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Ascertaining serum levels of trace elements in melanoma patients using PIXE and HR-ICPMS

S. Bernardes [a,*], M.H. Tabacniks [a], I.D.A.O. Santos [b], A.F. Oliveira [b], J.N. Shie [b], J.E.S. Sarkis [c], T. Oliveira [c]

[a] Physics Institute, University of São Paulo, Brazil
[b] São Paulo Federal University (UNIFESP), São Paulo, Brazil
[c] Institute of Energy and Nuclear Research (IPEN), Laboratory of Chemical Characterization (LCQ), Center for Chemistry and the Environment (CQMA), São Paulo, Brazil

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ABSTRACT

Melanoma is a serious and deadly form of skin cancer. However, patients' chances of survival and recovery are considerably increased when it is diagnosed and treated in its early stages. In this study, trace element concentrations in serum samples from patients with melanoma were measured using PIXE (Proton Induced X-ray Emission) and HR-ICPMS (High-Resolution Inductively Coupled Plasma Mass Spectrometry), with the purpose of correlating these concentrations with the disease. Blood samples from 30 melanoma patients and 116 healthy donors were collected at São Paulo Hospital (protocol CEP 1036/08 UNIFESP). Relevant clinical information on the patients has also been included in the statistical analysis. Analysis of the control group showed different P and Mg concentrations in individuals above and below 40 years of age. P, S, Ca, Cu and Zn concentrations in healthy individuals differed according to gender, highlighting the necessity to include age and gender variables in the case-control analysis. There were also differences in K, S, Ca and Se concentrations between the control and melanoma groups.

1. Introduction

Bio-essential trace elements play a fundamental role in several biological or physiological functions. It is already well-known that various organic functions depend on concentration levels of several elements contained in cells or organs [1]. It is also known that alterations in the concentration of major elements in organic tissues and fluids can be linked to several diseases and can be used to evaluate the clinical picture of individuals, providing important data for the diagnosis of clinical disorders and intoxications [2–4]. It is also recognized that many elements may either be beneficial or harmful, depending on their concentration levels [5]. Furthermore, individual factors such as gender, age, place of residence and nutritional habits also influence the levels of these elements and must therefore be taken into account and, if possible, filtered out for a reliable diagnosis [6]. Some studies have also established a link between trace elements and cancer [4,7–10,11,14].

Melanoma is one of the most aggressive forms of cancer in humans, since it is highly invasive and progresses quickly [15]. Its aggressiveness is characterized by the ability of melanoma cells to invade tissues and to metastasize. In recent decades, the rate of melanoma has risen sharply among the world's population for reasons that are not entirely understood. It is known, however, that exposure to the sun and genetic factors are important precursors of melanoma [11–13].

In this study, concentrations of elements in serum samples from 30 melanoma patients and 116 healthy control donors living in the city of São Paulo, Brazil, were measured using PIXE and HR-ICPMS. The original objective of this study was to contribute to melanoma diagnosis and to search for correlations between trace element concentrations and melanoma, and/or the stage of the disease.

2. Materials and methods

2.1. Sample collection and storage

A total of 30 patients with melanoma and 116 healthy control subjects were selected for this case-control study. Blood samples were collected at São Paulo Hospital in 6 ml BD Vacutainer tubes without any additives. The melanoma group samples were collected immediately prior medication and surgery and the control blood samples were taken from the blood bank. Serum was obtained by centrifugation at 4500 rpm for 15 min. After separation, the serum samples were frozen at −20 °C and stored until sample preparation for analysis. All material used was sterile and disposable.

2.2. Sample preparation

The samples were prepared according to three different methods: external elemental standard addition, with and without acid

* Corresponding author. Tel.: +55 11 984907308.
E-mail addresses: suene@if.usp.br, suenebernardes@yahoo.com.br (S. Bernardes).

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Table 1
Comparison between measured and SRM certified values and the combined *z* score.

Element	Certified (range)	Measured (SD)[a], *N* = 10	*z*-score	Precision (%), *N* = 9
P (g kg^{-1})	10.4 (0.5)	10.4 (0.5)[d]	1.0	5
S	6.50 (6.00–7.00)	6.21 (0.14)[c]	2.0	3
K	2.50 (2.10–2.70)	2.17 (0.16)[c]	1.9	4
Ca	0.29 (0.23–0.33)	0.287 (0.014)[c]	−0.05	5
Cu (mg kg^{-1})	2.12 (0.11)	2.28 (0.28)[b]	−0.5	4
Zn	2.43 (0.22)	2.56 (0.03)[b]	0.6	9
Se	0.276 (0.019)	0.280 (0.012)[b]	0.2	4

[a] Mean (standard deviation).
[b] Serum reference material.
[c] Whole blood SRM.
[d] Bovine liver.

digestion for PIXE analysis, and dilution for HR-ICPMS. Reference materials and blank solutions were prepared in the same manner for control and calibration purposes.

