the efficacy of pyruvate in patients with different mitochondrial diseases. Considering that pyruvate can activate glycolysis even in cells without any mitochondria, as shown in ρ^0 cells, pyruvate therapy is a promising treatment for mitochondrial diseases. More clinical and biochemical studies are necessary to clearly prove the efficacy of this treatment.

Acknowledgments

The authors would like to thank Dr. Ichizo Nishino of the Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Japan, for the histopathological analysis of the muscle biopsy specimens, and Dr. Akira Ohtake of the Department of Pediatrics, Saitama Medical University, Moroyama, Saitama, Japan, for the biochemical and molecular analyses. This work was supported in part by a Grant-in-Aid for Scientific Research (A-22240072 to M.T.), a Grant-in-Aid for the Global COE (Sport Sciences for the Promotion of Active Life to Waseda University) from the Ministry of Education, Culture, Sports, Science and Technology (to M.T.), and a grant for scientific research from The Takeda Science Foundation (to M.T.).

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European Neurology, Original Paper

Evaluation of systemic redox states in patients carrying MELAS A3243G mutation in mitochondrial DNA

Masamichi Ikawa[a] Kenichiro Arakawa[b] Tadanori Hamano[a] Miwako Nagata[c] Yasunari Nakamoto[a] Masaru Kuriyama[a] Yasutoshi Koga[d] Makoto Yoneda[a]

[a]Second Department of Internal Medicine, Faculty of Medical Sciences, University of Fukui, Fukui, Japan

[b]Department of Cardiology, Faculty of Medical Sciences, University of Fukui, Fukui, Japan

[c]Department of Neurology, Nakamura Hospital, Fukui, Japan

[d]Department of Pediatrics and Child Health, Kurume University School of Medicine, Fukuoka, Japan

Short title: Redox states in patients carrying A3243G in mtDNA

Corresponding author: Makoto Yoneda, MD, PhD

Second Department of Internal Medicine, Faculty of Medical Sciences, University of Fukui

23-3 Shimoaiduki, Matsuoka, Eiheiji-cho, Fukui 910-1193, Japan

Tel. +81 776 61 8351, Fax +81 776 61 8110

E-Mail myoneda@u-fukui.ac.jp (M. Yoneda)

Abstract

Background/Aims: To clarify the change of systemic redox states in patients carrying A3243G mutation in mitochondrial DNA (A3243G), we evaluated oxidative stress and antioxidant activity in the serum of patients. ***Methods:*** Oxidative stress and antioxidant activity in the serum samples obtained from 14 patients carrying A3243G and 34 healthy controls were analyzed using the the Diacron-Reactive Oxygen Metabolites (d-ROMs) and Biological Antioxidant Potential (BAP) tests, respectively. ***Results:*** The mean d-ROMs level of all patients was significantly greater than that of the controls ($P < 0.005$), and the mean BAP/d-ROMs ratio of all patients was significantly lower than that of the controls ($P < 0.02$). In the patients with a history of stroke-like episodes (n = 10), both mean d-ROMs and BAP levels were increased compared with those of the controls (both $P < 0.01$). The mean BAP level of the patients without a history of stroke-like episodes (n =4) was significantly decreased compared with that of the controls ($P < 0.001$), but the mean d-ROMs levels were not significantly different. ***Conclusion:*** d-ROMs and BAP tests indicated that patients carrying A3243G are always exposed to underlying oxidative stress, even though at a remission state of stroke-like episodes.

Key Words

MELAS; A3243G mutation; mitochondrial DNA; oxidative stress; antioxidant activity; redox states; d-ROMs test; BAP test

Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome is the most common type of mitochondrial disease, and is mainly caused by an A-to-G transition mutation at nucleotide position 3243 (A3243G) in mitochondrial DNA (mtDNA) [1]. MELAS is characterized by stroke-like episodes that occur repeatedly and provoke neurological symptoms (*e.g.* headache, epilepsy, hemiparesis and dementia) due to "stroke-like" brain lesions [2]. In other words, stroke-like episodes are diagnostic symptoms of MELAS, and are crucial factors determining the prognosis of patients with this syndrome [2].

In addition, A3243G is responsible for not only stroke-like episodes but also mitochondrial cardiomyopathy or diabetes mellitus (DM) [1, 3-6]. Conversely, some patients carrying A3243G present with typical MELAS syndrome with stroke-like episodes, and others present with only cardiomyopathy or DM without stroke-like episodes. However, the pathophysiological difference of phenotypes between the presence and absence of stroke-like episodes in patients carrying A3243G remains obscure.

