

Nickel-Related Intestinal Mucositis in IBS-Like Patients: Laser Doppler Perfusion Imaging and Oral Mucosa Patch Test in Use

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Received: 20 January 2016 / Accepted: 12 February 2016
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Abstract Nickel (Ni) is often the trigger of irritable bowel syndrome (IBS)-like gastrointestinal disorders: its ingestion may cause allergic contact mucositis, identifiable by means of oral mucosa patch test (omPT). OmPT effectiveness has been proven, but it is still an operator-dependent method. Laser Doppler perfusion imaging (LDPI) was tested to support omPT in Ni allergic contact mucositis diagnosis. Group A: 22 patients with intestinal/systemic symptoms related to the ingestion of Ni-containing foods. Group B: 12 asymptomatic volunteers. Ni-related symptoms and their severity were tested by a questionnaire. All patients underwent Ni omPT with clinical evaluation at baseline (T_0), after 30 min (T_1), after 2 h (T_2), and after 24–48 h (T_3). LDPI was performed to evaluate the mean mucosal perfusion at T_0 , T_1 , and T_2 . Statistical analysis was performed by ANOVA test and Bonferroni multiple-comparison test. All 22 Ni-sensitive patients (group A) presented oral mucosa hyperemia and/or edema at T_2 . Eight out of the same 22 patients presented a local delayed vesicular reaction at T_3 (group A1), unlike the remaining 14 out of 22 patients (group A2). All 12 patients belonging to control group B did not show any alteration. The mean mucosal perfusion calculated with LDPI showed an increase in both subgroups A1 and A2. In group B, no significant perfusion variations were observed. LDPI may support omPT for diagnostic purposes in Ni allergic contact

mucositis. This also applies to symptomatic Ni-sensitive patients without aphthous stomatitis after 24–48 h from omPT and that could risk to miss the diagnosis.

Keywords Nickel · Adverse effects to food · Patch test · Irritable bowel syndrome · Laser Doppler

Introduction

Nickel (Ni) is present in group VIII of the periodic table, and it is widely distributed in nature. The possible routes of exposure for humans are inhalation, contact with the skin, and ingestion [1].

According to the European Surveillance System of Contact Allergy (ESSCA), the prevalence of epicutaneous patch test (ePT) positive to nickel is about 20 % in Europe and, in particular, Italy holds the highest prevalence (32.1 %) [2]. Allergic contact dermatitis and systemic nickel allergy syndrome (SNAS) are well-known manifestations of Ni allergy.

Allergic contact dermatitis is characterized by local eczematous lesions found on the skin in close contact with Ni-containing objects, such as earrings, watches, and necklaces. The face, ear lobes, neck, and wrists are the most commonly involved sites. The diagnosis is made by means of the ePT, able to provoke delayed lesions, as a type IV hypersensitivity reaction [3].

On the other hand, also the gastrointestinal tract can represent a route of exposure to Ni [4] inducing SNAS, which is characterized not only by diffused eczematous lesions called systemic contact dermatitis but also by extracutaneous signs and symptoms. Apart from less common respiratory and neurological manifestations, Ni-related symptoms in SNAS are mainly gastrointestinal SNAS. Specifically, Ni-rich foods include tomato, cocoa, beans, mushrooms, broad-leaved

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vegetables, whole-wheat flour, corn, onion, garlic, shellfish, nuts, food in an aluminum box, and many more [5]. These considerations can lead to a heated debate, especially among gastroenterologists and clinical nutritionists since Ni-containing foods may be considered responsible for irritable bowel syndrome (IBS)-like symptoms and thus deserves an accurate differential diagnosis.

Recently, a Ni oral mucosa patch test (omPT) has been proposed and revealed useful in SNAS diagnosis. It induces Ni-related manifestations, such as edema and hyperemia, on labial mucosa after 2 h of exposure, and aphthous/vesicular lesions may appear even after 24–48 h [6]. The first alterations seem related to a toll-like receptor (TLR)4-dependent innate immune response, whereas the latest could be identified as a type IV hypersensitivity reaction. These mucosal abnormalities have been defined as allergic contact mucositis [7, 8].

Currently, an objective tool for the quantification of Ni-induced lesions still lacks since both Ni ePT and omPT are operator-dependent.

