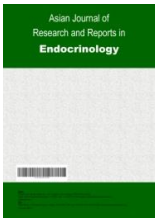

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Evaluation of Oxidative Stress and DNA Damage in Acromegaly Patients in a Follow-up Study at a Public Tertiary Health Service

A. C. M. Bonini[1], C. D. L. Oliveira[1], L. C. Talon[2], D. T. Pierine[2], F. V. Francisqueti[2], C. R. Correa[2] and V. S. Nunes-Nogueira[1*]

[1]Department of Internal Medicine, Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil.
[2]Department of Pathology, Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil.

Authors' contributions

This work was carried out in collaboration among all authors. Author VSNN designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors FVF, DTP and CRC performed the statistical analysis. Authors ACMB, CDLO and LCT managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

We evaluated oxidative stress (OS) and DNA damage in acromegaly patients in a follow-up study at a public tertiary health service. Acromegaly patients in follow up at a public tertiary health service from 2012 to 2013 were enrolled in this study. Healthy volunteers were selected as control group. OS was evaluated in plasma and peripheral lymphocytes by determining total antioxidant capacity (TAP), lipophilic antioxidant levels and lipid peroxidation through malondialdehyde (MDA) levels. DNA damage was determined using the comet test. Fifteen acromegaly patients and 19 healthy individuals were included. Considering IGF1 levels<1.3× the upper limit of the normal range as disease under control, 20% of individuals were uncontrolled for the disease. No significant difference in total carotenoid levels, TAP, or DNA damage was found between groups

**Corresponding author: Email: vania.nunes-nogueira@unesp.br;*

(respectively, 62.8 and 46, p=0.13; 51.3 and 43.5, p=0.093; 41.4.0 and 47.2, p=0.15). On the other hand, median MDA levels in the acromegaly group was significantly lower than that in the control group (0.44 and 0.977, respectively; p<0.001, Mann-Whitney test). A subgroup comparison within acromegaly patients revealed no statistically significant difference (p>0.05) between those with normal or increased IGF1 levels, with or without metabolic syndrome, or with or without use of statin (0.4 vs. 0.436, 0.42 vs. 0.5 and 0.533 vs, 0.413, respectively). Acromegaly patients when compared with a healthy control group did not present significantly higher levels of OS, antioxidants and DNA damage. However, most patients included in our study achieved disease control.

Keywords: Acromegaly; oxidative stress; antioxidant; DNA damage; comet test.

1. INTRODUCTION

Acromegaly is a chronic disease caused by excessive production of growth hormone (GH), and its main cause is a GH-secreting pituitary macroadenoma. Several studies have shown a mortality rate in acromegaly patients two to four times higher than that in the general population with the same gender and age [1], mainly owing to cardiovascular complications [2]. These complications are probably a result of hypertension, glucose intolerance, and lipid abnormalities, which together lead to early atherosclerosis in these patients. Atherosclerosis, in turn, is understood as a dynamic and progressive process, originating from endothelial dysfunction and inflammation, and that has oxidative stress (OS) as one of the fundamental mechanisms in its pathology [3].

OS is defined as an event resulting from the imbalance of magnitude between oxidizing substances and antioxidants [4,5]. Both substances (oxidants and antioxidants) are generated in oxidoreduction reactions, where oxidation and reduction correspond to gain and loss of electrons, respectively. Because the generation and action of oxidants and antioxidants depend on this oxidoreduction system, many authors have used the term redox system imbalance to refer to OS [6]. Popularly called free radicals, oxidizing substances include reactive oxygen species (ROS) and reactive nitrogen species (RNS). Reactive species are identified as substances that perform lipid and glucose oxidation (lipoxidation and glycation, respectively). The products generated in lipoxidation are malondialdehyde, glyoxal, acrolein and 4-hydroxynonenal, and those generated in glycation are glyoxal and methylglyoxal. These compounds bind to amino acids, resulting in highly reactive final products of glycation (AGEs) and lipoxidation (ALEs) [7]. AGEs are involved in common events related to obesity (type 2 diabetes mellitus, inflammation, and insulin resistance) and may be an important activator of the inflammatory process in several tissues [8,9].

