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Life Sciences, Vol. 58, No. 8, pp. 691-699, 1996

0024-3205/96 $15.00 + .00

0024-3205(95)02338-0

INCREASE OF BLOOD NAD+ AND ATTENUATION OF LACTACIDEMIA DURING NICOTINAMIDE TREATMENT OF A PATIENT WITH THE MELAS SYNDROME

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(Received in final form December 7, 1995)

Summary

Decreased activity of complex I (NAD:ubiquinone oxidoreductase) is the most frequent biochemical finding associated with the mutation at the base pair 3243 of the mitochondrial DNA. The mutation has been previously shown to lead to a defective translation. We hypothesized that due to an imperfect assembly of complex I subunits the substrate affinity of this enzyme may be lowered and this may be counteracted by increasing the mitochondrial NAD+NADH concentration. Therefore, we studied the effect and mechanism of action of nicotinamide treatment in a MELAS patient with the base pair 3243 mutation. Nicotinamide treatment was initiated after his first stroke-like episode. The blood NAD concentration (representing the intracellular concentration in erythrocytes) increased linearly being 24-fold at 6 weeks of treatment. Blood lactate and pyruvate concentration decreased by 50 % within three days and 24 h urine lactate content within 2 weeks and we observed a clinical improvement together with a decrease in the lesion volume in magnetic resonance imaging within the first month. The cellular NAD increase upon nicotinamide administration was probably universal, because it occurred in a time and dose-dependent manner in cultured fibroblasts from both the patient and the controls. Alleviation of the lactate accumulation during the nicotinamide treatment suggests that an increase in the cellular NAD+NADH concentration leads to enhancement of the oxidation of reducing equivalents. However, the Km of complex I for NADH in skeletal muscle from the patient was similar to that of controls. This may indicate that physiologically mitochondrial complex I operates at non-saturating substrate concentration, and this may explain the effect of nicotinamide treatment.

Key Words: **mitochondrial encephalomyopathy, point mutation, complex 1 activity, skin fibroblast culture, lactate, vitamin E therapy**

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MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) is one of the clinically defined mitochondrial encephalomyopathies (1-3). Several different mutations in the mitochondrial gene coding for the $tRNA^{Leu(UUR)}$ have been characterized in cases of MELAS (4), the most common mutation being found at the base pair 3243 of mtDNA. This mutation leads to a decreased rate of mitochondrial translation, decreased levels of the translation products and increased level of 19S RNA (16S ribosomal RNA + $tRNA^{Leu(UUR)}$ + ND1) (5). This impaired translation appears to affect specifically the large subunits of the mitochondrial respiratory complexes I, III and IV. Although ribosomal dysfunction is expected to lead to a generalized impairment of translation, a decrease in complex I activity is the most prevalent finding among patients with MELAS (6). Altogether seven of the 41 subunits of complex I are coded by the mitochondrial genome (7).

Complex I (NADH:ubiquinone oxidoreductase) is the first enzyme complex of the mitochondrial respiratory chain. It accepts electrons from NADH and passes them to ubiquinone (coenzyme Q). Impairment of mitochondrial reoxidation of NADH inhibits pyruvate oxidation and functioning of the substrate shuttles for reoxidation of cytosolic NADH, resulting in lactate accumulation (8).

At least two rationales based on the function of complex I have been used in the treatment of MELAS. One uses the electron acceptor site and involves administration of ubiquinone (or its analogue), and the other uses the NADH oxidation site and involves administration of nicotinamide and riboflavin. Ubiquinone and idebenone (ubiquinone analog) have been used in one patient with improvement in clinical and neurophysiological parameters (9). In another patient, treatment with nicotinamide and riboflavin has been reported to result in improvement in clinical condition, muscle metabolism and nerve conduction velocity (10). In experimental animals administration of a large dose of nicotinamide results in a several fold increase in tissue NAD concentration in 16 hours (11,12). We hypothesized that the decrease in complex I activity in patients with the MELAS syndrome is due to a defective enzyme with decreased affinity to its substrate NADH. If nicotinamide treatment increases the total cellular concentration of NAD+NADH, it would also increase the substrate availability to the enzyme. An open-label treatment with peroral nicotinamide was initiated to a patient with MELAS in the course of his first stroke-like episode. Prospective follow-up demonstrated a favourable clinical and biochemical effect but the fatal outcome of the disease could not be prevented.