Recent studies using cells cultured *in vitro* demonstrated increased oxidative stress in cells with impaired mitochondria due to A3243G [7-10]. Oxidative stress is provoked by reactive oxygen species (ROS) generation exceeding antioxidant defenses, such as manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase (GSH-Px), and damages nucleic acids, proteins and lipids, which leads to cellular dysfunction. Indeed, previous pathological or imaging studies demonstrated enhanced regional oxidative stress in lesions of both stroke-like episodes and cardiomyopathy in patients carrying A3243G [11-13]. Therefore, there is a high possibility that oxidative stress participates in the pathogenesis caused by A3243G, and influences the phenotypic diversity. In other words, redox (reduction-oxidation) states should be evaluated in patients carrying A3243G both with and without a history of stroke-like episodes to clarify the role of oxidative stress in the emergence of stroke-like episodes.

To perform such an investigation, a rapid and reliable method of evaluating redox states in patients carrying A3243G is needed. Direct measurement of oxidative stress and antioxidant activity in living humans has been difficult; redox states have thus not been clearly evaluated in patients carrying A3243G to date. Recently, the Diacron-Reactive Oxygen Metabolites (d-ROMs) and Biological Antioxidant Potential (BAP) tests have been used to evaluate redox states in serum. The d-ROMs level reflects the intensity of oxidative stress, and the BAP level indicates the activity of endogenous antioxidants [14, 15]. Their effectiveness as clinical markers

has been reported in various diseases [16-22]. We evaluated redox states in fresh serum of both patients carrying A3243G and healthy volunteers using d-ROMs and BAP tests, and clarified the change of redox states due to A3243G and the pathophysiological difference in phenotypes with or without stroke-like episodes.

Materials and methods

Subjects

Fourteen Japanese patients (7 men and 7 women; mean age 32.1 ± 14.7) carrying A3243G were recruited at the University of Fukui Hospital and Kurume University Hospital, Japan (table 1). Patients were classified by the presence or absence of stroke-like episodes into 'stroke type' and 'non-stroke type'. Ten patients with a history of stroke-like episodes were categorized as 'stroke type', and the other 4 patients who presented with mainly cardiomyopathy without a history of stroke-like episodes were categorized as 'non-stroke type'. Eleven patients were treated by antioxidant therapy such as coenzyme Q_{10} (CoQ_{10}) (daily dose: 30 - 90 mg) and/or vitamin E (daily dose: 100 mg) administration; 10 of these patients were 'stroke type', and the other one was 'non-stroke type'. Eight patients of 'stroke type' were also treated with an oral administration of L-arginine (daily dose: 14 - 21 g). All patients were in remission, free from exacerbation of symptoms or acute stroke-like episodes, when they were examined. Functional status was evaluated using the Performance Status rating (mean rating: 1.3 ± 1.0). In 'stroke type' patients, the mean age of the first stroke-like episode was 21.1 ± 15.2 years old and the mean duration between the examination and the last stroke-like episode was 14.2 ± 9.2 months. 'Stroke type' patients had headaches and/or vomiting on average twice a month, but almost none had convulsions. Thirty-four Japanese healthy volunteers (20 men and 14 women; mean age 34.6 ± 7.4) were also recruited as normal controls from the local community (table 1). This study was approved by the Ethics Committee of the University of Fukui. All subjects provided written informed consent to participate.

Measurement of oxidative stress levels

The oxidative stress levels were evaluated by measuring the quantity of hydroperoxides (R-OOH) in fresh serum samples using the d-ROMs test with the Free Radical Analytical System 4 (FRAS4^{R}; H&D srl, Parma, Italy) automatically [14]. Blood sampling was performed at fasting and at rest. Hydroperoxides consist of dehydrogenized and peroxidized proteins, lipids and fatty acids produced by ROS. In the d-ROMs test,

hydroperoxides are turned to radicals by the Fenton reaction in an acid medium, and these generated radicals oxidize *N,N*-diethyl-para-phenylenediamine (DEPPD). Oxidized DEPPD quantity is determined by an absorbency measurement (white light 505 nm). The sequence of these methods is automated, and oxidative stress levels can be evaluated easily and quickly. The values are expressed as U.CARR, where 1 U.CARR corresponds to 0.8 mg/L H_2O_2.

