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Immunological characterization of the allergic contact mucositis related to the ingestion of nickel-rich foods

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ABSTRACT

Background: The ingestion of nickel (Ni)-rich foods may result in allergic contact mucositis (ACM), a not yet well defined condition identifiable by oral mucosa patch test (omPT). Our aim was to characterize immunologically the ACM taking advantage from the allergen exposure that occurs during the omPT for Ni.

Methods: Thirty-seven symptomatic patients underwent to omPT for Ni. Before and after omPT, serum and urine Ni concentrations were determined by mass spectrometry, the white blood cells were counted by hemochromocytometric assay, the peripheral lymphocyte typing was carried out by flow cytometry, total IgE and cytokine serum concentrations were measured by immunoenzymatic assays. The local lymphocyte typing was performed by immunohistochemistry only after omPT.

Results: According to the omPT outcomes, 25 patients were defined as Ni-sensitive and the remaining 12 as controls. After omPT, serum and urine Ni concentrations increased significantly in all patients, while a significant increment of circulating lymphocytes and neutrophils was highlighted, respectively, in Ni-sensitive and control patients. Consistently, the Th and Tc circulating lymphocytes, as well as the Th/Tc ratio increased significantly in Ni-sensitive patients after omPT. No noteworthy increment in serum concentrations of total IgE and selected cytokines was observed in any patient after omPT. The presence of CD3+, CD4+, and CD8+ cells was highlighted on the oral mucosa biopsy samples taken from Ni-sensitive patients after omPT.

Conclusions: In patients with ACM, a local adaptive response with increased lymphocyte trafficking appears to be the most likely mechanism of reaction to Ni administered with the omPT.

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Abbreviations: A_{450nm}, absorbance at 450 nm; ACD, allergic contact dermatitis; ACM, allergic contact mucositis; Ags, antigens; ANOVA, analysis of variance; APC, allophycocyanin; DTH, delayed-type hypersensitivity; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ePT, epicutaneous patch test; EQA, external quality assessment; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; ICDRG, International Contact Dermatitis Research Group; ICP-MS, inductively coupled plasma-mass spectrometry; IQA, internal quality assessment; mAbs, monoclonal antibodies; Ni, nickel; NK, natural killer; omPT, oral mucosa patch test; PBS, phosphate buffered saline; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SD, standard deviation; SSC, side light scatter; Tc, T suppressor/cytotoxic; Th, T helper/inducer; TLR, Toll-like receptor; WBC, white blood cells.

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Introduction

Nickel (Ni) is a transition metal widespread in the environment, foods, drugs, chemicals, and metallic utensils which absorption may occur by inhalation, ingestion, or through the skin. In the blood, Ni is mainly bound to albumin while kidneys are the primary route of elimination (Barceloux, 1999).

This metal is an essential microelement for the homeostasis of the human organism however, it has also carcinogenic properties, can act as contact allergen and high concentrations may cause an acute toxic reaction (Barceloux, 1999; Magaye et al., 2012). In 2008, the American Contact Dermatitis Society has defined Ni as the “Contact Allergen of the Year” (Kornick and Zug, 2008). The prevalence of Ni allergy is indeed growing in many countries and represents a major health and socioeconomic issue. Researches published over the last forty years from North America to Western Europe highlight a median prevalence of 8.6%, with values higher among women than men (17.1% vs. 3%) (Darlenski et al., 2012; Schram et al., 2010; Thyssen et al., 2007).

The main clinical manifestation caused by skin contact with Ni is an allergic contact dermatitis (ACD), for which the epicutaneous patch test (ePT) is considered the gold standard for diagnosis (Mark and Slavin, 2006). Similarly, Ni exposure through the intestinal mucosa can result in a systemic hypersensitivity with dermatologic manifestations or symptoms such as diarrhea, headache, and general discomfort (Darlenski et al., 2012; Hsu et al., 2011; Matiz and Jacob, 2011). The allergic contact mucositis (ACM) has been recently described as a systemic Ni hypersensitivity identifiable by the oral mucosa patch test (omPT). In patients with ACM, the ingestion of Ni-rich foods causes the onset of intestinal and/or extraintestinal manifestations while, after a reasonable period of low-Ni diet, they report an improvement in symptoms and restoring of well-being (Picarelli et al., 2011). Nevertheless, further studies are necessary to confirm and extend these findings and their implications on human health.

Ni allergy is a T lymphocyte-driven delayed-type hypersensitivity (DTH) characterized by leukocyte infiltration at sites of allergen exposure. In detail, Ni ions bind directly to Toll-like receptor 4 (TLR4) expressed on monocytes, dendritic and endothelial cells providing a molecular signal that, in synergy with the DTH response triggered by Ni-haptenated proteins, leads to Ni allergy (Rothenberg, 2010; Schmidt and Goebeler, 2011; Wang and Dai, 2013). However, the current knowledge come from studies performed on patients with ACD and therefore, the immunological mechanisms underlying systemic Ni hypersensitivity still need to be characterized.

Based on these observations, our aim was to characterize immunologically the ACM related to the ingestion of Ni-rich foods by evaluating the DTH response typical of ACD, an eventual immediate hypersensitivity reaction, and the expression of TLR4 and specific cytokines after the allergen exposure that occurs during the omPT for Ni.

Materials and methods

Patients

A total of 37 consecutive outpatients (2 males/35 females, mean age 37.5, range 19–58 years), who from January to December 2012 were referred to our Gastroenterology Unit with intestinal and/or extraintestinal symptoms (e.g. diarrhea, abdominal pain and swelling, hives, itching, and headache) referable to the ingestion of Ni-rich foods, were enrolled in the study. In detail, the relationship between reported symptoms and Ni-rich food ingestion was highlighted by an anamnestic card that every patient has compiled at the time of study entry. The presence of pathological conditions that could justify the clinical picture, such as celiac disease, lactase deficiency, and allergic disorders (from the classic IgE-mediated allergies to ACD caused by metals other than Ni), was also considered.

The main clinical data of the patients being studied are summarized in Table 1.