For this purpose, Valentine-Thon et al. [9] used memory lymphocyte immunostimulation assay to detect Ni-sensitive patients. Other authors evaluated the Ni-induced cytokine production by enzyme-linked immunospot [10] or used Raman spectroscopy of the skin [11]. Unfortunately, both memory lymphocyte immunostimulation assay and enzyme-linked immunospot may be affected by many independent variables; furthermore, the first implies a non-negligible risk of radioactive exposure for laboratory personnel and Raman spectroscopy is currently limited to a few highly specialized centers, despite being a non-invasive *in vivo* assessment. Recently, according to Di Tola et al. [12], the calculation of post/pre Ni to omPT cell ratio in peripheral blood lymphocyte typing can support omPT in allergic contact mucositis diagnosis, with quantitative and objective data. In another study, the same authors [7] proposed lymphocyte typing and evaluation of specific TLRs by immunohistochemistry performed on biopsy specimens of labial mucosa after positive Ni omPT. In these biopsies, CD3+ T cells were evidenced in all mucosal layers, together with their intra-epithelial migration towards the omPT application site. It was also observed the presence of CD4+ T cells in the intermediate layers of the oral mucosa, together with CD8+ T cells in the capillaries and numerous epithelial cells expressing TLR2 and TLR4. Other authors [13] described similar results from patients affected by allergic contact dermatitis to Ni, performing immunohistochemical staining of skin biopsy specimens obtained after ePT. They demonstrate a significant infiltration of CD4+ T lymphocytes that secrete IL-17, IL-22, and IFN- γ in patients with positive ePT. They also typed the peripheral blood lymphocytes after ePT, showing an increase in $\gamma\delta$ T cells in peripheral blood, although there were no significant differences in the distribution of lymphocyte subtypes among patients with positive ePT and controls. All these results support the hypothesis that a

delayed-type hypersensitivity (type IV hypersensitivity) may be implied in both Ni allergic contact mucositis and allergic contact dermatitis [7, 13]. The above listed techniques actually revealed useful, despite some of them require skilled laboratory personnel and/or imply invasive procedures, such as labial mucosa biopsy.

Laser Doppler perfusion imaging (LDPI) has been recently presented as a non-invasive, rapid, and repeatable technique that quantifies the degree of hyperemia during acute inflammatory response. It uses a low-intensity monochromatic laser beam to scan across a tissue surface and determine flux of blood cells moving through the dermal microvasculature [14, 15]. For this reason, LDPI revealed to be a precious evidence-based adjunct to clinical evaluation of burn depth [16], as well as alterations due to systemic sclerosis [17], scleroderma, and primary Raynaud's phenomenon [18].

Moreover, literature reports that LDPI has been already used to evaluate the increased cutaneous blood flow in allergic contact dermatitis, with a correlation between LDPI itself and visual scoring [19–21].

On these premises, we tested LDPI as a diagnostic tool able to objectify Ni-related alterations after Ni omPT in allergic contact mucositis diagnosis.

Materials and Methods

Population

A total of 35 consecutive patients referring to our gastroenterology unit were recruited between December 2013 and July 2014 because of food-related gastrointestinal and/or extra-intestinal symptoms. Celiac disease, lactase deficiency, inflammatory bowel disease, cancer, infectious, autoimmune, and IgE-mediated diseases were the exclusion criteria; thus, 13 out of 35 patients have been excluded. The remaining 22 patients (1 male and 21 females, aged 18–65, mean age 39.5) were included in the study (group A) as they reported gastrointestinal and/or extra-intestinal symptoms related to ingestion of a Ni-containing food. A specific alimentary-symptom questionnaire attested their presence, as well as their severity.

Twelve healthy patients [5 males and 7 females, aged 18–65, mean age 41] were also included in the study as control population (group B). None of these patients suffered from gastrointestinal or extra-intestinal disorders, especially Ni-related discomforts, as proved by the above-mentioned specific questionnaire.

Questionnaire and Visual Analog Scale

Dietetic assessment with a specific alimentary-symptom questionnaire was performed by a medical doctor and an expert dietician at the entry of the study, after at least 6 weeks of Ni-

containing diet, to support the hypothesis of Ni dependence of symptoms. Ni-related gastrointestinal symptoms (abdominal swelling, abdominal pain, meteorism, constipation, belching, diarrhea, dyspepsia, nausea, stomatitis, vomit) and/or Ni-related extra-intestinal symptoms (asthenia, headache, dizziness, itch, eczematous lesions, erythema) were specifically tested in patients from both groups A and B. The perceived severity of symptoms was quantitatively evaluated by means of a visual analog scale (VAS): it is a 10-cm straight line with two ends that match to “no symptoms” and “maximum possible” (or the maximum of which has had experience). It is a one-dimensional tool that quantifies what the patient subjectively perceives. Both the questionnaire and VAS have been elaborated according to the Salerno Experts’ Criteria in the diagnosis of non-celiac gluten sensitivity [22].