2.2.1. Standard addition

The samples for PIXE analysis were prepared by adding 80 μl of Gallium (certified 1020 mg L^{-1}) to 1 ml of serum. After mixing, 8 μl of the Gallium-doped-serum-sample was micropipetted onto a 10 μm thick Nuclepore film, previously stretched and glued onto 25 mm diameter plastic rings. The pipetted samples were left to dry at room temperature on a clean bench with a laminar airflow (HEPA filtered), and subsequently analyzed by PIXE for identification of S, Cl, K, Ca, Cu, Zn and Br.

2.2.2. Acid digestion

Acid digestion was used to decrease the amount of organic compounds, thus concentrating the trace elements in the sample [16,17]. The disadvantages of this technique include the possibility of significant contamination and loss of volatiles such as Br and Cl. Serum left over from the first preparation (serum with Ga) was freeze-dried and digested by adding 1 ml of 14 mol L^{-1} nitric acid, encapsulated in a Teflon digestion cell at 110 °C for 12 h. Eight microlittre of the digested samples was pipetted onto Kimfol film (220 μg cm^{-2} Kimberly–Clark) and analyzed by PIXE for P, S, K, Cu and Zn.

Table 2
Control group stratification by age according to *t* and *U* test on serum analysis.

Element	Group 1 (<40, age = 28 ± 6) Mean (SD)	*N*	Group 2 (>40, age = 50 ± 7) Mean (SD)	N	*t* test(*p*)
Mg (mg kg^{-1})	17.5 (1.6)	57	18.3 (1.4)	32	0.02
P (mg kg^{-1})	87 (12)	73	93 (14)	43	0.02

N is the number of samples.

2.2.3. Dilution for HR-ICPMS

Serum samples and calibration solutions were diluted to 1:20 in 1% HNO_3. All solutions were spiked with a standard elemental mixture to correct for non-spectral interferences and to control signal stability. Hence, the final solution also contained 10 μg L^{-1} of Sc, 1 μg L^{-1} of Rh, and 1 μg L^{-1} of In. The Rh was used for Se signal correction, the In for Cu and Zn corrections and Sc for Mg correction.

2.3. Analytical techniques

PIXE analysis was carried out in the Laboratory for Material Analysis (LAMFI-USP), with a 5SDH Pelletron accelerator using a 2.4 MeV proton beam. The X-rays were measured with two Kevex Si (Li) detectors with a 65 m Be filter and a 290 m Mylar filter, respectively, generating low-energy and high-energy X-ray spectra. The samples were measured for 10 min with a 3 nA beam current and 8 elements were detected (P, S, Cl, K, Ca, Cu, Zn and Br). The X-ray spectra were analyzed using AXIL [18].

HR-ICPMS analysis was carried out at the Chemical Characterization Laboratory (LCQ–CQMA), Institute of Energy and Nuclear Research (IPEN) in São Paulo, Brazil, using a high resolution double-focusing magnetic sector ICP mass spectrometer (Element-1, Finnigan MAT, today Thermo Fisher Scientific, Bremen, Germany),

Table 4
Factorial analysis correlations from the control group.

Element	Control Group	
	F1	F2
S	0.84	
Ca	0.75	
Zn	0.70	
Cu	−0.60	
Gender	−0.60	
Mg		0.72
Age		0.66
KMO = 0.62		

Table 3
Control group stratification by gender according to *t* and *U* test on serum analysis.

Element	Male (age = 39 ± 15, *N* = 56) Mean (SD)	Female (age = 42 ± 15, *N* = 60) Mean (SD)	*t* test (*p*)
P (mg kg^{-1})	86 (13)	92 (12)	<0.01
S (g kg^{-1})	1.00 (0.07)	0.96 (0.08)	<0.01
Ca (mg kg^{-1})	91 (4)	89 (5)	<0.01
Cu (mg kg^{-1})[a]	0.92 (0.52–2.53)	1.23 (0.74–2.46)	<0.01
Zn (mg kg^{-1})	0.84 (0.16)	0.76 (0.12)	<0.01

N is the number of samples.
[a] Median (range) values and *U* test of Mann–Whitney.

Table 5
MANOVA analysis of melanoma and control groups, including clinical information.