Case Report

The patient was a 27-year-old man, who came to our attention because of an unprovoked generalized epileptic seizure. The combination of epilepsy with symmetric basal ganglia calcifications, sensorineural hearing loss and a slight elevation in blood glucose suggested the diagnosis of MELAS that was further confirmed by muscle histology and elevated blood and CSF lactate. Carbamazepin was started for epilepsy. Three months later he experienced a sudden onset of visual problem and a right homonymous hemianopia was observed. Magnetic resonance imaging (MRI) demonstrated a left occipitoparietal signal change. Nine days later his neurological condition progressed and a mild right hemiparesis, sensory dysphasia and a slightly reduced state of consciousness were noted with concomitant increase in the MRI-observed lesion. Two weeks after the onset of the stroke-like episode peroral nicotinamide was started at a dose of 1 g 4 times daily. After 1 month the preparation was changed from suspension to capsules keeping the dosage unchanged. At 4 months of treatment he began to experience episodic headaches and within 1 week onset of left homonymous hemianopia. A new lesion in

the right occipital area was verified by MRI. At 5 months of treatment he suffered from a severe headache for 12 h that was followed by cardiopulmonary arrest refractory to resuscitation.

Methods

Nicotinamide treatment. Peroral nicotinamide 1 g 4 times daily was initiated at 2 weeks after the onset of the stroke-like episode. Blood lactate and pyruvate, 24 h urine lactate and clinical status (Scandinavian Stroke Scale, SSS) were followed, as well as CSF lactate and volume of the MRI-detectable lesion (1 Tesla, Magnetom; Siemens, Germany). T2-weighted 3 mm transaxial slices were used in the volumetric measurements that were carried out on a computer workstation. Blood samples were also obtained for NAD determination.

Molecular biology. Total DNA was extracted from blood, frozen skeletal muscle and frozen autopsy tissue samples by the standard SDS-proteinase K method. A 399-bp fragment was amplified by polymerase chain reaction (PCR) using primers corresponding to the nt positions 3153 to 3172 for the light strand and 3551 to 3531 for the heavy strand. The A to G transition at nt 3243 was detected by the presence of an additional site of Apa I (13) and confirmed by direct sequencing of the PCR product (ΔTaq Cycle-Sequencing, United States Biochemical). The ratio of mutant to normal DNA was ascertained by digesting the ^{35}S-CTP-labelled fragment with the restriction enzyme ApaI and performing electrophoresis through a 6% acrylamide gel. The gels were dried and autoradiographed at -72°C overnight using Kodak XAR-5 film with an intensifying screen. The films were analyzed with a Bioimage scanner and image-processing apparatus (Millipore; Ann Arbor, MI). The obtained percentage of mutant genome out of total was corrected for heteroduplex formation (14).

Mitochondrial oxidations and complex I activity. Mitochondria were isolated from a fresh biopsy of skeletal muscle according to Makinen and Lee (15). Mitochondrial respiratory chain activities and respiratory control were determined by oximetry in a sealed thermostated chamber fitted with an YSI 5775 oxygen electrode (Yellow Springs Instruments, Yellow Spring, OH). Complex I activity was measured in freeze-thawed, sonicated mitochondria using NADH as the electron donor and ubiquinone$_1$ as the electron acceptor (16). Substrate affinity of the enzyme was determined by analyzing the complete progress curve of the reaction (17).

Cell culture. Fibroblast culture was established from a skin biopsy specimen obtained from the lower leg. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum, 290 μg/ml of glutamic acid, 50 μg/ml of ascorbic acid, 100 U/ml of penicillin and 100 μg/ml of streptomycin (18). The cultures were used in passages 3-6. The cells were grown for variable time periods with varying concentrations of nicotinamide after which the total NAD was determined.

Measurement of NAD and metabolites. NAD and NADH were extracted from the blood samples with perchloric acid and potassium hydroxide, respectively, and measured by enzymatic spectrophotometric methods (19,20). NAD in the fibroblast cultures was determined by enzymatic cycling according to the principles described by Lowry and Passonneau (21) using glutamate dehydrogenase and lactate dehydrogenase (LDH) for cycling and LDH for the indicator reaction. Venous blood and CSF lactate and venous blood pyruvate were determined by a conventional enzymatic method.