Oral Mucosa Patch Test

A 5-mm filter paper disk saturated with a 5 % solution of Ni sulfate in Vaseline (0.4 mg Ni-sulfate/8 mg Vaseline) was applied on the lower lip mucosa after removal of excess saliva with sterile gauze. It was held in place by an adhesive transparent film (Film 3M Tegaderm; 3M SpA, Milan, Italy) covered with a small absorbent pad. It was performed on all patients at baseline (T_0). After 30 min (T_1), the patch was removed and the site of application was closely observed to determine the presence of any lesion or reaction. A new disk containing an equal amount of Ni sulfate was subsequently applied and then removed after 2 h from baseline (T_2). The application site was carefully observed by medical staff to detect erythematous, edematous, and/or vesicular lesions. Specifically, itch was considered as positive result too. After 24–48 h from baseline (T_3), any oral mucosa delayed reaction (aphthous/vesicular lesions) as well as gastrointestinal and/or general symptoms (asthenia, headache) were recorded. An identical omPT without Ni was simultaneously applied blindly in the opposite site, as negative control.

Laser Doppler Perfusion Imaging

LDPI was performed in both groups A and B on the lower lip mucosa, at baseline immediately before omPT application (T_0), at T_1 , and at T_2 . Alcohol, coffee, and smoking were forbidden 2 h before the test. The labial mucosa blood flow was detected by Lisca laser Doppler perfusion imager (Perimed AB, Stockholm, Sweden) at a low energy (wavelength = 670 nm). The scanner was positioned perpendicularly and at a distance of 15 cm from the labial mucosa, as indicated in the instruction manual provided by the manufacturer. The laser scans sequentially up to 4096 points (64×64 pixels) of the tissue analyzed, covering a maximum area of $12 \times 12 \text{ cm}^2$. The omPT application site was identified as a specific region of interest, whose mean mucosal perfusion was

expressed in perfusion units, directly proportional to the product between the average speed and red blood cell concentration. All perfusion signals were also converted into a color image, whose ends of the scale are represented by dark blue (minimum perfusion values) and red (maximum perfusion values) (Fig. 1a, b).

Statistical Analysis

Qualitative data were expressed as frequencies (absolute and relative) and processed by the Fisher exact test, chosen because at least one frequency resulted equal to zero.

Quantitative data were firstly analyzed by the D’Agostino-Pearson omnibus test to verify the hypothesis of normal distribution within each statistical sample. Since p values were not significant ($p > 0.05$), it was assumed that data obtained from each group of study participants fell into Gaussian distributions and, therefore, were expressed as mean values $\pm$ standard deviations. For the same reason, data were processed using parametric tests. Comparisons between the severity of each symptom assessed in patients belonging to groups A and B were performed by the Student t test for unpaired data. Comparisons among values of mean mucosal perfusion obtained before (T_0), after 30 min (T_1), and after 2 h (T_2) from omPT were carried out by the analysis of variance (ANOVA) for repeated measures where, for overall $p < 0.05$, the Bonferroni multiple comparison was used as post test. In all tests applied, p values < 0.05 were considered significant. Statistical evaluations were performed using the GraphPad Prism package version 5.2 (GraphPad Software Inc., San Diego, CA).