Studies on transgenic mice overexpressing bovine GH as a model of acromegaly have shown a deterioration of endothelial function [10], initially by OS and subsequently by other alterations in vascular function [11]. These mice show many of the complications common to patients with acromegaly, such as hypertension, dyslipidemia, and hyperinsulinemia, but without metabolic syndrome [12]. In addition, 3T3-L1 adipocyte cells treated with insulin-like growth factor 1 (IGF1) stimulated the production of ROS and inhibited insulin-dependent glucose uptake via ROS [13].

Anagnostis et al. evaluated the blood redox status and endothelial function in patients with acromegaly. They concluded that acromegalics as compared with controls had significantly lower levels of catalase activity and nitric oxide [14]. However, Ozkan et al. compared the levels of oxidized-LDL cholesterol and total antioxidant capacity in a group of acromegaly with a control group, and no significant differences were found between groups [15].

Considering this conflicting evidence in the literature, this research aimed to evaluate OS and DNA damage in acromegaly patients. Our hypothesis was that acromegaly patients, even without complications related to metabolic syndrome, have increased OS levels that contribute to endothelial dysfunction, which justifies the higher cardiovascular risk in these individuals.

2. MATERIALS AND METHODS

Acromegaly patients in follow-up at a public tertiary health service from 2012 to 2013 were

enrolled in this study. The health service is a pituitary disorder reference center for 68 cities in the state of São Paulo.

All included patients had the diagnosis of acromegaly confirmed by baseline GH levels greater than 1 ng/mL, lack of suppression of GH < 1 ng/mL after a glucose tolerance test (GTT), and high levels of IGF1 for age and gender. We excluded individuals with discordant GH and IGF1 levels at diagnosis, a daily alcohol intake higher than 60 g (≥5 drinks), use of antioxidant supplements, abnormal hematological and albumin parameters, renal and hepatic insufficiency, smoking, or other active neoplasms. Healthy, non-smoking volunteers without any disease and with a daily intake of alcohol of less than 60 g were recruited from donors of a local blood bank and used as a control group. All measurements for controls and patients were performed in the same assays.

2.1 Oxidative Stress Analysis

2.1.1 Evaluation of Total Antioxidant Capacity (TAP)

Serum TAP was determined using the method reported by Aldini and collaborators [16] and validated by Beretta and collaborators [17] for application of total antioxidant capacity in both hydrophilic and lipophilic serum components to high-throughput studies. This method measures the rate of oxidation of 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591), a lipid-soluble fluorescent probe and uses the lipid-soluble radical initiator 2,29-azobis(4-methoxy-2,4-dimethylvaleronitrile). Oxidation is determined by monitoring the appearance of green fluorescence of the oxidation product of BODIPY (lex=500 nm, lem=520 nm) using a 1420 multilabel counter (Wallac Victor 2, Perkin Elmer Life Sciences, Boston, Massachusetts, USA). Results are expressed as TAP values, which represent the percent inhibition of BODIPY oxidation in human serum with respect to that occurring in a control sample consisting of BODIPY 581/591 in phosphatidylcholine liposomes.

2.1.2 Malondialdehyde (MDA) level determination

A 100 μL aliquot of plasma was used for MDA analysis. Briefly, we added 700 μL of 1% orthophosphoric acid and 200 μL of thiobarbituric acid (42 mM) to the sample and then boiled it for 60 min in a water bath; the sample was cooled on ice immediately after that. Two hundred μL was transferred to a 2 mL tube containing 200 μL sodium hydroxide-methanol (1: 12 v/v). The sample was vortex-mixed for 10 s and centrifuged for 3 min at 13,000 ×g. The supernatant (200 μL) was transferred to a 300 μL glass vial and 50 μL injected onto the column. The HPLC was a Shimadzu LC-10AD system (Kyoto, Japan) equipped with a C18 Luna column (5 μm, 150 × 4.60 mm, Phenomenex Inc., Torrance, CA, USA), a Shimadzu RF-535 fluorescence detector (excitation: 525 nm, emission 551 nm), and 0.5 mL/min flow of phosphate buffer (KH2PO4 1 mM, pH 6.8) [18]. MDA was quantified by area determination of the peaks in the chromatograms relative to a standard curve (1,1,3,3 tetraethoxypropane (TEP)) of known concentrations.