Results

Effect of nicotinamide on lactate and NAD levels. The total time of nicotinamide treatment was 5 months. Nicotinamide treatment was initiated two weeks after the onset of the first stroke-like episode. Blood lactate and pyruvate declined rapidly during the first days of treatment reaching a 40 % and 50 % reduction at day 3 of treatment, respectively (Fig 1A). The amount of lactate in 24 h urine declined to 50 % in 2 weeks (Fig 1B). The CSF lactate was 7.03 mmol/l before treatment, 5.62 mmol/l at 2 weeks and 6.75 mmol/l at 4 weeks of treatment (reference interval below 2.8 mmol/l).

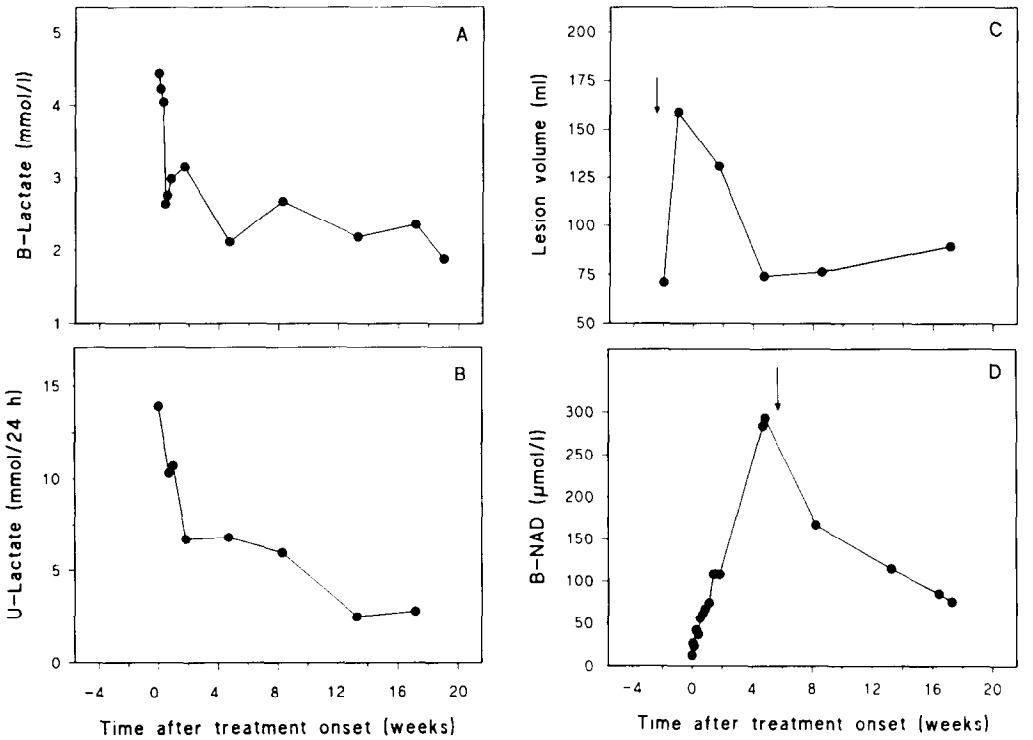

Fig. 1.
The course of nicotinamide treatment in a patient with MELAS after his first stroke-like episode. The onset of the stroke-like episode was at time point -2 weeks as indicated by the arrow in panel C. Treatment with peroral nicotinamide was initiated at time point 0 weeks. (A) The concentration of lactate in blood (reference interval 1.0 - 1.8 mmol/l). (B) The amount of lactate in 24 h urine sample (reference interval below 2.6 mmol/24 h). (C) The volume of the brain stroke-like lesion in MRI images. (D) The concentration of nicotinamide dinucleotides in blood (values in control subjects 3.73 ± 1.81 µmol/l, mean ± SD). The change in the form of the medication from suspension to capsules is indicated by arrow.

The SSS score was lowest at the time of the start of the medication being 46. At two weeks of treatment the score was 51 and improved up to 54 by the first month of treatment. The volume of the MRI-detectable lesion decreased from 159 ml to 74 ml during the first month of treatment (Fig 1C). Blood NAD increased linearly during the first month of treatment and was 24-fold compared to the initial value and 32-fold compared to the value in the control subjects (Fig 1D). The NADH content was below the detection limit of the assay. The NAD content in the cells, therefore, reflects the total NAD+NADH pool, as the NAD content in the cells is three orders of magnitude higher than the NADH content (22).