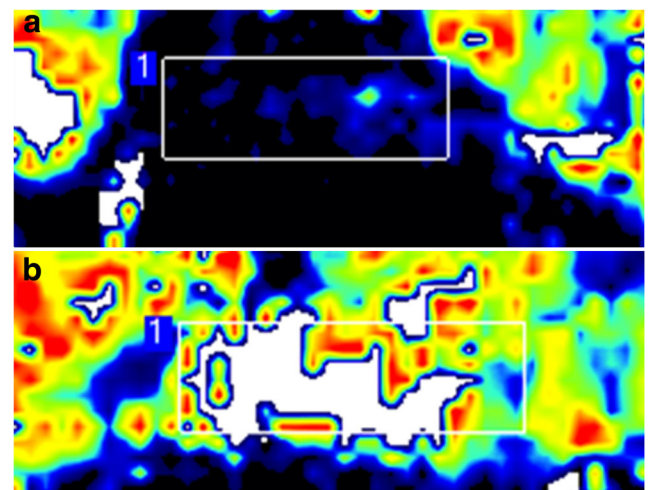


Fig. 1 Laser Doppler perfusion imaging. Perfusion signals are converted into a color image, whose ends of the scale are represented by dark blue (minimum perfusion values) and red (maximum perfusion values). **a** Imaging at baseline, **b** imaging after stimulation (Ni omPT). Ni nickel, omPT oral mucosa patch test

All procedures followed in this study were in accordance with the ethical standards of the institutional committee responsible for human experimentation. Furthermore, a written informed consent was obtained from each patient studied.

Results

Questionnaire Results

Abdominal swelling and pain, meteorism, headache, and asthenia were among the most frequent and the most severe Ni-related symptoms, according to patients belonging to group A. Patients belonging to group B did not complain any symptoms. All detailed results are reported in Table 1.

Oral Mucosa Patch Test Results

All the 22 Ni-sensitive patients belonging to group A showed no mucosal or clinical alterations at T₁, after omPT. However, the same 22 patients presented oral mucosa hyperemia and/or edema of the lower lip test site at T₂. According to the development of further mucosal lesions after omPT at T₃, the same 22 patients of group A were further divided into two subgroups: A1, consisting of 8 patients with local delayed vesicular reaction at T₃ (Fig. 2); A2, consisting of the remaining 14 patients without any local vesicular lesion at T₃. However, 6

out of these 14 patients presented delayed extra-intestinal symptoms.

All the 12 patients belonging to control group B did not show any local mucosal lesion or intestinal and extra-intestinal symptoms, during and after omPT (T₀–T₃).

Control tests (omPT without Ni) showed clear negative results in both groups A and B, in all times considered (T₀–T₃).

Laser Doppler Perfusion Imaging Results

With regard to group A, mean mucosal perfusion values were calculated in the above-mentioned region of interest, in Ni omPT application site. They showed an increase in both subgroup A1 and in subgroup A2. In particular, subgroup A1 showed a statistically significant increase in mean mucosal perfusion between T₀ and T₁ ($p < 0.05$) and between T₀ and T₂ ($p < 0.01$). The increase in perfusion observed between T₁ and T₂ resulted to be not statistically significant.

In subgroup A2, data showed a statistically significant increase in average mucosal perfusion only between T₀ and T₁ ($p < 0.05$); the increase observed between T₀ and T₂ as well as between T₁ and T₂ was not statistically significant.

In group B, mean mucosal perfusion did not undergo any significant change between the three times of observation. These results are expressed graphically in Fig. 3.

Table 1 Distribution of symptoms and assessment of their severity in patients belonging to groups A and B