2.1.3 Extraction of lymphocytes

To evaluate the levels of DNA damage (comet assay), we used peripheral blood lymphocytes. Blood samples (3 mL) were placed into tubes containing 3 mL of RPMI 1640 medium (Sigma-Aldrich) and then placed carefully on 3 mL Histopaque 1077 (Sigma-Aldrich). After centrifugation at 2500 rpm for 30 min at 10°C, the layer of lymphocytes was removed and mixed with 3 mL RPMI 1640 medium and centrifuged again at 1500 rpm for 15 min. The supernatant was then discarded, and lymphocytes were resuspended to be used for the evaluation of DNA damage in the comet assay.

2.2 Comet Assays

Comet assays were performed following the protocols described by Singh and collaborators [19] and Tice and collaborators [20], with some modifications. Clean slides were briefly dipped into a container containing standard melting point agarose (Sigma-Aldrich) diluted in 1.5% (300mg/20 mL) PBS buffer (Sigma-Aldrich) (free of Ca ++ and Mg ++). The slides were then left to dry at room temperature. The next day a 10-µL aliquot of lymphocytes was added to 120 µL of low melting point agarose, diluted in 0.5% (100 mg/20 mL) PBS buffer (Sigma-Aldrich) (free of Ca++ and Mg++). The suspension was placed on two previously prepared slides, identified, and then overlaid with cover slips (24 × 60 mm) and placed at 4 °C for 10 min to solidify the agarose. After this period, cover slips were removed and

slides were placed in containers containing ice-cold, freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, Triton X-100 and 1% DMSO), where they remained in the dark for 24 h at 4°C. To increase the specificity of oxidative DNA damage detection, two sheets per individual were treated with endonuclease III (endo III) and formamidopyrimidine-DNA glycosylase (FPG) (BioLabs, Ipswich, MA, USA), capable of detecting pyrimidines and oxidized purines, respectively [17]. After cell lysis, the slides were placed in a container containing PBS (Ca++ and Mg++ free) for 5 min and then transferred to a flask containing Flare 1× (40 mM HEPES, 0.1 M KCl, bovine serum albumin (BSA) buffer 0.2 mg/mL, and 0.5 mM EDTA pH 8 (Sigma-Aldrich)) for 5 min. This procedure was repeated three times. After being placed in a moist chamber, the slides were treated with 50 mL buffer (950 µL Milli-Q H_2O, 40 mL Flare 10× and 10 mL BSA, control), or with 50 mL endo III (1:1000 dilution) or 50 mL FPG (dilution 1: 1000), covered with a cover slip and incubated for 45 min at 37ºC. The slides were then placed in a refrigerator for 10 min to solidify the agarose. After this period, the cover slips were carefully removed and the slides transferred to an electrophoresis tank, filled with cold, freshly prepared alkaline buffer (1 mM EDTA and 300 mM NaOH, pH>13). After 40 min to denature the DNA, electrophoresis was performed at 25 V and 300 mA for 30 min. Next, the plates were placed for 15 min in a neutralization solution (0.4 M Tris, pH 7.5), fixed with 100% ethanol, and allowed to dry at room temperature.

At the time of analysis, the slides were stained with 70 µL solution of SYBR Gold (2:10000; Invitrogen, USA), covered with a cover slip, and nucleotides viewed with a fluorescence microscope (400× magnification) coupled within an image analysis system (Comet Assay II, Perceptive Instruments, UK). Fifty nucleotides per slide were analyzed. Tail intensity was used to assess DNA damage. The tests were performed in duplicate and analyzed blind with coded slides.

2.3 Biochemical and Hormonal Analysis

Total cholesterol and fractions, glycemia, serum aspartate transaminase (AST) and alanine transaminase (ALT), urea, creatinine, hemogram, TSH, and free T4 levels in acromegaly patients and healthy individuals were determined in a baseline visit. Acromegaly patients also had their GH and IGF1 serum levels determined at the time of collection, and those with IGF1 levels higher than 1.3× the upper limit of the normal range were classified as uncontrolled. Patients with pituitary macroadenoma were also evaluated for the presence of hypopituitarism.