After 1 month of treatment there was a trend for a further decline in the lactate in blood and 24 h urine, while no change in the volume of the MRI-detectable lesion or clinical status was observed. Blood NAD started to decline sharply coinciding with the change of the form of medication in an exponential fashion (r = 0.997 for the disappearance curve) with a disappearance half-life of 22 days (Fig 1D).

Molecular biology and biochemistry of tissue samples. Heteroplasmic digestion site of ApaI was demonstrated in the blood and muscle of the patient. The proportion of the mutant genome was 38 % in blood and 86 % in muscle. Samples of the brain were obtained at autopsy, and the proportion of the mutant genome was 89 % in two samples of the cerebral cortex, 85 % in the cerebellum and 83 % in frontal white matter.

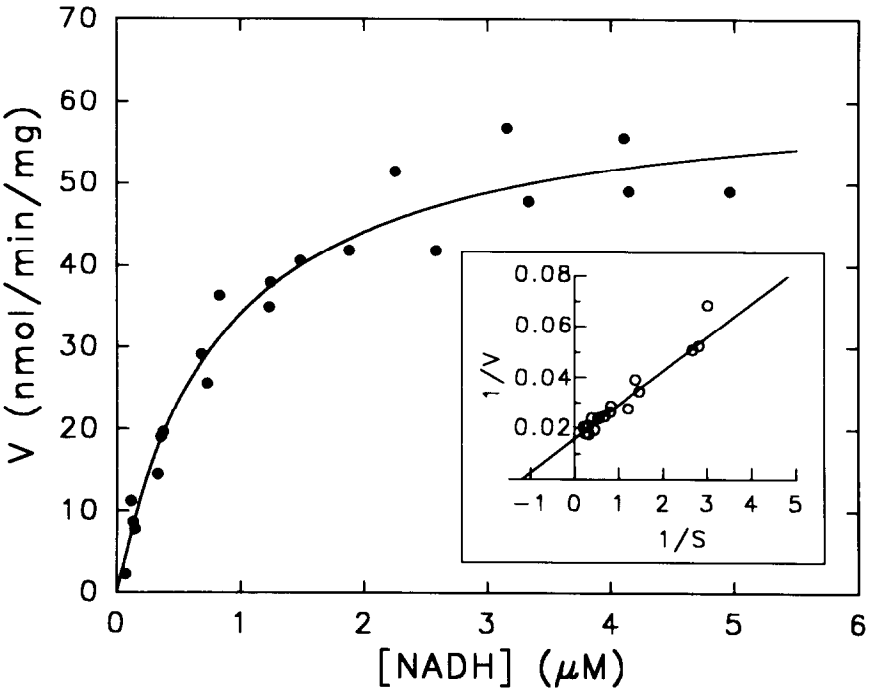

Fig. 2.
NADH concentration dependence of the NADH:ubiquinone oxidoreductase reaction in skeletal muscle mitochondria from the MELAS patient. The curve represents a non-linear fit to the datapoints and gives a K_m of 0.83±0.11μM (±SEM) and a V_{max} of 62.4 nmol/min/mg. The corresponding double resiprocal plot is shown in the inset.

The K_m of NAD for the complex I of muscle mitochondria was determined by analyzing the complete progress curve of the reaction (Fig 2). A K_m of 0.83 μM for NADH was obtained in the complex I of the patient and 0.88 μM in the controls. No difference was found in the V_{max} values.