Study design

For diagnostic purposes, each patient underwent to omPT for Ni. Both before and immediately after the omPT, every patient was submitted to blood sampling to assess the amount of Ni absorbed during the test as well as the cellular, humoral, and cytokine systemic response induced by Ni exposure. In order to evaluate the amount of Ni cleared following its eventual absorption, an urine/24 h collection was prescribed to each patient before and after the omPT. In 10 patients with positive omPT results, who have agreed to be subjected to additional procedures, a blood sample was also obtained at 48 h from the test to monitor the systemic cellular response induced by Ni exposure. In 4 patients with positive and another one with negative omPT results, who have agreed to be submitted to further procedures, an oral mucosa biopsy sample was taken immediately after the test to evaluate the local cellular response induced by Ni exposure. As a Ni-unexposed negative control, an oral mucosa biopsy sample was also taken from 3 healthy volunteers not subjected to omPT.

Table 1
Main clinical data of the patients being studied.

Intestinal symptoms		Extraintestinal symptoms		Other diseases	
	Pts/total (%)		Pts/total (%)		Pts/total (%)
Abdominal swelling	21/37 (56.8%)	Asthenia	16/37 (43.2%)	LD	12/37 (32.4%)
Abdominal pain	15/37 (40.5%)	Headache	15/37 (40.5%)	CD	7/37 (18.9%)
Meteorism	15/37 (40.5%)	Itching	12/37 (32.4%)	OAs	7/37 (18.9%)
Belching	14/37 (37.8%)	Hives	11/37 (29.7%)	T	7/37 (18.9%)
Diarrhea	13/37 (35.1%)	Dermographia	10/37 (27.0%)	ACD	5/37 (13.5%)
Dyspepsia	12/37 (32.4%)	Dizziness	10/37 (27.0%)	IgAD	2/37 (5.4%)
Stomatitis	11/37 (29.7%)	Conjunctivitis	7/37 (18.9%)	P	2/37 (5.4%)
Constipation	10/37 (27.0%)	Erythema	7/37 (18.9%)	AA	1/37 (2.7%)
Nausea	7/37 (18.9%)			V	1/37 (2.7%)
Vomiting	5/37 (13.5%)				

Each patient presented at least one intestinal symptom referable to the ingestion of Ni-rich foods, but not everyone had an extraintestinal Ni-related symptom or were suffering from another disease.

AA, alopecia areata; ACD, allergic contact dermatitis (to iron, nickel, palladium, and silver); CD, celiac disease; IgAD, IgA deficiency; LD, lactase deficiency; OAs, other allergies (to casein, cat's hair, dust, grains, latex, mites, parietaria, and pollen); P, psoriasis; T, thyreopathy; V, vitiligo.

All procedures followed in this study were in accordance with the ethical standards of the institutional committee responsible for human experimentation. An informed consent was also obtained from every patient at the time of study entry.

Identification of nickel-sensitive patients

As previously reported (Picarelli et al., 2011), the Ni-sensitive patients were identified by an omPT performed inside the upper lip after absorption of excess saliva with a sterile gauze. Briefly, a disk support consisting of filter paper (diameter: 5 mm) covered with a solution of Ni sulfate ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$) 5% in Vaseline (0.4 mg Ni sulfate/8 mg Vaseline) was placed on the test site. This support was applied through an adhesive film (3 M Tegaderm transparent film; 3 M SpA, Milan, Italy) that in turn, was supported by a small absorbent pad. The test duration was set at 2 h, after which the occurrence of any local and/or systemic reaction was evaluated. In particular, the omPT reactions were arbitrarily graded according to the International Contact Dermatitis Research Group (ICDRG) scoring system (Wilkinson et al., 1970), as following: type 0 (no reaction), type Ia (erythema and/or edema on the test site), type Ib (type Ia + any systemic reaction), type IIa (vesicles on the test site), type IIb (type IIa + any systemic reaction), type IIIa (confluent vesicles and/or ulcers on the test site), type IIIb (type IIIa + any systemic reaction). The systemic reactions included itching, headache, diarrhea, dizziness, abdominal pain and swelling, joint and muscle pain.

Determination of serum and urine nickel concentration

The whole blood specimens collected into tubes without anticoagulant were centrifuged for 8 min at 3000 rpm (1700 g). Sera were then gathered, subdivided in ten aliquots, and stored at -80°C until used. After gentle stirring, two aliquots for each urine/24 h collection were gathered and stored at -80°C until used.

The Ni concentration in serum and urine was determined by inductively coupled plasma-mass spectrometry (ICP-MS) which interfaces an inductively coupled plasma source with a mass spectrometer. The instrument used, a quadrupole mass spectrometer Thermo Scientific Instruments X-SeriesII ICP-MS (Thermo Fisher Scientific Inc., Waltham, MA), includes a sample injection system with nebulizer, an ICP torch that generates the plasma, an interface region that allows the connection between plasma and mass spectrometer, a lens system that focuses the ions into a beam that is sent to the quadrupole, and finally a detector. In general, the ions produced in the ICP torch are introduced into the quadrupole mass spectrometer that separates them according to each specific mass/charge ratio, while their detection and quantification is done into the detector. The spectra generated are composed of a series of isotopic peaks that can be used for both qualitative and quantitative measures.

Before analysis, both serum and urine samples were diluted 1:14 with double-distilled water acidified with nitric acid (HNO_3 2%). Five external standards (Carlo Erba reagents, Milan, Italy) were used to obtain a calibration curve able to correlate the signal intensities given by the instrument and the known Ni concentrations (1 – 5 – 10 – 20 – 50 $\mu\text{g/L}$). This curve was used by the system for data analysis to calculate the unknown Ni concentration in the samples analyzed. An internal standard, consisting of a monoisotopic element (^{103}Rh) with a mass not Ni-interfering, was used to correct any variations in the instrumental response.

Total and differential white blood cell count

In whole blood specimens collected into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant, the white

blood cells (WBC) and their components (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) were counted by hemochromocytometric assay on the automated cytometer ADVIA 2120 (Siemens Healthcare Diagnostics, Deerfield, IL) and expressed as cells/ μL . To ensure the achievement of correct results, this analytical system was constantly monitored by means of both internal and external quality assessment (IQA and EQA) programs.