Measurement of antioxidant activity levels

The antioxidant activity levels were evaluated by measuring the quantity of molecules with antioxidative potency in fresh serum samples using the BAP test in the FRAS4^R automatically [15, 17]. Blood sampling was performed at fasting and at rest. In the BAP test, serum molecules with antioxidative potency reduce and decompose compounds of ferric chloride ($FeCl_3$) and thiocyanate derivative (AT) to $FeCl_2$ and free AT. Free AT is achromatized and dissociates from compounds, and is quantified by an absorbency measurement (white light 505 nm). The sequence of these methods is automated, and antioxidant activity levels can be evaluated easily and quickly. The results are expressed as μmol/l.

Statistical analysis

The BAP-to-d-ROMs ratio (BAP/d-ROMs ratio) was calculated from the ratio of the BAP levels and d-ROMs levels for each subject. Data are presented as means ± standard deviations (SD). The resultant differences between normal controls and all patients were analyzed by means of a two-tailed Mann-Whitney *U* test. Since the subject number of each group was small, a non-parametric Kruskal-Wallis test was used for multiple data comparison and a post hoc Dunn test was performed to evaluate differences among normal controls, 'stroke type' patients and 'non-stroke type' patients. All statistical analyses were performed in SPSS Statistics Version 17.0 (SPSS Japan Inc., Tokyo, Japan), and $P < 0.05$ was considered significant.

Results

The levels of serum d-ROMs and BAP, and BAP/d-ROMs ratios of all the patients and controls are shown in fig. 1, and those of the 'stroke type' patients, 'non-stroke type' patients and controls are shown in fig. 2. The mean age of each group demonstrated no significant differences.

The mean d-ROMs level of all patients (332.6 ± 110.7 U.CARR) was significantly higher than that of the controls (259.1 ± 42.0 U.CARR) ($P < 0.005$) (fig. 1a). In particular, the mean d-ROMs level of the 'stroke type' patients (361.0 ± 119.6 U.CARR) was significantly greater than that of the controls ($P < 0.01$) (fig. 2a). Meanwhile, the mean d-ROMs level of 'non-stroke type' patients (261.5 ± 28.0 U.CARR) demonstrated no significant differences compared with those of the controls and 'stroke type' patients (fig. 2a).

The mean BAP level of all patients (2258.9 ± 517.7 μmol/l) was not significantly different compared with that of the controls (2057.6 ± 149.5 μmol/l) (fig. 1b). However, compared with the controls, 'stroke type' patients (2428.9 ± 523.1 μmol/l) demonstrated significantly high BAP levels ($P < 0.01$), and 'non-stroke type' patients (1834.0 ± 59.2 μmol/l) demonstrated significantly low BAP levels ($P < 0.001$) (fig. 2b). There was no significant difference between 'stroke type' patients and 'non-stroke type' patients in terms of the mean BAP levels.

The mean BAP/d-ROMs ratio of all patients (7.87 ± 5.05) was significantly lower than that of the controls (8.13 ± 1.30) ($P < 0.02$) (fig. 1c). However, there were no significant differences among the controls and patient groups (fig. 2c).

There was no relationship between the functional status evaluated by Performance Status rating and the d-ROMs level or BAP level or BAP/d-ROMs ratio.

Discussion

In the present study, the d-ROMs and BAP tests were applied to evaluate the redox states in serum of patients carrying A3243G. These tests demonstrated that oxidative stress represented by the d-ROMs levels was increased and redox balance represented by the BAP/d-ROMs ratios was decreased (tendency for oxidation) in the patients compared with those of the controls (fig. 1). These findings suggested that an imbalance of redox states due to mitochondrial dysfunction affects the pathogenesis in patients carrying A3243G.

In the 'stroke type' patients in particular, both d-ROMs levels (oxidative stress) and BAP levels (antioxidant activity) were increased compared with those of the controls (fig. 2a, b). *In vitro* studies previously demonstrated that A3243G enhances ROS generation leading to oxidative stress [7-10], and enhanced oxidative stress is proportional to mitochondrial dysfunction [7, 23]. In the present study, all of the 'stroke type' patients

had been treated with antioxidants, and 8 of 10 these patients were also treated with an oral administration of L-arginine. Although serum antioxidant activity may be increased by antioxidants and L-arginine therapy, serum oxidative stress was still increased in 'stroke type' patients. Increased oxidative stress even with increased antioxidant activity suggested a severe deterioration of mitochondrial function in patients with a history of stroke-like episodes, and that oxidative stress plays a crucial role not only in the brain lesions of stroke-like episodes [11, 12] but also systemically in these patients. In other words, a history of stroke-like episodes indicates that patients who have these episodes are exposed to underlying oxidative stress.

