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Environmental exposure to trace elements and risk of cutaneous melanoma

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Purpose: Our aim was to examine the risk of melanoma in association with exposure to trace elements of toxicological and nutritional interest.
Methods: We analyzed the concentrations of cadmium, lead, chromium, selenium, copper and zinc in toenails of 58 patients with newly diagnosed cutaneous melanoma as well as in 58 age- and sex-matched control subjects, randomly selected from the population of Modena province in northern Italy.
Results: Melanoma risk was substantially unrelated to toenail levels of cadmium, chromium, lead and selenium. Subjects with higher toenail copper levels showed an excess risk, both in the crude analysis and after adjusting for sun exposure and level of education, while in both analyses high iron concentrations were associated with a decreased risk of the disease. A weak direct association between zinc levels and melanoma risk also emerged in the multivariate analysis.
Conclusions: Overall, these results do not suggest an involvement of heavy metals in melanoma etiology, while they do give some support to a possible role of zinc and, in particular, copper and iron exposure in influencing disease risk. However, these findings must be evaluated with caution due to the limited statistical stability of the point estimates.
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Introduction

Epidemiologic studies indicate that melanoma incidence is still growing in Western countries, which is likely due to a real increase in disease occurrence and not just to early detection (Vinceti *et al*., 1999; de Vries *et al*., 2003; Lens and Dawes, 2004). This observation has prompted the search for environmental risk factors of the disease and apart from the effect of ultraviolet light, the possible role of exposure to chemicals has also been investigated (Linet *et al*., 1995; Desmond and Soong, 2003). Clearly, trace elements are also among these potential environmental risk factors, since exposure to minerals with both nutritional and toxicologic properties, such as zinc (Zn), copper (Cu), selenium (Sc) and iron (Fe), and heavy metal lead (Pb), have been linked to changes in melanoma risk (Bain *et al*., 1993; Nielsen *et al*., 1996; Ros-Bullon *et al*., 1998; Desmond and Soong, 2003). Very few epidemiologic studies have been conducted on these topics, however, and the results have been inconsistent particularly for selenium (Desmond and Soong, 2003).

We carried out a population-based case–control study to investigate the role of environmental exposure to a few trace elements of toxicologic and nutritional interest in melanoma etiology.

Methods

We performed a case–control study in the population of the province of Modena in northern Italy (around 650,000 inhabitants). For a consecutive period of 3 years, we recruited all the patients newly diagnosed with cutaneous melanoma attending the Dermatologic Clinic of Modena University Hospital, which is the only center for diagnosis, therapy and follow-up of the disease within the province. Inclusion criteria were residence in Modena province and a diagnosis of histologically based cutaneous melanoma but in the absence of clinical evidence of metastasis. The fifty-nine out of the eighty-two patients (72.0%) eligible to participate in the study accepted to do so.

For each patient included in the study, we randomly extracted from the database of all residents in Modena Province 10 subjects having the same gender and born ± 5 years around the year of birth of the patient. The database

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for Modena province residents was provided by the Local Health Authority, and the data extraction was carried out by using a computer-generated algorithm for random sampling, with the iterative procedure for each case–control data set being repeated 10 times. We then contacted the eligible controls by telephone, proceeding chronologically through the list until one could be recruited in the study. The percentage of controls able to be contacted and who eventually participated in the study was 56.3% of all the subjects we attempted to enrol.

All case and control subjects were enrolled after having been informed of the protocol and the aims of the study and having given informed written consent to their participation. The study protocol included sampling of blood and toenails, a dermatological examination and administration of questionnaires on anamnestic data, their regular diet and lifestyle habits (including detailed information about sun exposure, such as total number of days with sun exposure, history of sunburn, usage of sunscreens). Socioeconomic status was estimated by collecting data on education attainment and current and previous occupational status. Questionnaires were administered with face-to-face interviews except for the dietary questionnaire, which was given to the patients for self-completion. We attempted to enrol each of the study control subject no later than 1 month following the enrolment date of the matched patients, although in some cases the controls were recruited up to 3 months after the corresponding cases.

Toenail clippings were kept separate for the two feet in nonreactive plastic envelopes and stored at room temperature until analysis. We used inductively coupled plasma optical spectrometry to measure Cd, Pb and Cu concentrations in left foot toenails. Specimens were washed using a Triton X-100 (5% in deionized water) solution for 15 min in an ultrasonic sonicator, and then deionized water for the same time. After filtration on paper filter, the toenails were dried at 100°C for 2 h. The samples, weighing from 100–200 mg, were digested with 2.5 ml HNO_3 (nitric acid) + 7.5 ml of deionized water in a Perkin–Elmer Multiwave microwave digestion system, using PTFE/TFM vessels and operating condition of 170°C and 15 bar. The period of digestion was about 1 h. Cooled samples were transferred into 10 ml measuring flasks, diluted as needed, and analyzed for Cd, Pb and Cu content using inductively coupled plasma optical spectrometry, using a Perkin-Elmer Optima 3000 XL spectrometer equipped with a Perkin-Elmer AS91 autosampler and a CETAC U-5000T + Ultrasonic Nebulizer.