2.4 Lipophilic Antioxidants Analysis

Carotenoids (lutein, cryptoxanthin, alpha carotene, beta carotene and lycopene), retinol, and α-tocopherol were measured in 100 µL of plasma using reversed-phase HPLC (Waters Alliance 2695 Separation Module, Waters, Wilmington, MA, USA). A C30 column was used (Waters Alliance, YMC carotenoid; 4.6 × 150 mm; 3.0 µm). The measurements were performed as previously described by Yeum and collaborators [21].

2.5 Statistical Analysis

Comparisons between groups were done using ANOVA for one classification factor, followed by the Tukey multiple comparison test for the data that presented symmetrical distribution. For asymmetrical distributions, a generalized linear model with gamma distribution was adjusted followed by the Wald multiple comparison test. Data are presented as mean and standard deviation. A significance level of 5% or the corresponding p-value was considered statistically significant. Analyzes were performed using the software SAS for Windows v. 9.3.

3. RESULTS AND DISCUSSION

3.1 Results

Of the 28 acromegaly patients followed up in our service during the study period, 15 patients were included (Table 1), of which eight were female and seven were male, between 32 and 67 years of age. Four individuals were excluded owing to smoking and nine did not attend the collection (they were called more than twice). Of the 15 included patients, 40% reached cure criteria (normal IGF1 levels for age and gender and random GH levels of less than 1 µg/L), 4 individuals (26.7%) had discordant GH and IGF1 levels, and the remainder (33.3%) were uncontrolled. Considering IGF1 levels<1.3× the upper limit of the normal range as disease under control, 20% of individuals were uncontrolled (Table 1). A total of 73 % of acromegaly patients were taking somatostatin analogs (27% in association with cabergoline), and 27% of individuals achieved disease control after trans-

sphenoidal surgery. No patient was taking pegvisomant.

Diabetes, carbohydrate intolerance, and altered fasting glycemia were found in 40%, 6.7% and 20% of patients, respectively, whereas the remainder (33.3%) showed normal fasting glycemia. Mixed dyslipidemia was observed in 46.7% of patients, which were treated with statin. No patient showed renal or hepatic insufficiency. The presence of central hypothyroidism was found in 33.3% of patients, which was compensated by the chronic use of levothyroxine. The remaining patients (66.7%) showed normal TSH levels and free T4 levels greater than 1 ng/dL. Three patients (20%) were diagnosed with central adrenal insufficiency, all of whom were in chronic use of prednisone. Three women and two men were diagnosed with hypogonadism, but none were under hormone replacement therapy. Carotenoid (lutein, cryptoxanthin, alpha-carotene, beta-carotene, and lycopene), retinol, alpha tocopherol, TAP, and MDA levels of acromegaly and control groups are shown in Table 2.

Comparison of the two groups (Table 2) showed that the median lutein levels were significantly higher in the control than in the acromegaly group (5.24 and 1.9, respectively; $p<0.001$, Mann-Whitney test). Median retinol levels were also significantly higher in the control group (64.13 and 2.82; $p<0.001$, Mann-Whitney test). In contrast, lycopene median levels in the acromegaly group was significantly higher than those in the control (38 and 2.8, respectively; $p<0.001$, Mann-Whitney test). Mean alpha-tocopherol levels were also significantly higher in the acromegaly group (992.4 and 423.7; $p <0.001$, Student's t-test). No significant difference between groups was found in total carotenoids or TAP levels (Table 2).

Median MDA levels in the acromegaly group were significantly lower than those in the control group (0.4 and 0.977, respectively; $p<0.001$, Mann-Whitney test; Table 2). A subgroup comparison within acromegaly patients found no statistically significant difference ($p>0.05$) between those with normal or increased IGF1 levels, with or without metabolic syndrome, and with or without use of statin (0.4 vs. 0.436, 0.42 vs. 0.5, and 0.533 vs, 0.413, respectively). Comet test results showed that the percentage of DNA damage in acromegaly patients was not significantly different from that in the control group (41.4 and 47.2, $p=0.15$; Student's t test).

3.2 Discussion

Acromegaly is characterized by high morbimortality mainly owing to cardiovascular complications, but it has been increasingly associated with other neoplasias that, together, may contribute to a higher mortality in these individuals [2]. OS and endothelial dysfunction are fundamental mechanisms of atherosclerosis, and therefore contribute to an increase in cardiovascular disease incidence. DNA damage in turn contributes to the onset of cancers.