In the 'non-stroke type' patients, the mean d-ROMs level (oxidative stress) was not significantly different compared with that of the controls (fig. 2a). Meanwhile, the BAP levels (antioxidant activity) were significantly decreased (fig. 2b). Only one of 4 patients was treated with antioxidants, and antioxidant therapy may not affect antioxidant activity in 'non-stroke type' patients. These findings may reflect that antioxidants are consumed in order to prevent increase of oxidative stress in these patients. In addition, the difference of profiles in redox states between 'stroke type' and 'non-stroke type' suggested phenotypic diversity in patients carrying A3243G

In the present study, we presented redox states in the serum of patients carrying A3243G using the d-ROMs and BAP tests. Rapid evaluation of redox states in serum has been difficult to date. To assay oxidative stress in serum, the spin trap method using electron spin resonance (ESR) has been the most reliable method [24]. However, performing ESR is cumbersome, thus it is difficult to apply this method in clinical practice. The d-ROMs test can evaluate oxidative stress in serum by measuring oxides due to hydroperoxides, and this test has been validated by ESR [25]. Likewise, each endogenous antioxidant can be measured, but there has been no method estimating the whole activity of endogenous antioxidants in serum to date. The BAP test provides a reliable indicator of the antioxidant activity in serum by measuring the ability to reduce ferric to ferrous ions [15]. Moreover, the d-ROMs and BAP tests only need a small amount of blood, and require only 15 minutes for measurement. Therefore, these methods are prompt and reliable, and suitable for evaluating redox states in patients.

Previous studies using postmortem organs or positron emission tomography (PET) imaging have demonstrated regional enhancement of oxidative stress in the brain lesions of stroke-like episodes and the heart lesions of cardiomyopathy in patients carrying A3243G [11-13]. Although enhanced oxidative stress due to A3243G has been proven in these lesions, systemic oxidative stress in patients carrying A3243G has not been evaluated to date. The present study demonstrated a systemic and underlying imbalance of redox states in

these patients.

The present study has some limitations as follows; 1) The 'non-stroke type' group included only 4 patients; 2) The mean age of 'non-stroke type' patients was likely older than that of 'stroke type' patients; 3) The 'stroke type' group included only 2 of 10 patients with cardiomyopathy or diabetes, which might affect the systemic redox states; 4) All of the 10 'stroke type' patients received antioxidant therapy, but only 1 of the 4 'non-stroke type' patients received; 5) This study did not show any significant difference in either value of oxidative stress or antioxidant activity between the 'stroke type' and 'non-stroke type' groups; 6) The possibility that the 'non-stroke type' patients in this study will also subsequently develop stroke-like episodes cannot be ruled out. Further studies are necessary to confirm our preliminary results.

Taken together, the d-ROMs and BAP tests clearly demonstrated an abnormality of redox states in patients carrying A3243G. In particular, enhanced oxidative stress in patients with a history of stroke-like episodes may reflect severe mitochondrial dysfunction, which would contribute to the emergence of stroke-like episodes. In addition, in patients without stroke-like episodes, consumption of antioxidant activity may indicate latent oxidative stress. These findings suggested that patients carrying A3243G are always exposed to underlying oxidative stress, and further antioxidant therapy would be beneficial to prevent an intensification of the symptoms.

Acknowledgements

This study was funded in part by Scientific Research on Innovative Areas (2020021) from the Japan Society for the Promotion of Science.

Conflict of interest statements

The authors report no conflicts of interest.

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Table

Table 1. Demographic characteristics of the patients and controls

Subject	Patients			Normal controls
	All	'Stroke type'	'Non-stroke type'	
Number	14	10	4	34
Gender (males/females)	7/7	5/5	2/2	20/14
Mean age at examination (years)	32.1 ±14.7	27.8 ± 12.5	42.8 ±15.8	34.6 ±7.4
Clinical features				
Stroke-like episodes (n)	10	10	0	0
Cardiomyopathy (n)	6	2	4	0
Diabetes (n)	3	0	3	0
Under antioxidant therapy (n)	11	10	1	0