Instrumental neutron activation analysis was used to determine the elemental concentrations of Chromium (Cr), Fe, Se and Zn. Toenail clippings from the right foot were cleaned with diethyl-ether, bidistilled water and acetone; after the cleaning process, the samples were dried at 50°C for 72 h. The samples (dry weight ranging from 10 to 30 mg) were then irradiated at the reactor BERII of the Hahn-Meitner Institute in Berlin. The samples were irradiated for 7 days at a position with a thermal flux density of $6 \times 10E^{12}$ $1/cm^2 s$ in precleaned quartz vials. After a cooling time of about 4 days, the activity was measured by means of a high-purity Ge-detector.

Suitable multielement standards, made of highly pure chemicals, were used to calculate the elemental concentrations. For quality control, we used the human hair GBW 09101 and the bovine liver NIST 1577b as reference material. All measurements were carried out by simultaneously analyzing the matched sets, that is, a case with the corresponding control, in the same batch. Owing to problems in sensitivity of the analytical methodology, we were unable to measure levels of Cd, Pb and Cu in 36, nine and two subjects, respectively. In these individuals, we inputted half of the lowest detectable value as defined by the sampling and the analytical method for the corresponding element. Analytical determination of a few elements measured through instrumental neutron activation analysis could not be performed in one control subject, due to the loss of the samples during their preparation. In another control, the extremely high values obtained in the determination of Cd, Pb, and Cu led to the exclusion of the individual from the entire analysis.

We computed the odds ratio of melanoma, as an estimate of the relative risk, according to the toenail trace element concentrations, using a conditional logistic regression model with age and gender as matching variables. We divided the subjects into two categories using as cutoffs the median of distribution of trace elements, based on distribution in controls. We also entered the trace elements in the model concentrations as continuous variables to compute *P*-values for linear trend. We repeated these calculations including the analysis of educational attainment as a proxy of socioeconomic status, total number of atypical nevi and amount of sun exposure (estimated as total number of days of high sun exposure per year for leisure or occupational reasons). Since we could not rule out a possible relation between trace element exposure and risk of atypical nevi, to avoid the risk of overmatching we also carried out the multivariate analysis by controlling only for education and sun exposure.

Results

We report in Table 1 the 25th, 50th and 75th percentiles of the distribution of the trace element content determined in toenails of the study subjects. Concentrations of trace elements were roughly comparable between cases and controls, although patients exhibited higher median levels of Cu and lower concentrations of Fe compared with the control group. Table 2 shows the odds ratio of melanoma according to toenail trace element content category, with and without adjustments for potential confounders. No substantial association was found between disease risk and

Table 1. Centile distribution of trace elements concentrations[a] in toenails of melanoma patients and population controls.

Element	N	25th	Centile 50th	75th
Cadmium				
Controls	58	0.0142	0.0226	0.0406
Cases	58	0.0096	0.0178	0.0298
Chromium				
Controls	58	0.53	1.40	3.78
Cases	55	0.36	1.20	3.17
Copper				
Controls	57	1.099	2.797	4.186
Cases	58	3.071	3.537	3.950
Iron				
Controls	57	18.0	23.0	32.0
Cases	55	9.0	14.0	22.5
Lead				
Controls	50	0.201	0.513	1.079
Cases	46	0.185	0.368	0.897
Selenium				
Controls	58	0.57	0.65	0.73
Cases	55	0.57	0.64	0.71
Zinc				
Controls	58	102.8	112.0	127.3
Cases	55	106.0	116.0	124.5

[a]Concentrations expressed as μg/g.

Table 2. Risk of melanoma according to toenail concentrations of trace elements.