Our initial hypothesis was that lower levels of antioxidant markers would be found in acromegaly patients, and OS would be higher when compared to that in the control group. However, our results show that the levels of two antioxidant markers, lycopene and alpha tocopherol, were significantly higher in the acromegaly group, whereas the OS evaluated by lipid peroxidation (MDA dosage) was higher in the control group. We initially thought that the observed difference in antioxidant levels could be justified by a richer diet in these vitamins in the acromegaly group, but this was not confirmed in the food recall of these patients. Another possibility would be that the somatostatin analogue octreotide, used by most patients (76 %), had antioxidant properties. It has been described that Wistar rats with acute pancreatitis induced by sodium taurocholate were treated with octreotide, which led to a significant suppression of MDA levels and a significant increase in the levels of antioxidant enzymes superoxide dismutase and glutathione peroxidase [22]. We therefore compared MDA levels in patients with and without use of octreotide, and each group with the control. In the first comparison, MDA levels were lower in patients using the analogue, but the difference was not significant (0.360 x 0.429, $p>0.05$). It is possible that the small number of patients prevented detection of a significant difference. In the last two comparisons, a significant difference favoring the acromegaly group was observed.

A published cross-sectional study also evaluated OS in acromegaly, comparing 15 patients with 15 control subjects [14]. Antioxidant levels, catalase activity, glutathione concentration, and TAP, nitric oxide, total oxidized glutathione, and TBARS levels were evaluated. The authors demonstrated that acromegaly is associated with significantly elevated OS levels and significantly lower levels of antioxidants. Except for the comparison of TAP levels, which also did not

Table 1. Characteristics of acromegaly patients

P	Gender	Age	IGF1 (ng/mL)	RV of IGF1	GH (µg/L)	FG (mg/dL)	T/TC/HDL (mg/dL)	Statin use	Urea (mg/dL)	Creatinine (mg/dL)	AST (U/L)	ALT (U/L)	DM	DNA Damage (%)
1	M	53	290	87-238	0,07	164	89/142/32	No	44	1,1	17	43	Yes	33.9
2	M	61	227,7	75-212	2,9	101	75/182/60	No	34	1,1	23	27	No	80.5
3	F	37	135,4	109-284	0,94	78	84/148/48	No	28	0,6	29	32	No	22.6
4	F	62	548	75-212	5,8	112	192/230/60	Yes	47	0,8	18	23	Yes	41.9
5	F	35	55	115-307	0,14	100	218/266/47	Yes	29	1,6	32	21	Yes	52.7
6	F	32	137	107,8-246,7	0,9	83	324/201/36	No	26	0,8	-	-	No	36.3
7	F	62	222	54-204	0,83	91	165/249/64	Yes	43	1,01	-	-	Yes	42.8
8	F	52	216	87-238	0,27	104	89/171/84	Yes	41	0,9	16	21	No	28.9
9	M	62	<25	75-212	3,1	91	48/126/58	No	52	0,9	-	-	No	27.2
10	M	63	244,1	75-212	1,3	86	104/162/62	No	42	1,3	25	24	No	47.4
11	M	48	196	94-252	0,326	77	179/232/61	No	39	0,8	31	50	No	53.8
12	F	60	239,7	81-225	0,219	102	140/169/44	Yes	44	0,9	19	22	No	38.3
13	F	67	201,2	69-200	0,854	111	153/178/43	yes	34	0,8	-	-	No	36.4
14	M	62	327	75-212	1,03	148	97/145/53	Yes	38	1	14	6	Yes	40.6
15	M	47	380,6	88,3-209,9	1,8	79	84/210/49	No	36	0,8	-	-	No	37.8

P: patient; M: male; F: female; RV: reference value; GH: growth hormone; FG: fasting glucose; T: triglycerides; TC: total cholesterol; AST: serum aspartate transaminase; ALT: alanine transaminase, reference value of hepatic aminotransferases (ALT and AST) for men and women 17-59 and 14-36 U/L; 21-72 and 9-52 U/L, respectively; DM: Diabetes Mellitus; -: not available