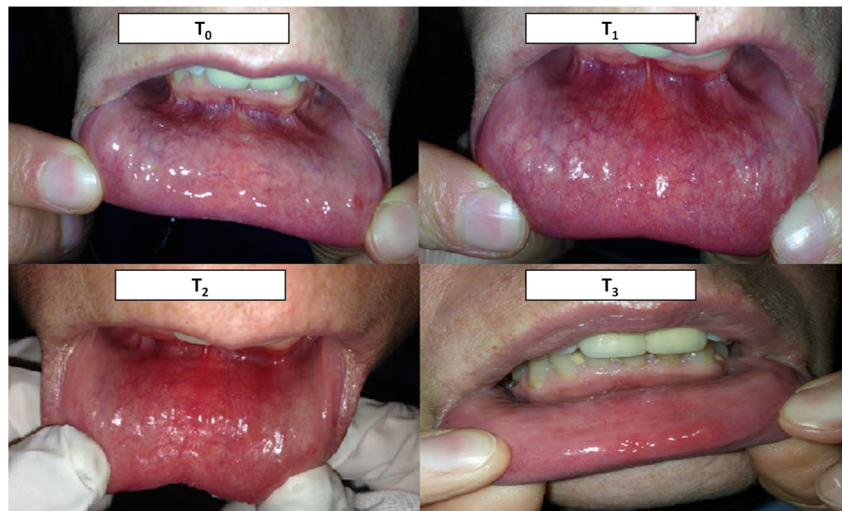
Symptoms	Symptom distribution [no./tot (%)]			Symptom severity [mean VAS ± SD]		
	Group A	Group B	<i>p</i> value ^a	Group A	Group B	<i>p</i> value ^b
Abdominal swelling	20/22 (90.9 %)	0/12 (0.0 %)	<0.0001	5.7 ± 1.2	0.0 ± 0.0	<0.0001
Abdominal pain	17/22 (77.3 %)	0/12 (0.0 %)	<0.0001	3.2 ± 1.2	0.0 ± 0.0	0.0068
Meteorism	17/22 (77.3 %)	0/12 (0.0 %)	<0.0001	5.9 ± 1.1	0.0 ± 0.0	<0.0001
Constipation	15/22 (68.2 %)	0/12 (0.0 %)	0.0001	3.8 ± 1.3	0.0 ± 0.0	0.0034
Belching	12/22 (54.5 %)	0/12 (0.0 %)	0.0018	1.6 ± 1.0	0.0 ± 0.0	ns
Diarrhea	12/22 (54.5 %)	0/12 (0.0 %)	0.0018	1.9 ± 0.9	0.0 ± 0.0	0.0287
Dyspepsia	12/22 (54.5 %)	0/12 (0.0 %)	0.0018	2.8 ± 1.2	0.0 ± 0.0	0.0151
Nausea	10/22 (45.5 %)	0/12 (0.0 %)	0.0058	2.7 ± 1.2	0.0 ± 0.0	0.0181
Stomatitis	10/22 (45.5 %)	0/12 (0.0 %)	0.0058	2.5 ± 1.3	0.0 ± 0.0	0.0364
Vomit	5/22 (22.7 %)	0/12 (0.0 %)	ns	1.2 ± 1.1	0.0 ± 0.0	ns
Asthenia	21/22 (95.4 %)	0/12 (0.0 %)	<0.0001	5.3 ± 1.0	0.0 ± 0.0	<0.0001
Headache	20/22 (90.9 %)	0/12 (0.0 %)	<0.0001	5.2 ± 1.2	0.0 ± 0.0	0.0001
Dizziness	15/22 (68.2 %)	0/12 (0.0 %)	0.0001	1.4 ± 0.5	0.0 ± 0.0	0.0028
Itch	15/22 (68.2 %)	0/12 (0.0 %)	0.0001	3.1 ± 1.1	0.0 ± 0.0	0.0035
Eczematous lesions	12/22 (54.5 %)	0/12 (0.0 %)	0.0018	1.5 ± 0.8	0.0 ± 0.0	0.0486
Erythema	10/22 (45.5 %)	0/12 (0.0 %)	0.0058	2.2 ± 1.2	0.0 ± 0.0	ns

ns not significant, SD standard deviation, VAS visual analog scale

^a Fisher exact test

^b Student *t* test for unpaired data

Fig. 2 Oral mucosa alterations obtained at baseline (T_0), after 30 min (T_1), after 2 h (T_2), and after 24–48 h (T_3) from omPT in patients presenting Ni-related symptoms. All 22 patients with Ni-related symptoms showed lower lip mucosa hyperemia and/or edema after 2 h (T_2) from Ni omPT. In addition, eight of these 22 patients had a delayed mucosal lesion (aphthous lesion) after 24–48 h (T_3) from Ni omPT. *Ni* nickel, *omPT* oral mucosa patch test



All patients belonging to both groups A and B did not undergo any significant change in the mean mucosal perfusion on the control-test site (omPT without Ni) during the whole time of observation (T_0 – T_2).