Peripheral blood lymphocyte typing

The venous blood lymphocyte typing was performed by using the BD Multitest IMK kit for direct immunofluorescence on the BD fluorescence-activated cell sorter (FACS)Canto I flow cytometer (Becton Dickinson – BD Biosciences, San Jose, CA), able to determine the absolute number of total lymphocytes ($\text{CD}45^+$) and their subsets: T lymphocytes ($\text{CD}3^+$), T helper/inducer or Th ($\text{CD}3^+\text{CD}4^+$), T suppressor/cytotoxic or Tc ($\text{CD}3^+\text{CD}8^+$), B lymphocytes ($\text{CD}19^+$), and natural killer or NK cells ($\text{CD}16^+$ and/or $\text{CD}56^+$). In detail, the above mentioned kit contains: $\text{CD}3/\text{CD}8/\text{CD}45/\text{CD}4$ and $\text{CD}3/\text{CD}16^+\text{CD}56/\text{CD}45/\text{CD}19$ reagents encompassing the monoclonal antibodies (mAbs) specific for each lymphocyte subset conjugated with four different fluorochromes [fluorescein isothiocyanate (FITC)-labeled anti- $\text{CD}3$ (clone SK7), phycoerythrin (PE)-conjugated anti- $\text{CD}8$ (clone SK1), peridinin chlorophyll protein (PerCP)-labeled anti- $\text{CD}45$ (clone 2D1 – HLe-1), allophycocyanin (APC)-conjugated anti- $\text{CD}4$ (clone SK3), PE-labeled anti- $\text{CD}16$ (clone B73.1) + anti- $\text{CD}56$ (clone NCAM 16.2), and APC-conjugated anti- $\text{CD}19$ (clone SJ25C1)]; fixing/lysing solution containing formaldehyde (<15%) and diethylene glycol (<50%); Trucount disposable tubes with a lyophilized pellet of fluorescent microspheres.

The cell surface marking was performed by adding 50 μL of whole blood in EDTA anticoagulant and 20 μL of $\text{CD}3/\text{CD}8/\text{CD}45/\text{CD}4$ or $\text{CD}3/\text{CD}16^+\text{CD}56/\text{CD}45/\text{CD}19$ reagent on the bottom of each Trucount tube using the reverse pipetting technique. After shaking on vortex at low speed, the samples were incubated in the dark for 15 min at room temperature. Subsequently, 450 μL of fixing/lysing solution were added in each Trucount tube. After shaking and incubation carried out as described above, the marked samples were acquired and analyzed on the flow cytometer. In particular, the whole blood specimens were labeled directly into Trucount tubes causing the release of a known number of fluorescent microspheres from the lyophilized pellet, that during the analysis on BD FACSCanto software version 2.1 (BD Biosciences) was compared with the number of positive cells for single fluorescent labeling (positive cells/ μL). Combined analysis of $\text{CD}45$ expression and side light scatter (SSc), which assesses the internal complexity of the cells, allowed to only include lymphocytes in the analysis by applying a gate on the $\text{CD}45^{\text{bright}}$ and SSc^{low} events. At least twenty thousand lymphocytes were tested for each sample, while the lymphosum (T + B + NK lymphocytes) was always $100 \pm 2\%$.

Determination of total IgE and cytokine serum concentration

Seven serum aliquots were used to determine the concentration of total IgE, IL-2, IL-4, IL-12, IL-15, IFN- γ , and TNF- α by sandwiched enzyme-linked immunosorbent assay (ELISA)-based kits (total IgE: Alpha Diagnostic, San Antonio, TX; IL-2, IL-15, IFN- γ , and TNF- α : BioSource Inc., Camarillo, CA; IL-4 and IL-12p70: Bender MedSystems GmbH, Vienna, Austria). In detail, the serum samples were incubated in ELISA plates previously seeded with mAbs against the interest proteins and, therefore, the excess antigens (Ags) were removed by washing. Horseradish peroxidase (HRP)-conjugated secondary Abs were added to allow the formation of Ab-Ag-Ab sandwiches. After another washing step, a chromogenic substrate

was added and thus, the enzymatic reactions were arrested with a specific stop solution (H_2SO_4 1.8 N). The ELISA plates were analyzed by colorimetric reading at 450 nm, while the unknown concentration of the interest proteins was extrapolated by reference to calibration curves obtained by linking the absorbance values ($A_{450\text{nm}}$) and the known concentrations of specific standards.

Local lymphocyte typing

The oral mucosa biopsy samples were taken from the test site of patients submitted to the omPT, as well as from an analogous tissue site of the healthy volunteers not subjected to omPT, by using a disposable biopsy punch (diameter: 4 mm) after local anesthesia with lidocain 1%. These samples were then oriented, embedded in OCT compound (Tissue-Tek; Miles Inc., Elkhart, IN), snap frozen in liquid nitrogen, and stored at -80°C until used.

The oral mucosa lymphocyte typing was performed by a HRP two-steps amplified immunohistochemical method able to detect and quantify T (CD3+), Th (CD4+), and Tc (CD8+) lymphocytes, as well as to evaluate the expression of specific TLRs. In detail, serial cryostat sections (7 μm) were cut frozen, coated on polylysine pre-treated slides, air-dried overnight at room temperature, and then fixed in 100% cold acetone for 10 min at 4°C . After washing in phosphate buffered saline (PBS), the slides were pretreated for 10 min at room temperature with a peroxidase-blocking reagent (DAKO Corp., Carpinteria, CA) to suppress the endogenous peroxidase and pseudo-peroxidase activity, washed again in PBS, and then incubated in a humid chamber for 1 hr at room temperature with the following mouse anti-human primary mAbs: anti-CD3 (Dilution 1:20; DakoCytomation, Milan, Italy), anti-CD4 (Dilution 1:20; DakoCytomation), anti-CD8 (Dilution 1:20; DakoCytomation), anti-TLR1 (Dilution 1:50; R&D Systems Inc., Minneapolis, MN), anti-TLR2 (Dilution 1:50; R&D Systems Inc.), anti-TLR3 (Dilution 1:50; R&D Systems Inc.), and anti-TLR4 (Dilution 1:50; R&D Systems Inc.). As a negative control, the primary mAbs were substituted with buffer. Thereafter, the slides were rinsed twice in PBS and incubated for 30 min at room temperature with goat anti-mouse secondary Abs conjugated to a HRP-labeled polymer (EnVision+, HRP; DAKO Corp.). After washing in PBS, the revealing reaction was carried out using 3,3'-diaminobenzidine and 3-amino-9-ethylcarbazole (liquid DAB+ and AEC+ substrate-chromogen systems; Dako Corp.) as chromogenic substrates. The slides were washed again in PBS, counterstained with hematoxylin, and viewed microscopically (Magnification 40 $\times$) by means of the Leitz Dialux 20 microscope.