Element	Odds ratio[a]	*P*	*P* trend[b]
Cadmium			
Crude	0.8 (0.4–1.6)	0.494	0.181 (+)
Adjusted[c]	0.7 (0.4–1.4)	0.347	0.196 (+)
Adjusted[d]	0.7 (0.3–1.9)	0.518	–[e]
Chromium			
Crude	0.8 (0.3–1.8)	0.533	0.475 (–)
Adjusted[c]	0.8 (0.3–2.0)	0.660	0.654 (–)
Adjusted[d]	0.9 (0.2–3.2)	0.812	0.663 (–)
Copper			
Crude	6.0 (2.1–17.3)	0.001	0.145 (+)
Adjusted[c]	9.8 (2.6–36.2)	0.001	0.135 (+)
Adjusted[d]	15.5 (1.7–142.6)	0.015	0.123 (+)
Iron			
Crude	0.3 (0.1–0.6)	0.002	0.312 (–)
Adjusted[c]	0.3 (0.1–0.6)	0.007	0.414 (–)
Adjusted[d]	0.4 (0.1–1.4)	0.151	0.395 (–)
Lead			
Crude	0.6 (0.2–1.4)	0.207	0.541 (–)
Adjusted[c]	0.6 (0.3–1.3)	0.194	0.444 (–)
Adjusted[d]	0.7 (0.2–2.8)	0.573	0.990 (–)
Selenium			
Crude	0.8 (0.4–1.7)	0.565	0.274 (–)
Adjusted[c]	0.7 (0.3–1.7)	0.463	0.407 (–)
Adjusted[d]	0.7 (0.3–2.0)	0.548	0.381 (–)
Zinc			
Crude	1.7 (0.7–3.8)	0.226	0.437 (–)
Adjusted[c]	1.8 (0.7–4.3)	0.212	0.403 (–)
Adjusted[d]	3.5 (1.0–12.6)	0.056	0.477 (+)

[a]Odds ratios comparing the category having toenail concentrations $\geq$median levels vs. the remaining category; 95% confidence limits are given in parentheses.
[b]*P* for linear trend (direction of the association is given in parentheses: "+" for direct and "–" for inverse relation).
[c]Adjusted for education and sun exposure.
[d]Adjusted for education, sun exposure and total number of atypical nevi.
[e]Computationally invalid estimate.

concentrations of Cd, Cr, Pb, and Sc, both in the unadjusted and adjusted analyses. We detected an excess melanoma risk among subjects included in the highest category of Cu exposure, the adjusted point estimates being higher than the unadjusted one. The risk was also increased in subjects with the highest Zn toenail content, particularly when all the potential confounders were included in the analysis. On the contrary, toenail Fe levels equal to or higher than the median were associated with a decreased melanoma risk, although adjustment for a number of atypical nevi considerably reduced the statistical precision of the estimates obtained in the crude analysis or in a simpler multivariate model. When toenail trace element concentrations were entered in the logistic regression model as continuous variables, no clear association with disease risk emerged. In this analysis, however, we were unable to obtain a computationally valid estimates for Cd.

Discussion

Among the limitations of the current study, we outline its low statistical power, which is reflected in the wide confidence intervals of the point estimates. This appears to be mainly a consequence of the limited study size, and suggests the need to confirm the results of our investigation in larger studies, which might also allow detailed analyses in specific subgroups of subjects. Another potential limitation of the study was the rather low participation rate of the population controls, despite our effort to keep it as low as possible, although this rate was similar to that obtained in prior population-based case–control investigation carried out in Modena province (Bergomi *et al.*, 2002).

We also acknowledge the risk of some exposure misclassification in the present investigation. We chose toenails as biomarkers of exposure considering the suitability of this indicator for epidemiologic research and its potential ability

to reflect overall mid-term exposure, ranging from 6 to 18 months for the different trace elements (Wilhelm *et al*., 1991; Bergomi *et al*., 2002). However, the little epidemiologic evidence available so far suggests that toenails may be a suitable indicator of mid-term intake of Cu, Cr, Zn, Fe (Garland *et al*., 1996) and Se (Krogh *et al*., 2003), while sparse data are available on the relation between Cd exposure and nail concentrations (Wilhelm *et al*., 1991). Conflicting results have also been obtained concerning the use of toenails as biological indicators of Pb exposure (Wilhelm *et al*., 1991; Bu-Olayan *et al*., 1996; Gulson, 1996). We also recognize that toenails may not reflect long-term exposure to trace elements, particularly several years before their sampling, an important limitation for subjects experiencing marked changes in exposure over time.

Two strengths of the present study are the population-based design and the use of a biomarker likely reflecting overall environmental exposure. No population-based study investigation seems to have so far examined environmental exposure to heavy metals and other trace elements as a risk factor of melanoma, with the exception of some prospective studies examining association between Se exposure and subsequent melanoma risk. Furthermore, controls in previous case–control investigations were hospital-referred subjects, blood donors or so-called ''healthy subjects'', and such control groups are at risk of selection bias. Moreover, most studies evaluated body levels of trace elements using short-term indicators of exposure, such as serum or blood concentrations, or assessment of dietary intake. However, diet is not the only source of exposure for most elements of nutritional and toxicological interest, and in some cases, exposure through other sources (dermal and respiratory routes) may be of great importance. So, misclassification of exposure might have been responsible for the heterogeneous results obtained in previous investigations.