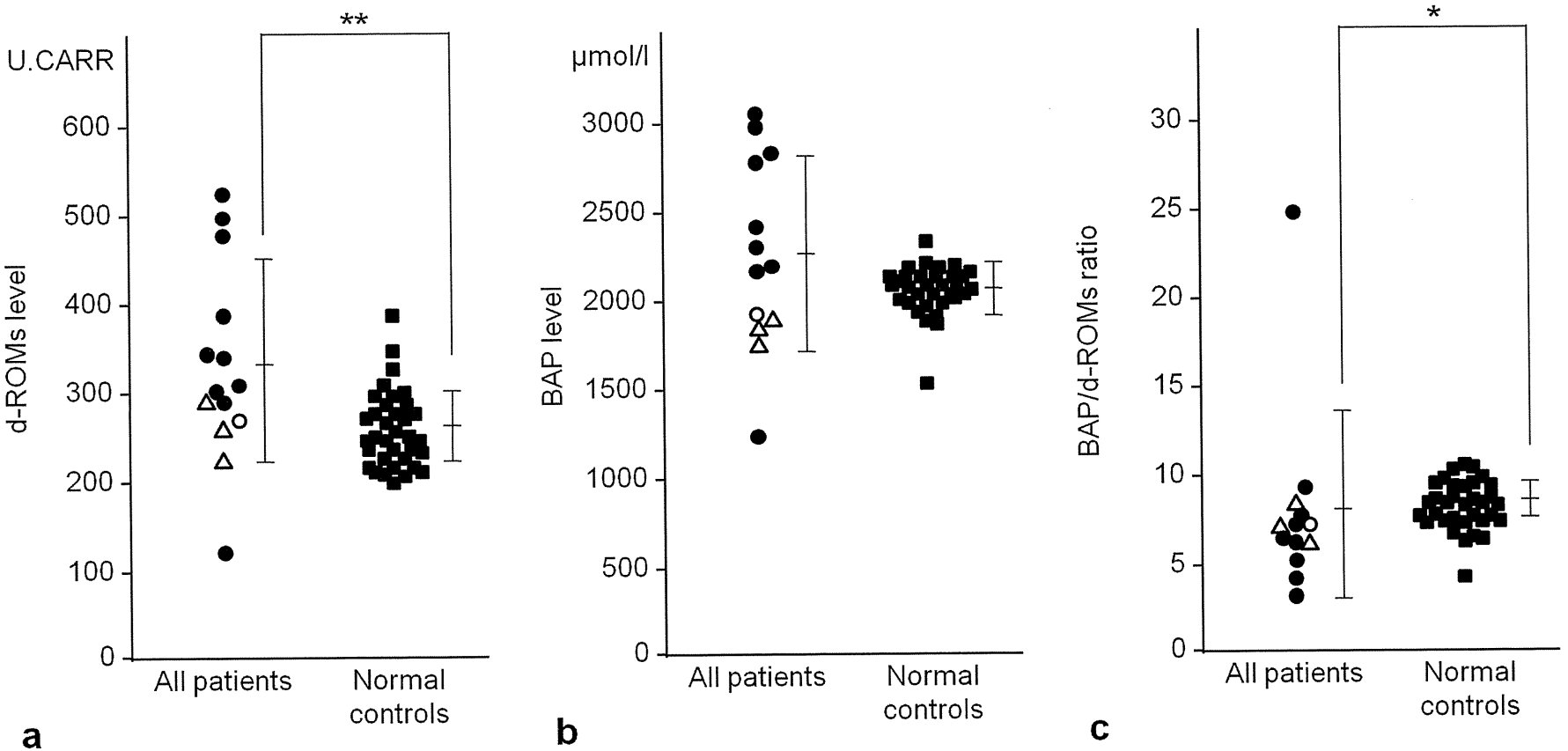
Values are mean ± S.D.

Figure Legends

Fig. 1. Scatter plots portraying the levels of d-ROMs (**a**) and BAP (**b**) and BAP/d-ROMs ratios (**c**) in all the patients and controls. In the patients, circles and triangles correspond to the patients with and without antioxidant administration, respectively. In addition, closed and open diagrams correspond to the 'stroke type' patients and 'non-stroke type' patients, respectively.

*$P < 0.02$, **$P < 0.005$, according to two-tailed Mann-Whitney U test. Bars indicate mean ± S.D.