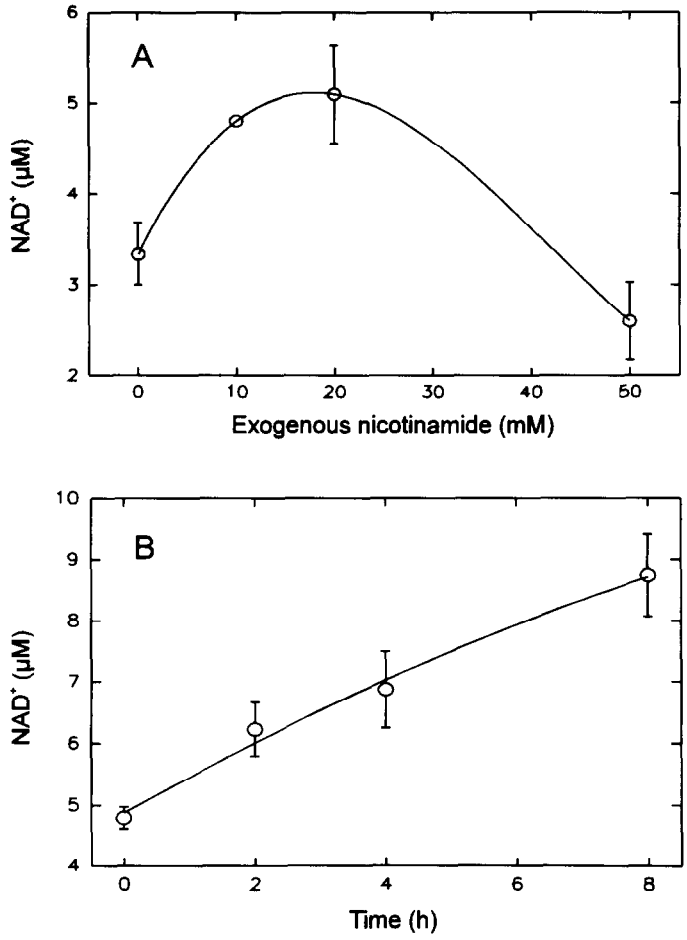

Fig. 3.
Effect of various concentrations of nicotinamide on NAD concentration of cultured skin fibroblasts in 24 h incubation (A). Effect of time on NAD concentration increase in cultured skin fibroblasts incubated in the presence of 20 mM nicotinamide (B).

Effect of nicotinamide on NAD concentration in cultured skin fibroblasts. Since there are no previous data on the effect of nicotinamide administration on NAD content of human cells, the effect of nicotinamide was studied in cultured skin fibroblasts of the patient and control subjects. Incubation of confluent cells in the presence of various concentrations of nicotinamide demonstrated a concentration-dependent increase in cellular NAD content up to nicotinamide concentrations of 20 mM. At higher concentrations there was no effect on the NAD content (Fig 3A). A time-dependent increase in the cellular NAD concentration was observed when the cells were incubated in the presence of 20 mM nicotinamide. The NAD concentration increased linearly, being two-fold at 8 h of incubation (Fig 3B). No differences were found in these experiments between the fibroblasts obtained from the control persons and the patient.

Discussion

We observed an initial linear increase in blood NAD and a rapid clearance of blood and urine lactate in a patient with the MELAS syndrome during peroral nicotinamide treatment. The clinical effect of the treatment was, however, temporary and the disease eventually progressed to death. In a previous report, a patient with clinically definite MELAS and with the bp 3243 mutation in the gene for the $tRNA^{Leu(UUR)}$ was treated with nicotinamide and riboflavin (10). There was a clinically beneficial effect of the treatment, which was further confirmed by demonstrating a temporal relationship between the discontinuation of treatment and the clinical variables and skeletal muscle high-energy phosphate levels and kinetics as measured by ^{31}P magnetic resonance spectroscopy, respectively. We chose to use only nicotinamide in the treatment, because we hypothesized that a decrease in the mitochondrial translation products (5) may lead to a compromised assembly of complex I. Such a defect in complex I would then affect the affinity of its substrate NAD(H) more than its prosthetic group FMN even though the NADH and FMN binding sites both reside in a 51-kD subunit coded by a nuclear gene (for references see (23)). Recently also, riboflavin has been shown to be ineffective in the rat in the prevention of striatal lesions induced with malonate, a mitochondrial toxin, while nicotinamide and coenzyme Q_{10} proved to be effective (24).