Table 2. Difference In lipophilic antioxidants, total antioxidant capacity, malondialdehyde and DNA damage between control and acromegaly groups

	Control (19)		Acromegaly (15)		p-value
Lutein #	5.24	(4.2- 8.0)	1.902	(0.8-3.4)	<0.001
Cryptoxanthin #	7.62	(4.85-29.4)	4.067	(1.57-14.5)	0.022
α-Carotene *	4.89	2.864	5.62	1.938	0.404
β-Carotene#	8.1	(5.58-10.62)	9.39	(6.95-12.55)	0.367
Lycopene#	2.8	(0.52-9.7)	38.01	(16.42-49.34)	<0.001
Carotenoids*	46.03	32.653	62.79	29.847	0.133
Retinol#	64.13	(46.82-88.12)	2.82	(1.68-3.72)	<0.001
α-Tocopherol *	423.7	228.992	992.4	386.786	<0.001
TAP (%)*	43.50	14.716	51.29	10.446	0.093
MDA#	0.977	0.648-1.177	0.400	0.326-0.475	<0.001
DNA damage (%)*	47.2	8.7	41.4	13.8	0.15

** Test t of Student, mean (standard deviation)*
 # Test of Mann Whitney, medium (25% - 75%)
TAP, total antioxidant capacity; MDA, malondialdehyde
Unit: µg/dL

present a significant difference between the studied groups, the reported results are opposite to those obtained in our study. Despite our study population has similar cardiovascular risk than this cross-sectional study (frequency of arterial hypertension, diabetes mellitus and dyslipidemia), its percentage of uncontrolled acromegaly patients was higher than in our study (80% vs. 20%). This difference between the two studies may have been responsible for the discordant OS values. Another study also measured the OS levels in the serum of patients with acromegaly, and the authors concluded that the increased levels of IGF-I were associated with enhanced OS [23]. Additionally, in the cross-sectional study 30% of the patients were smokers, whereas in our study smoking patients were excluded.

DNA damage in acromegaly patients was assessed in a study in which genotoxicity and mutagenicity in peripheral lymphocytes from acromegaly patients (with disease in activity) were determined using cytokinesis-block micronucleus assay (CBMN cyt) and evaluated oxidative DNA damage [24]. The authors showed that the frequency of micronucleus (a biomarker of breakage and/or loss of chromosomes), nucleoplasmic bridges (chromosomal rearrangement biomarker), and nuclear buds (a biomarker of amplified DNA elimination and/or DNA repair complexes) was significantly higher in the patients than in the control group ($p<0.001$, $p<0.001$ and $p<0.001$, respectively). The frequency of apoptotic and necrotic cells was also significantly higher in acromegaly patients. The authors conclude that these findings may predict an increased risk of cancer in this population. However, our results show that, compared with that in healthy individuals, DNA damage in acromegalic patients did not increase. This probably occurred because most acromegalic patients included in our study (80%) achieved disease control (IGF1 levels <1.3× the upper normal limit).

Ozkan et al. investigated the markers of early atherosclerosis, OS and inflammation in acromegaly. They showed that compared with a control group acromegaly is associated with early atherosclerosis (decreased flow mediated dilatation and increased carotid intima media thickness), but without increasing OS and inflammation parameters. These results suggest that inflammation and OS do not seem to contribute to the development of atherosclerosis in these patients [15].

Several epidemiological studies have shown that acromegaly patients have mortality rates higher than those of the general population, specially owing to cardiovascular comorbidities [1]. However, a recently published systematic review pointed out that this mortality decreased in the last decade as a result of more frequent use of SAs as adjuvant therapy in patients not controlled with surgery [25]. Similarly, our results show that negative outcomes in acromegaly are strongly related to disease control.

4. CONCLUSION

In conclusion, acromegaly patients did not present significantly higher levels of OS, antioxidants, and DNA damage. However, most patients included in our study achieved disease

control, which in fact influenced these findings. As the present work was performed in a small cohort, these results should be confirmed in a larger number of patients.

CONSENT AND ETHICAL APPROVAL

This study was approved by the institutional ethics committee and participants provided informed consent. All procedures performed in the participants were in accordance with the ethical standards of institutional and national research committees (reference number 4130-2012) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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