All samples (male and female)						
Element	Melanoma (mean error)	Control (mean error)	Variable[a]	*F* test	*p* value	*N* (M/C)[a]
K (mg kg^{-1})	144 ± 7	133.4 ± 1.0	TNM_4	2.62	0.03	5/116
	146 ± 4		TH_4	3.04	0.02	8/116
Se (μg kg^{-1})	62 ± 4	70.8 ± 1.6	Mit	6.24	0.01	18/89
		Male				
S (g kg^{-1})	0.90 ± 0.05	0.999 ± 0.009	TNM_2	3.29	0.02	3/56
	0.78		L	4.00	0.01	8/56
	0.93 ± 0.04		Mit	6.31	0.02	9/56
Ca (mg kg^{-1})	86.6 ± 1.6	91.2 ± 0.5	Group	11.4	<0.01	14/56
	85.6 ± 2.3		TNM_3	4.10	<0.01	7/56
	85 ± 3		TH_3	3.82	0.01	5/56
	86.0 ± 2.1		B_3	3.51	0.02	8/56
	86.4 ± 1.3		L_1	5.95	<0.01	5/56
	84 ± 6		L_2	5.95	<0.01	2/56
	86.7 ± 2.2		U	7.97	<0.01	10/56
	85.3 ± 2.0		Mit	13.80	<0.00	9/56
		Female				
Ca (mg kg^{-1})	88.8 ± 0.6	82 ± 3	B_3	3.01	0.036	3/60

[a] TH = histological type of tumor, TNM = level of melanoma, B = breslow depth scale, L = number of lymph nodes, Mit = mitosis, U = ulceration, N = number of sample, M = melanoma and C = control.

coupled to a cyclonic spray chamber and a Meinhard nebulizer for sample injection. Operational conditions were 1.1 kW r.f. power, and 1.2 L min^{-1} Argon carrier, and an electron multiplier detector (for low and high counting rates).

2.4. Certification

The analytical methods were assessed by analyzing reference materials (CRMs) from three different sources: IAEA A-13 Freeze Dried Animal Blood, QMEQAS08S-06 liquid Human Serum (National Institute of Public Health – Québec, Canada), and Bovine Liver (NIST 1577b). PIXE and HR-ICPMS results of the CRMs are presented in Table 1. Experimental standard deviations were calculated over replicated samples of the CRMs, as described in Table 1. Analytical precision ranged from 3% to 9%. *Z*-scores of CRM measurements are between −0.5 and 2.0.

3. Results and discussion

Evaluation of the control group showed differences in P and Mg concentrations among individuals above and below 40 years of age (see Table 2). Additionally, P, S, Ca, Cu and Zn concentrations in healthy individuals showed statistically significant differences according to gender, highlighting the need for including age and gender variables in case-control analyses (Table 3). A factorial analysis was executed to complement the control group evaluation. The results indicated correlations between gender and S, Ca, Cu and Zn concentrations (Table 4). Moreover, correlations between Mg and age were also observed. The possibility of applying factorial analysis to our data was ascertained using the Kaiser-Meyer-Olkim test (KMO), which needs to be greater than 0.5.

The comparison between the selected trace elements measured in serum in the melanoma and control groups is summarized in Table 5. The following clinical information on the patients was also included in the statistical analysis: histological tumor type, level of melanoma, Breslow depth scale, number of lymph nodes, presence or absence of mitosis and/or ulceration. MANOVA analysis indicated a minor difference between the melanoma and control data for K and Se. For the male cohort, a difference between S and Ca levels was found. Furthermore, the female cohort presented differences between the melanoma and control groups in Ca levels only, considering the Breslow scale ($p < 0.05$).

4. Conclusions

There are still few studies correlating trace element concentrations in human fluids and tissues with melanoma. While Bergimi et al. observed high Cu levels and low Fe values in the toenails of melanoma patients [11], Zn measured in serum has been linked to the disease by Ros-Bullon et al. [14]. In this study, P and Mg concentrations in the control group proved to be age-dependent, while P, S, Ca, Cu and Zn concentrations in healthy individuals proved to be gender-dependent. Both observations stress the necessity of inserting *age* and *gender* variables into the case-control statistical analysis, thus reinforcing data from Bárány et al. [6] regarding this aspect.

The present study shows that the concentrations of K, S, Ca and Se differ between the control and melanoma groups, when considering the clinical variables of the patients ($p < 0.05$). The consequent need to stratify the database by *age*, *gender* and *stage* of cancer critically reduced the already limited number of samples, thus compromising the interpretation of the small differences found in the concentrations of trace elements. In spite of the importance of this kind of research, which complements and adds to established knowledge about the disease, the results proved to be of limited use in the positive diagnosis of the illness. Furthermore, the differences between this work and literature should be investigated more thoroughly.

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