Statistical analysis

Data achieved in this study were firstly analyzed by the D'Agostino–Pearson omnibus test to verify the normal distribution hypothesis within each statistical sample. Given that some resulting *p* values were significant ($p < 0.05$), it is reasonable to assume that not all data obtained from every group of patients fall into Gaussian distributions and therefore, were synthesized as mean values $\pm$ standard deviations (SD) and processed by means of parametric tests, the results of which were verified by the relative non-parametric tests. Comparisons between the parameters measured before and immediately after the omPT for Ni were performed by using the Student *t*-test for paired data confirmed by the Wilcoxon rank test. Differences among the parameters determined before, immediately after, and after 48 h from the omPT for Ni were assessed by the analysis of variance (ANOVA) for repeated measures where, for *p* values ≤ 0.05 , the Bonferroni multiple comparison was used as post test. Results of the latter tests were verified, respectively, by the Friedman test and Dunn multiple comparison. Comparisons between the parameters measured in different groups or subgroups of patients were performed by using the

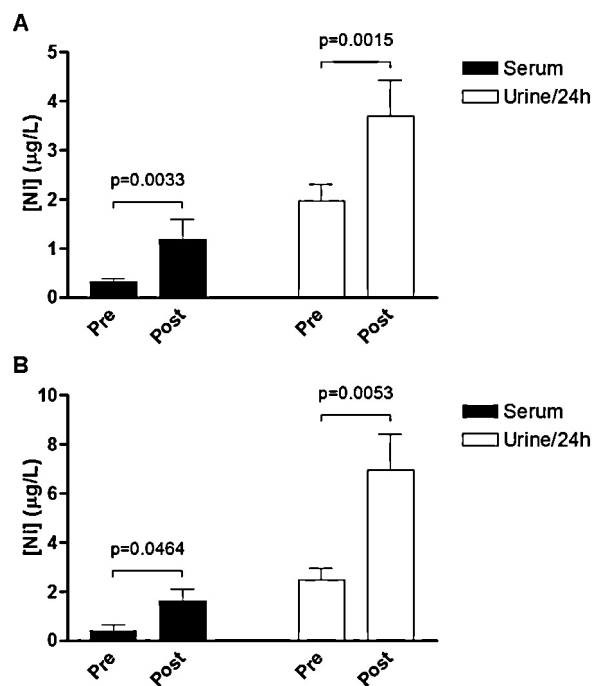


Fig. 1. Ni concentrations determined in serum and urine of Ni-sensitive patients (A) and control patients (B) before (pre) and after (post) the omPT for Ni. Serum and urine Ni concentrations, expressed in $\mu\text{g/L}$, were measured by ICP-MS. The *p* values reported in figure refer to Student *t*-test for paired data confirmed by the Wilcoxon rank test and applied to evaluate differences between the parameter determined before and after the omPT for Ni. ICP-MS, inductively coupled plasma-mass spectrometry; Ni, nickel; omPT, oral mucosa patch test.

Student *t*-test for unpaired data confirmed by the Mann–Whitney test. The relationship between different parameters was assessed by the Bravais–Pearson linear correlation that in turn, was verified by the Spearman rank correlation.

In all tests applied, the *p* values ≤ 0.05 were considered significant. The statistical evaluations were carried out by using the GraphPad Prism package version 5.2 (GraphPad Software Inc., San Diego, CA). Given that in literature there are no investigations comparable to the present study, it was not possible to perform a power analysis to determine the sample size.

Results

Patients' assessment

Out of the 37 symptomatic patients underwent to omPT for Ni, 25 (67.6%) showed at least a local and/or systemic reaction within 2 h of the test whereas, at the same time, the remaining 12 (32.4%) did not develop any response. Therefore, the first 25 were defined as Ni-sensitive patients and the remaining 12 as control patients. According to the omPT reaction grading used in this study, all 12 control patients belonged to 0 type, 9 out of the 25 Ni-sensitive patients (36%) to Ia type, 7 (28%) to Ib type, 5 (20%) to IIa type, 3 (12%) to IIb type, 1 (4%) to IIIa type and finally, none of them belonged to IIIb type.

Systemic response to nickel

The Ni concentrations determined in serum and urine of Ni-sensitive patients after the omPT were significantly higher ($p = 0.0033$ and $p = 0.0015$, respectively) than those measured in the same samples/patients before the test (Fig. 1A). Even for

Table 2

Total and differential WBC count performed in Ni-sensitive and control patients before (pre) and after (post) the omPT for Ni.

Cells/ μ L	Ni-sensitive patients (n = 25)			Control patients (n = 12)		
	Pre	Post	p	Pre	Post	p
WBC	6332.8 $\pm$ 1561.4	6547.6 $\pm$ 1470.5	NS	5603.3 $\pm$ 923.5	5720.0 $\pm$ 1094.9	NS
Neu	3865.2 $\pm$ 1188.3	3849.6 $\pm$ 1075.2	NS	3188.3 $\pm$ 712.6	3416.7 $\pm$ 850.8	0.0246
Lymph	1937.2 $\pm$ 497.6	2204.4 $\pm$ 604.3	0.0003	1921.7 $\pm$ 498.7	1855.0 $\pm$ 449.7	NS
Mono	350.0 $\pm$ 125.6	333.6 $\pm$ 110.1	NS	326.7 $\pm$ 110.3	316.7 $\pm$ 102.3	NS
Eos	132.0 $\pm$ 105.1	110.0 $\pm$ 85.7	NS	136.7 $\pm$ 93.7	110.0 $\pm$ 85.3	NS
Baso	26.4 $\pm$ 17.3	26.0 $\pm$ 16.6	NS	30.0 $\pm$ 16.0	21.7 $\pm$ 12.7	NS

Total and differential WBC count/ μ L whole blood was carried out by hemochromocytometric assay. The p values reported in table refer to Student *t*-test for paired data confirmed by the Wilcoxon rank test and applied to evaluate differences between the parameters determined before and after the omPT for Ni.