The blood lactate and pyruvate concentrations declined rapidly within three days after the introduction of treatment, and a more sustained decline was observed in the 24 h urine lactate excretion over two weeks. Within the first month of treatment we also observed an improvement of the clinical condition of the patient and a decrease in the volume of the MRI-detectable brain lesion. These findings are compatible with a favourable clinical effect and may suggest an improvement in the cellular energy metabolism. ^{31}P magnetic resonance spectroscopy measurements in a patient with the MELAS syndrome have demonstrated an improvement in the phosphocreatine/ATP recovery rate following exercise during nicotinamide treatment (10). In experimental animals coenzyme Q_{10} and nicotinamide prevent ATP depletions produced by a mitochondrial toxin malonate (24). These lines of evidence suggest that nicotinamide indeed acts by improving the efficiency of the electron transfer chain.

The latter part of the treatment failed and the patient died after 5 months of treatment at a time when his blood NAD was approximately 6-fold compared to the control values and the pre-treatment value of the patient. The blood NAD concentration began to decrease after 6 weeks of treatment with an apparent half-life of 22 days. The decline may have been due to an altered metabolism of the drug induced by high nicotinamide concentrations or an increased consumption of NAD due to the progression of the disease. The first possibility may be supported by the findings on cultured cells. At concentrations higher than 20 mM no increase in cellular NAD was observed. Interestingly, a similar concentration-dependency has been observed in experiments where malonate-induced striatal lesions in the rat were blocked with different concentrations of nicotinamide (24). It was, however, intriguing to observe the coincidence between the change in the form of nicotinamide medication from suspension to capsules and the abrupt decline in the blood NAD content. The shape of the decline curve and the temporal relationship suggest that the change led to an altered bioavailability of nicotinamide. The basic pharmacological data on the absorption of nicotinamide indicate that the drug is effectively and completely absorbed throughout the length of the gastrointestinal tract, and thus a delay in the release of the drug from the capsules should not affect its absorption (25,26). These findings may suggest that any treatment efforts of the MELAS syndrome with nicotinamide should include

dosage testing and monitoring of the plasma levels of the drug itself or of the blood concentration of NAD.

Previous data suggest that nicotinamide improves the efficiency of the electron transfer chain (10,24), but the basic mechanism of nicotinamide action is unknown. Nicotinamide may exert its pharmacological action as a vasoactive agent or as a precursor for the NAD synthesis. Based on its vasoactive property, nicotinamide is an effective radiosensitizer of murine tumours, functioning by improving tumour perfusion (27) and enhancing tissue oxygenation. The second possible mechanism is based on findings that administration of nicotinamide results in an increase in the NAD content suggesting that the uptake of nicotinamide in tissues is followed by an incorporation into NAD (11,12). We hypothesized that the substrate affinity of complex I for NAD is decreased in MELAS and that an increase in this substrate concentration would enhance the enzyme activity. We observed a definite lactate clearance in our patient, but the hypothesis was, however, refuted by the findings that the substrate affinity of complex I appeared to be normal in the muscle mitochondria, and no decrease was observed in the specific activity of the complex. The present findings of improved NADH oxidation during elevated cellular NAD(+NADH) may indicate that under physiological conditions the reaction catalyzed by complex I may indeed operate at submaximal substrate saturation. Thus, it would be merely the increase in the available substrate that would increase the activity of complex I. It is noteworthy that the malonate toxicity (24) represents a situation where the mitochondrial dysfunction is a result of impairment of complex II (succinate:ubiquinone oxidoreductase) with normal complex I. If nicotinamide supplementation is effective in this condition, an enhancement of the activity of complex I with normal K_m must occur. This is in good agreement with the present findings where an increase in NAD (+NADH) concentration is metabolically favourable although an obvious defect of complex I was not detectable.

The biochemical effect of nicotinamide treatment in our MELAS patient suggests an improvement of the energy metabolism that may be attributed to increased complex I activity. An ultimate experiment to prove the hypothesized mechanism of the effect of nicotinamide treatment would be the "in situ" measurement of intramitochondrial free NADH in normal and diseased cells or tissues. Although the compartmentation of the total nucleotide content is possible to measure with the currently available methodology (28), the levels of the free nucleotides remain elusive. Therefore, further studies will be required to establish the mechanism of nicotinamide action in MELAS and, moreover, the relapsing and remitting nature of the syndrome necessitates treatment studies with larger patient groups.

Acknowledgements

This work was supported by grants from the Sigrid Juselius Foundation and the Medical Research Council of the Academy of Finland. The authors gratefully acknowledge the expert technical assistance of Miss Anja Roukala and Mrs. Maija-Leena Lehtonen.

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