n=1$)	0.20	0.14	0.70
Suspected ($n=5$)	0.26±0.12 (0.17–0.40)	0.21±0.07* (0.13–0.26)	0.97±0.56* (0.33–1.39)
Affected ($n=4$)	0.75±0.40 (0.17–1.07)	0.72±0.12* (0.66–0.90)	1.60±1.59* (0.62–3.95)
Control ($n=20$)	0.46±0.29 (0.10–0.94)	0.06±0.05 (0.02–0.20)	0.18±0.12 (0.04–0.49)

[a] SD, standard deviation; $\bar{X}$, mean.
*Statistically significant difference ($P<0.05$).

Table 4
The concentrations of free sphingosine (So), sphinganine (So) and Sa/So ratio in urine samples of women from the endemic nephropathy area in Croatia in comparison with healthy women from the non-endemic area[a]

Group of subjects	Sphingosine (pmol/ml) $\bar{X}$±SD (range)	Sphinganine (pmol/ml) $\bar{X}$±SD (range)	Sa/So $\bar{X}$±SD (range)
At risk ($n=5$)	2.30±2.79 (0.33–4.27)	0.97±1.16 (0.16–1.79)	0.44±0.03* (0.42–0.47)
Suspected ($n=5$)	2.59±1.15 (0.88–3.40)	1.75±1.02 (0.56–2.90)	0.66±0.17* (0.44–0.85)
Affected ($n=5$)	2.58±1.59 (0.40–4.27)	0.32±0.25 (0.07–0.66)	0.14±0.08 (0.03–0.25)
Control ($n=20$)	17.18±13.84 (0.87–41.77)	3.60±3.08 (0.20–11.58)	0.25±0.08 (0.16–0.46)

[a] SD, standard deviation; $\bar{X}$, mean.
*Statistically significant difference ($P<0.05$).

pairment could be postulated as an early indicator of EN. To confirm the above hypothesis, analysis of sphingoid bases should be repeated in the same individuals, accompanied by thorough information on their present health state.

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