Baso, basophils; Eos, eosinophils; Lymph, lymphocytes; Mono, monocytes; Neu, neutrophils; Ni, nickel; NS, not significant; omPT, oral mucosa patch test; WBC, white blood cells.

control patients, the Ni concentrations determined in serum and urine after the omPT were significantly higher ($p=0.0464$ and $p=0.0053$, respectively) than those measured in the same samples before the test (Fig. 1B).

The WBC count highlighted a statistically significant increase ($p=0.0003$) in the number of circulating lymphocytes after the omPT carried out in Ni-sensitive patients, as well as a statistically significant increase ($p=0.0246$) in the number of circulating neutrophils after the omPT performed in control patients (Table 2). Consistently, the number of total, T, Th, Tc, and B lymphocytes determined in whole blood of Ni-sensitive patients after the omPT was significantly higher ($p<0.0001$ for the first three, $p=0.0071$ and $p=0.0003$ for the last two lymphocyte subsets, respectively) than that measured in the same samples/patients before the test (Fig. 2A). Also the Th/Tc lymphocyte ratio determined in the same samples/patients after the omPT (1.81 ± 0.57) was significantly higher ($p=0.0119$) than that measured before the test (1.69 ± 0.62). The increase in the number of total, T, Th, Tc, and B lymphocytes/ μ L whole blood observed in Ni-sensitive patients after the omPT ($\Delta_{\text{post-pre}}$) was not significantly correlated with the $\Delta_{\text{post-pre}}$ serum Ni concentrations highlighted in the same patients ($r=0.0813$, $r=0.0457$, $r=0.1329$, $r=0.2707$ and $r=0.0429$, respectively). No statistically significant increase in the number of total lymphocytes and their subsets was observed in whole blood of control patients after the omPT (Fig. 2B). The number of total, T, Th, Tc, and B lymphocytes determined in whole blood of 10 Ni-sensitive patients immediately after the omPT was significantly higher than that measured in the same samples/patients both before ($p<0.01$ for the first three and $p<0.05$ for the last two lymphocyte subsets) and after 48 h ($p<0.01$ for the first three, $p<0.05$ and $p<0.01$ for the last two lymphocyte subsets, respectively) from the test (Fig. 3).

Total IgE concentrations determined in serum of Ni-sensitive patients after the omPT were comparable to those measured in the same samples/patients before the test. Even for control patients, total IgE concentrations determined in serum after the omPT were comparable to those measured in the same samples before the test (Fig. 4). Total IgE serum levels determined in Ni-sensitive patients both before and after the omPT tended to be higher than those measured in control patients at the same times. However, subdividing the Ni-sensitive patients in allergic ($n=7$) and non-allergic ($n=18$), total IgE serum concentrations determined in the first subgroup before (226.9 ± 67.35) and after (214.3 ± 76.28) the omPT were significantly higher ($p=0.0015$ for both) than those measured in the second subgroup before (15.94 ± 4.095) and after (15.71 ± 3.697) the test.

The concentrations of IL-2, IL-4, IL-12, IL-15, IFN- γ , and TNF- α determined in serum of Ni-sensitive patients after the omPT were comparable to those measured in the same samples/patients before the test. Even for control patients, the cytokine concentrations determined in serum after the omPT were comparable to those measured in the same samples before the test, with the exception

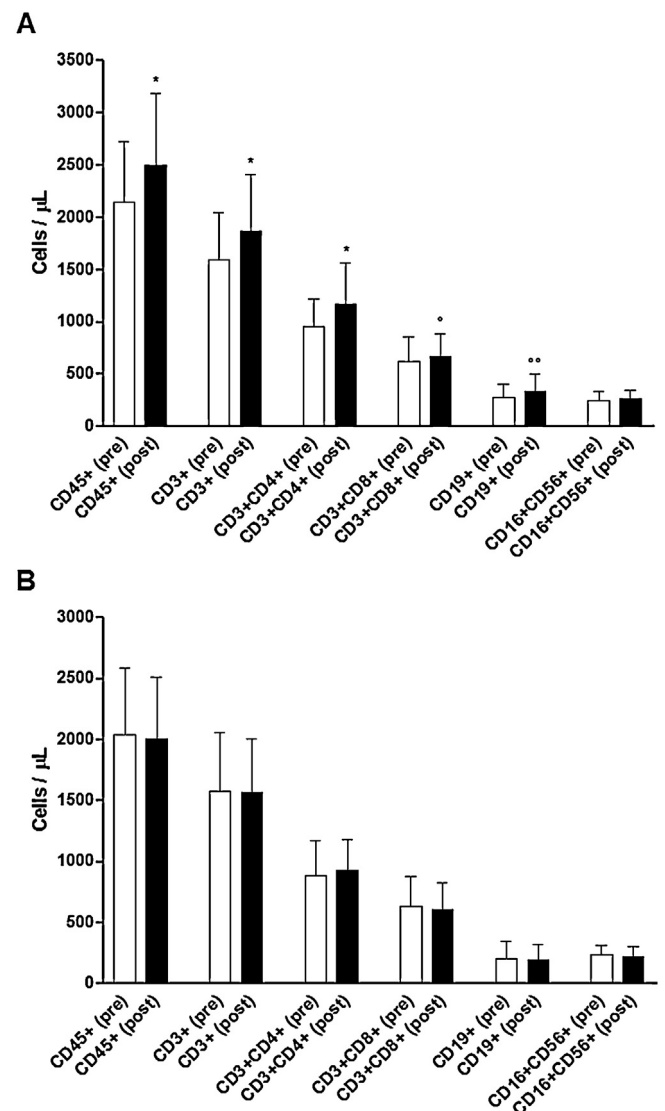


Fig. 2. Peripheral blood lymphocyte typing performed in Ni-sensitive patients (A) and control patients (B) before (pre) and after (post) the omPT for Ni.

This lymphocyte typing, laying down the number of total (CD45+), T (CD3+), Th (CD3+CD4+), Tc (CD3+CD8+), B (CD19+), and NK (CD16+ and/or CD56+) lymphocytes/ μ L whole blood, was carried out by flow cytometry (Truocount system). The symbols reported in figure refer to Student *t*-test for paired data confirmed by the Wilcoxon rank test and applied to evaluate differences between the parameters determined before and after the omPT for Ni (* $p<0.0001$ vs. pre; ° $p=0.0071$ vs. pre; °° $p=0.0003$ vs. pre).