Even if the present investigation has a case–control design, we consider it unlikely that some common limitations of this type of study might have affected our results. First, we selected patients clinically free of metastasis, to reduce the risk of disease-induced changes in tissue mineral content. There is also no reason to hypothesize that the diagnosis of melanoma induced changes in patients' dietary habits or in lifestyle, thus modifying the usual exposure to trace elements, since diet and lifestyle habits (apart from sun exposure) are not generally believed to play any role in melanoma etiology.

Although there is some indication from the epidemiologic literature of increased melanoma risk among subjects employed in the chemical industry (Linet *et al*., 1995), including lithographers (Nielsen *et al*., 1996) and subjects exposed to welding and soldering fumes and therefore to Pb (Rockley *et al*., 1994), no convincing evidence of the environmental toxins potentially involved has so far emerged. Results of our study do not support the hypothesis that exposure to some heavy metals, namely Cd, Pb, and Cr, may enhance melanoma risk, although the above-mentioned limitation of the study suggests that caution should be exercised in evaluating these findings.

We did, however, find an excess melanoma risk in subjects having the highest toenail Cu levels. Serum Cu levels in patients were found to be unchanged compared to ''healthy subjects'' (Ros-Bullon *et al*., 1998) or to blood donors (Kolaric *et al*., 1975), and they appeared to increase in relation with degree and extent of melanoma activity (Fisher *et al*., 1981). Our findings concerning Cu, when exposure was categorized into two categories, must be evaluated with caution, also considering that there was limited evidence of a linear association between toenail levels and disease risk. Despite these caveats, in view of the established ability of Cu to induce oxidative stress, a possible pathogenetic mechanism of melanoma (Farmer *et al*., 2003; Sander *et al*., 2004), an association between excess Cu exposure and melanoma risk should also be considered to explain our findings. Our findings might also be explained by an abnormal metabolism of this element, since in melanoma patients it has been described as an abnormal expression of metallothioneins, ubiquitous proteins with high affinity for metals such as Cu, whose higher concentrations might increase body content of this element (Weinlich *et al*., 2003).

We also observed that an association between high Zn exposure and melanoma risk, although this relation was statistically unstable, could be detected only in the multivariate analysis, after controlling for potential confounders including number of atypical nevi, and was not present in the trend analysis. In previous studies, serum Zn concentrations were found to be increased (Siu *et al*., 1991; Ros-Bullon *et al*., 1998) or decreased (Horcicko and Pantucek, 1983) in melanoma patients. Since Zn may have pro-oxidant effects, the possibility that an excess exposure to this element or its abnormal metabolism, as previously hypothesized for Cu, might enhance melanoma risk should be considered while explaining our findings.

Se is a trace element of both nutritional and toxicological interest, whose involvement in cancer etiology and prevention has been the focus of great scientific interest (Vinceti *et al*., 2000). With specific reference to melanoma, two prospective studies, one carried out in an area very close to Modena province (Vinceti *et al*., 1998) and a report from the Nurses' Health Study (Garland *et al*., 1995), suggested that excess exposure to this element might enhance the risk of this site-specific cancer. However, other case–control studies were consistent with the lack of association between this metalloid and disease risk, or with a beneficial effect of Se in melanoma prevention (Vinceti *et al*., 2000). Our results do not lend support to an involvement of Se intake in modifying melanoma risk. However, nearly all analytical methodologies, including our own, used to determine Se levels in biological matrices can measure only overall Se concentrations, and total tissue Se content has been shown to poorly

reflect intake of some chemical forms of Se (Vinceti *et al.*, 2000), each of which has distinctive toxicological and nutritional characteristics. For example, the excess melanoma risk detected in a prior study (Vinceti *et al.*, 1998) was associated to exposure to the inorganic hexavalent form of this element, selenate, whose intake induces peripheral tissues Se concentrations considerably lower than do equivalent amounts of organic Se species (Vinceti *et al.*, 2000). This suggests that toenail and more generally overall tissue Se content has a strong potential for exposure misclassification when looking at exposure to single Se species.

The reduced melanoma risk in subjects having the highest toenail Fe concentrations is interesting. A case–control study carried out in the US suggested an inverse association between Fe intake through food and disease risk (Stryker *et al.*, 1990), though overall Fe intake (i.e., intake from both food and dietary supplements) was no longer associated with disease risk. Another case–control investigation from Australia also found an inverse association between dietary Fe intake and melanoma risk (Bain *et al.*, 1993). In our study, the inverse association between toenail Fe concentrations and disease risk was considerably reduced in the multivariate analysis when the number of atypical nevi was controlled for, possibly indicating their involvement in the causal pathway between Fe exposure and disease onset, and the risk of overmatching when adjusting for this factor. Further studies on the possible relation between reduced iron status and melanoma etiology appear to be warranted.

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