Fig. 2. The mean d-ROMs (**a**) and BAP (**b**) levels and mean BAP/d-ROMs ratio (**c**) in the 'stroke type' patients (black), 'non-stroke type' patients (grey) and controls (white). ***$P < 0.01$, ****$P < 0.001$, according to Dunn test. Bars indicate mean + S.D.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U.CARR
d-ROMs level
600
500
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200
100
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All patients
Normal controls
a
μmol/l
BAP level
3000
2500
2000
1500
1000
500
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All patients
Normal controls
b
*
BAP/d-ROMs ratio
30
25
20
15
10
5
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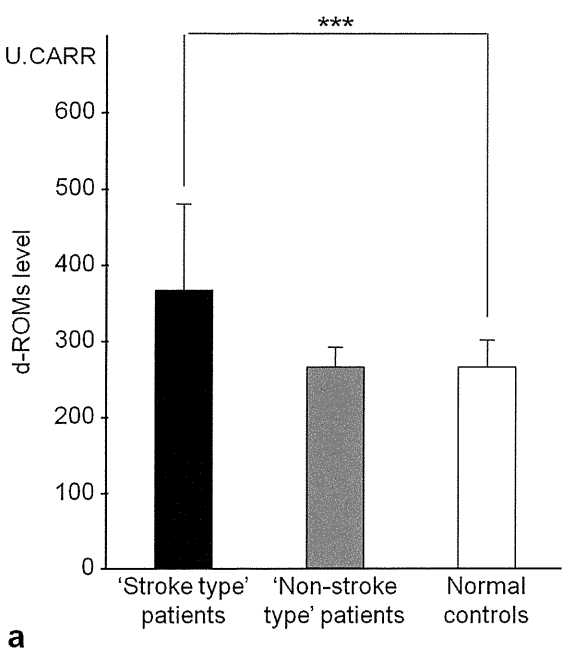
All patients
Normal controls
c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U.CARR
d-ROMs level
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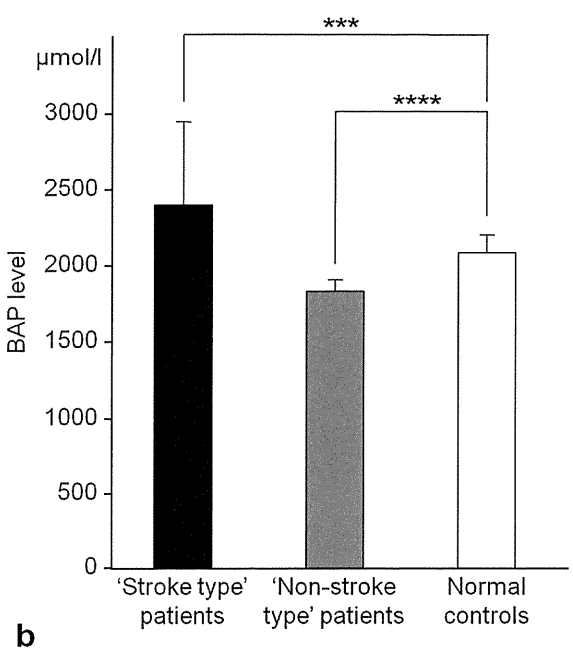
'Stroke type' patients
'Non-stroke type' patients
Normal controls

a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µmol/l
BAP level
3000
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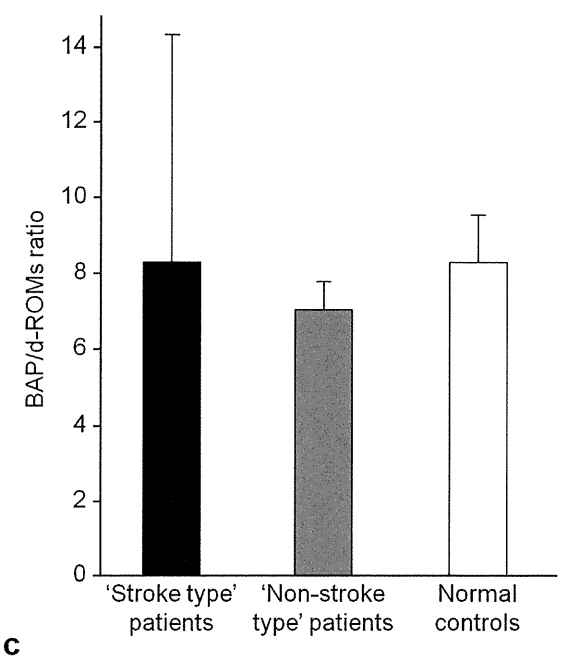
'Stroke type' patients
'Non-stroke type' patients
Normal controls

b


BAP/d-ROMs ratio
14
12
10
8
6
4
2
0
'Stroke type' patients
'Non-stroke type' patients
Normal controls

c