NK, natural killer; Ni, nickel; omPT, oral mucosa patch test; Tc, T suppressor/cytotoxic; Th, T helper/inducer.

Table 3
Cytokine concentrations measured in serum of Ni-sensitive and control patients before (pre) and after (post) the omPT for Ni.

CTKs (pg/mL)	Ni-sensitive patients (n = 25)			Control patients (n = 12)		
	Pre	Post	p	Pre	Post	p
IL-2	1.699 ± 0.206	1.553 ± 0.185	NS	1.728 ± 0.258	1.277 ± 0.325	0.0250
IL-4	0.278 ± 0.046	0.326 ± 0.051	NS	0.153 ± 0.048	0.287 ± 0.048	0.0446
IL-12	7.865 ± 2.184	8.115 ± 3.329	NS	8.940 ± 3.128	8.350 ± 3.251	NS
IL-15	114.1 ± 44.49	124.7 ± 49.69	NS	16.42 ± 12.39	15.49 ± 12.48	NS
IFN-γ	9.732 ± 6.684	11.60 ± 7.940	NS	3.630 ± 1.533	3.098 ± 0.991	NS
TNF-α	5.799 ± 1.531	6.133 ± 2.156	NS	5.680 ± 2.616	4.623 ± 1.541	NS

Serum cytokine concentrations, expressed in pg/mL, were determined by ELISA. The p values reported in table refer to Student *t*-test for paired data confirmed by the Wilcoxon rank test and applied to evaluate differences between the parameters determined before and after the omPT for Ni. CTKs, cytokines; ELISA, enzyme-linked immunosorbent assay; Ni, nickel; NS, not significant; omPT, oral mucosa patch test.

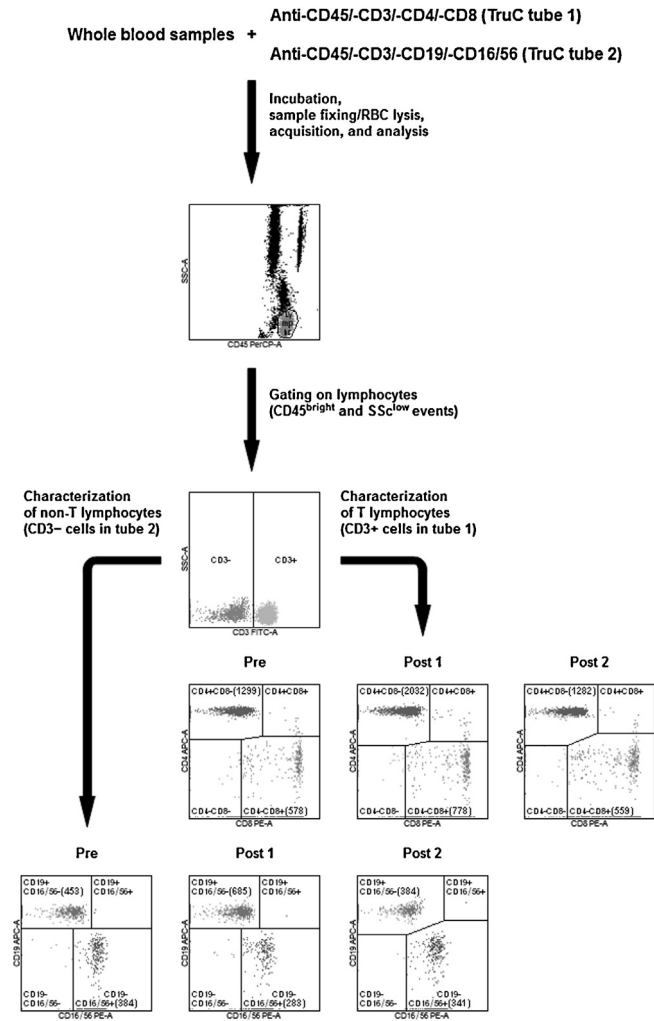


Fig. 3. Peripheral blood lymphocyte typing performed in some Ni-sensitive patients before (pre), immediately after (post 1), and after 48 h (post 2) from the omPT for Ni. This lymphocyte typing, laying down the number of total (CD45+), T (CD3+), Th (CD3+CD4+), Tc (CD3+CD8+), B (CD19+), and NK (CD16+ and/or CD56+) lymphocytes/ μ L whole blood, was carried out by flow cytometry (TruCount system) in 10 Ni-sensitive patients. The upper plots describe both the flow cytometry panel and the gating strategy, while the lower plots refer to characterization of T, B, and NK lymphocyte subsets in a representative case belonging to the same group of patients. APC, allophycocyanin; FITC, fluorescein isothiocyanate; NK, natural killer; Ni, nickel; omPT, oral mucosa patch test; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; RBC, red blood cells; SSC, side light scatter; Tc, T suppressor/cytotoxic; Th, T helper/inducer; TruC, TruCount.

of a moderate decrement ($p=0.0250$) of IL-2 and a slight increment ($p=0.0446$) of IL-4 (Table 3). The IL-15 and IFN- γ serum levels determined in Ni-sensitive patients both before and after the omPT tended to be higher than those measured in control patients at the same times.

Local response to nickel

The expression analysis of T-cell surface markers on the oral mucosa biopsy samples taken from 4 Ni-sensitive patients after the omPT showed widespread presence of CD3 in all mucosal layers (Fig. 5A and B), intraepithelial migration of CD3+ cells toward the site of Ni exposure (Fig. 5B), abundant expression of CD4 in the intermediate layers of the mucosa (Fig. 5C), and moderate presence of CD8 limited to the capillaries (Fig. 5D). No significant expression of CD3, CD4, and CD8 was highlighted on the oral mucosa biopsy samples obtained from one control patient after the omPT, as well as from 3 healthy volunteers not subjected to omPT.

The expression analysis of TLRs on the oral mucosa biopsy samples taken from 4 Ni-sensitive and one control patients after the omPT showed clear presence of TLR2 (Fig. 5E) and TLR4 (Fig. 5F) in the epithelium. Conversely, no relevant expression of TLR1 and TLR3 was observed in the same samples/patients. No significant expression of any TLR was finally highlighted on the oral mucosa biopsy samples obtained from 3 healthy volunteers not subjected to omPT.

Discussion

In the present study, we have firstly confirmed that the omPT is able to identify Ni sensitivity in over 60% of symptomatic patients

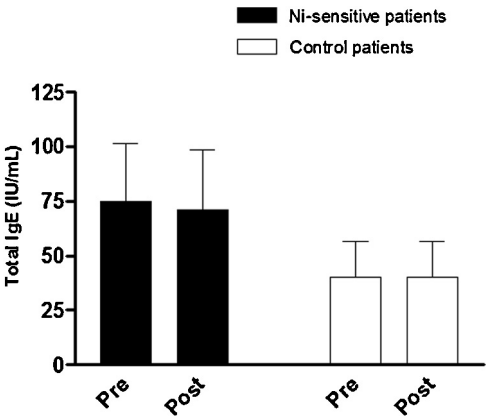


Fig. 4. Total IgE concentrations measured in serum of Ni-sensitive and control patients before (pre) and after (post) the omPT for Ni. Serum concentrations of total IgE, expressed in IU/mL, were determined by ELISA. ELISA, enzyme-linked immunosorbent assay; Ni, nickel; omPT, oral mucosa patch test.

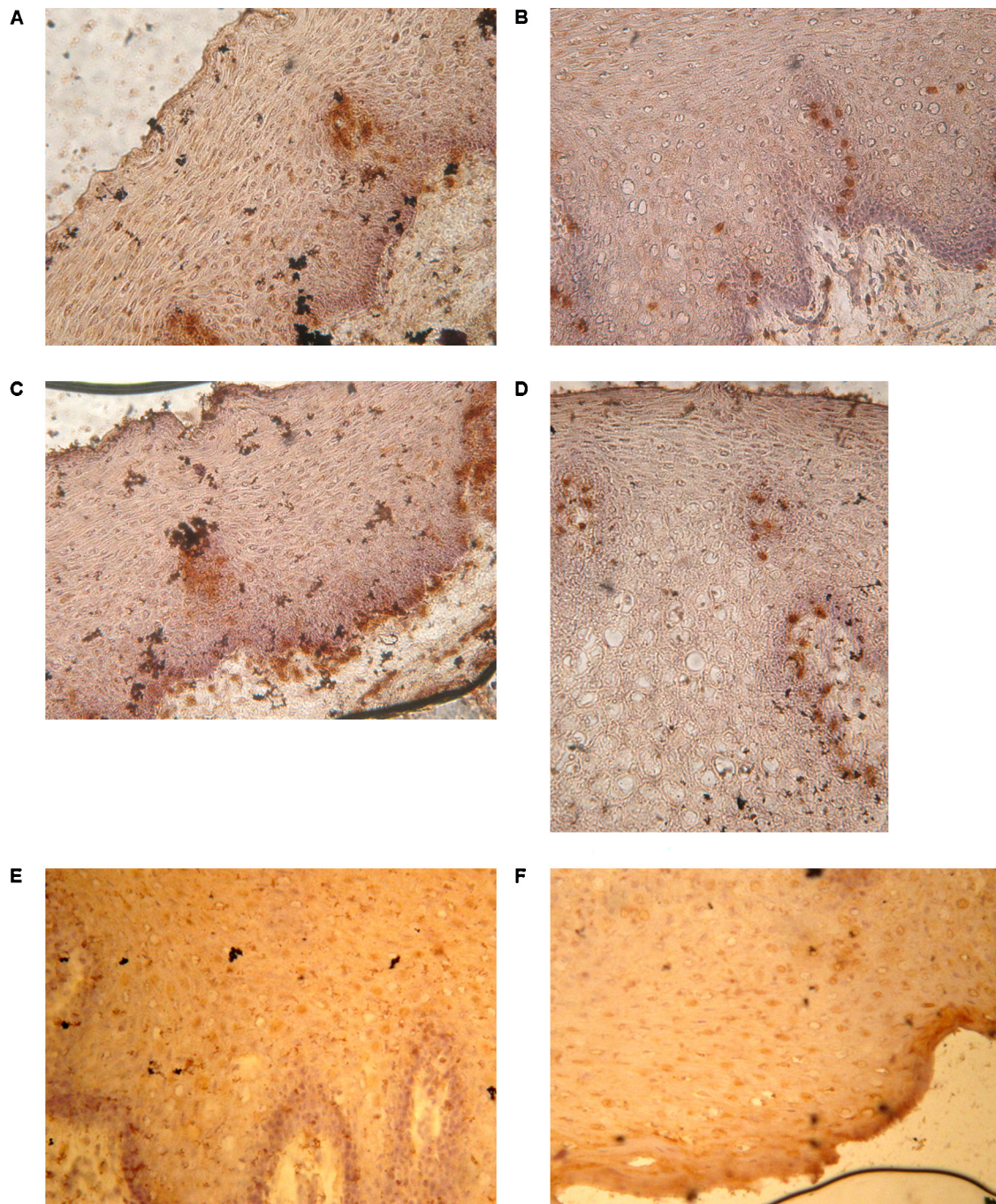


Fig. 5. Expression of T-cell surface markers and TLRs on the oral mucosa biopsy samples taken from some study participants after the omPT for Ni. The expression analysis of T-cell surface markers, performed by immunohistochemistry, showed widespread presence of CD3 in both epithelial and submucosal compartments (A and B), migration of CD3+ cells across the epithelial layers toward the site of Ni exposure (B), abundant expression of CD4 in the interface between the epithelial and submucosal compartments (C), and moderate presence of CD8 limited to the vessel walls (D) of the oral mucosa biopsy samples taken from some Ni-sensitive patients after the omPT. The expression analysis of TLRs showed clear presence of TLR2 (E) and TLR4 (F) in the epithelial compartment of the oral mucosa biopsy samples taken from some Ni-sensitive and one control patients after the omPT. Ni, nickel; omPT, oral mucosa patch test; TLR, Toll-like receptor.

(Picarelli et al., 2011). Accordingly, this procedure has been used to divide the study participants into Ni-sensitive and control patients. We have further provided an omPT reaction grading based on the well-established ICDRG scoring system (Wilkinson et al., 1970) which could be useful, in the future, for both clinical/diagnostic and research purposes.

Taking advantage from the allergen exposure that occurs during the omPT, this procedure has been also used to characterize

immunologically the ACM related to the ingestion of Ni-rich foods. In particular, the significant increase in serum and urine Ni concentrations highlighted in both Ni-sensitive and control patients after the omPT indicates that Ni is rapidly absorbed during the test, transferred in the circulation and eliminated within 24 h. This finding suggests that no study participant had alterations in Ni metabolism and, in agreement with a recent investigation showing that the ePT administered to patients with or without ACD may cause a transient

systemic Ni exposure (Darsow et al., 2012), does not exclude the possibility of a systemic response to Ni coming from the chemical patch applied on the oral mucosa.

Another finding emerging from our study is the significant increase in the number of circulating lymphocytes observed in Ni-sensitive patients after the omPT. As occurs in the DTH reaction typical of ACD (Wang and Dai, 2013), this increment is suggestive of an adaptive response that in turn, could be systemic or local with increased lymphocyte trafficking due to a chemotactic recall from the site of Ni exposure. On the contrary, the significant increase in the number of circulating neutrophils highlighted in control patients after the omPT is suggestive of a non-MHC-restricted response probably not related to Ni allergy. Although this finding is consistent with previous studies showing that the intratracheal instillation of Ni oxide nanoparticles leads to neutrophil infiltration and expression of cytokine-induced neutrophil chemoattractants in the rat lung (Morimoto et al., 2011, 2012), further investigations are necessary to understand the role of this response in subjects not sensitive to Ni. The peripheral blood lymphocyte typing has firstly confirmed the omPT-dependent increase of circulating lymphocytes observed in Ni-sensitive patients, with return to normal within 48 h. The Th and Tc lymphocytes appear to be the subsets mainly involved in this response, with a greater contribution of the first cells than the latter as also demonstrated by the omPT-dependent increase of Th/Tc lymphocyte ratio. On the other hand, the absence of a significant correlation between the increments of circulating lymphocytes and serum Ni concentrations highlighted in Ni-sensitive patients after the omPT supports the hypothesis of a local reaction, at the expense of a systemic response to Ni administered with the chemical patch.

In spite of the moderately increased number of circulating B lymphocytes observed in Ni-sensitive patients after the omPT, no concomitant increment in total IgE serum concentrations has been found, thus excluding the possibility of an IgE-dependent immediate hypersensitivity reaction. Consistently, the slightly higher total IgE serum levels highlighted in Ni-sensitive patients than in controls have been ascribed to the presence of IgE-mediated allergies to casein, cat's hair, dust, grains, latex, mites, parietaria, and pollen. As recently supposed (Walsh et al., 2010), these data leave open the possibility that Ni may act as a mast cell discharger on a non-immunological basis and accordingly, further studies should be addressed to clarify this issue as well as the omPT-dependent increase of circulating B lymphocytes observed in Ni-sensitive patients.

No significant increment in serum concentrations of selected cytokines has been found in Ni-sensitive patients after the omPT. Furthermore, the slightly higher serum levels of IL-15 and IFN- γ highlighted in Ni-sensitive patients than in controls could be ascribed to the concomitant presence of other pathological disorders such as celiac disease, a systemic autoimmune disorder mainly linked to the production and release of the above mentioned cytokines (Sollid and Jabri, 2013). Also these data support the hypothesis of a local reaction, at the expense of a systemic response to Ni coming from the omPT. Conversely, the moderate decrement of IL-2 and the slight increment of IL-4 observed in serum of control patients after the omPT seem to suggest a non-specific response to Ni with a systemic component, even if further investigations are needed to confirm and explain the cytokine imbalance induced by Ni exposure in non-sensitive subjects.

The lymphocyte typing performed on the oral mucosa biopsy samples taken from Ni-sensitive patients after the omPT showed widespread CD3+ cells with images of intraepithelial migration toward the site of Ni exposure, abundant CD4+ cells in the intermediate layers of the mucosa, and some CD8+ cells in the capillaries. This immunohistochemical picture overlaps with the peripheral blood lymphocyte typing showing Th and Tc involvement with a

greater contribution of the first cells than the latter, supporting further the hypothesis of a local adaptive response with increased lymphocyte trafficking due to a chemotactic recall from the site of Ni exposure. In addition, the perivascular localization of CD8+ cells could explain why the reactions observed on the omPT site are mainly of vascular type. Similar conclusions have been reached in a very recent study by performing both a local and peripheral lymphocyte typing in a series of ACD patients stimulated with the ePT for Ni (Dyring-Andersen et al., 2013). On the other hand, the expression analysis of TLRs showed clear presence of TLR2 and TLR4 on the oral mucosa biopsy samples taken from Ni-sensitive and control patients after the omPT, but not from healthy volunteers not subjected to the test. This finding does not exclude the possibility of a TLR4-mediated innate response to Ni administered with the omPT, as recently demonstrated in case of ACD (Schmidt et al., 2010), and opens opportunities to investigate the role of TLR2 and TLR4 in either Ni-sensitive or non-sensitive individuals exposed to Ni-rich foods. It would be interesting to verify, for example, if the local expression of TLR2 and/or TLR4 is related to the increasing number of circulating neutrophils and to the cytokine imbalance observed in subjects not sensitive to Ni after the omPT. Furthermore, since for ethical reasons the immunohistochemical data have been obtained on the oral mucosa biopsy samples taken from 4 Ni-sensitive patients, one control patient and 3 Ni-unexposed healthy volunteers, further studies are necessary to extend these observations to a greater number of cases.

In conclusion, we confirmed that the omPT is able to identify Ni sensitivity in symptomatic patients, and provided an omPT reaction grading that could be useful for both clinical/diagnostic and research purposes. In the attempt to characterize immunologically the ACM related to the ingestion of Ni-rich foods, we highlighted that a local adaptive response with increased lymphocyte trafficking is the most likely mechanism of reaction to Ni administered with the omPT. However, further studies should be warranted to confirm and extend our observations.

Conflict of interest

The authors declare that they have no conflict of interest.

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