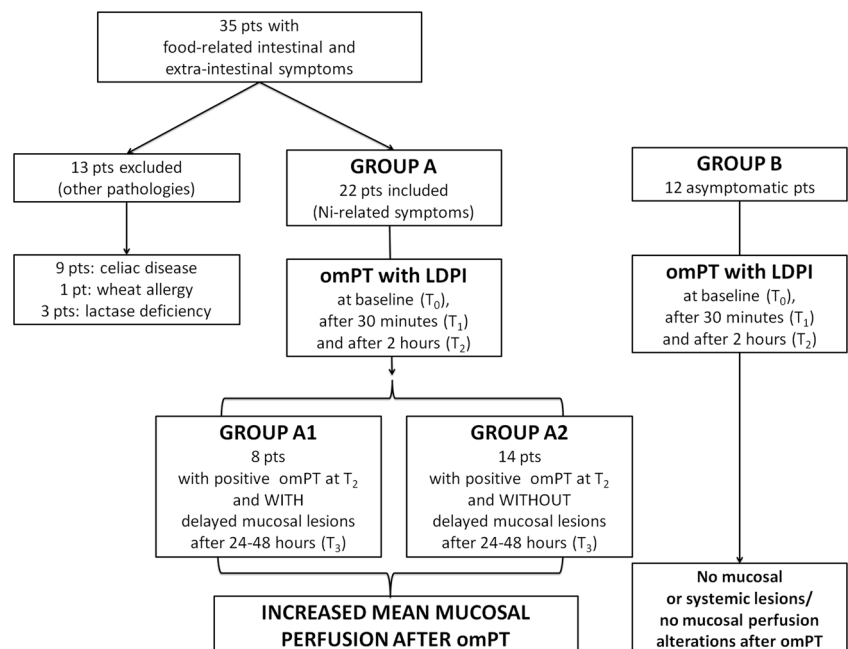
Discussion

There is growing awareness that some gastrointestinal IBS-like symptoms, as well as extra-intestinal manifestations, may be induced by the ingestion of Ni-containing food [4, 23]. It is also well known that foods rich in Ni come mainly from the Mediterranean diet inscribed on the Intangible Cultural

Heritage List of UNESCO, therefore making this issue global. Ni allergy (and specifically allergic contact mucositis) can be considered the cause of these alterations, and omPT has been proved useful for its diagnosis. However, omPT is based on operator-dependent assessment of oral mucosa lesions and their severity [6]. This limitation of accuracy has led authors to search for more objective methods for Ni allergy diagnosis.

Recently, several authors proposed laboratory tests based on peripheral or mucosal biopsy lymphocyte subsets. Valentine et al. [9] used memory lymphocyte immunostimulation assay, an optimized lymphocyte transformation test for detecting and monitoring hypersensitivity to metals, including Ni: starting from samples of peripheral

Fig. 3 Mean mucosal perfusion obtained before (T_0), after 30 min (T_1), and after 2 h (T_2) from omPT. The ANOVA for repeated measures applied among values of mean mucosal perfusion obtained in different time-points was significant in omPT-positive patients (overall $p = 0.0027$) and in those showing omPT-negative results (overall $p = 0.0350$), but not in healthy volunteers. The p values reported in the graph refer to Bonferroni multiple-comparison test. ANOVA analysis of variance, *omPT* oral mucosa patch test, pU perfusion unit



blood, it identifies a type IV metal-induced hypersensitivity response. Other studies [10] focused on peripheral blood mononuclear cells from Ni-sensitive patients in short-term cultures in the presence of optimized concentrations of Ni sulfate, detecting the production of IFN-gamma and IL-10 by means of the enzyme-linked immunospot assay. These methods resulted not exhaustive, are expensive, and require highly specialized laboratory staff. On the other hand, literature reports that LDPI has already been used in studies on patients with allergic contact dermatitis, showing an increased cutaneous blood flow that correlates with visual scoring [19–21]. On these premises, since Ni omPT has been revealed useful in allergic contact mucositis diagnosis, we tested LDPI in Ni-sensitive patients after Ni omPT, as a non-invasive, rapid, and repeatable diagnostic approach to Ni allergy.

Data showed an increased average mucosal perfusion of the lower lip after omPT in the Ni-sensitive group (group A): it has been seen not only in subgroup A1 with aphthous stomatitis but also in subgroup A2, where the aphthous lesion was absent. In particular, LDPI performed in subgroup A1 evidenced a statistically significant increase in mucosal perfusion between T_0 and T_1 as well as between T_0 and T_2 . Increased vascularization between T_1 and T_2 has also been observed, although it was not statistically significant, probably due to too-low sample size. In subgroup A2, our data showed a statistically significant increase in average mucosal perfusion only between T_0 and T_1 . Also in this case, the limited sample size has probably vitiated the assessment among the other time points. Control patients (group B) did not undergo any significant change in mucosal perfusion in all the three timeframes examined, confirming the absence of alterations.

All the diagnostic workup is summarized in the specific flow chart (Fig. 4).

The increased mucosal perfusion seen in Ni-sensitive patients may be due to a vascular reaction caused by activation

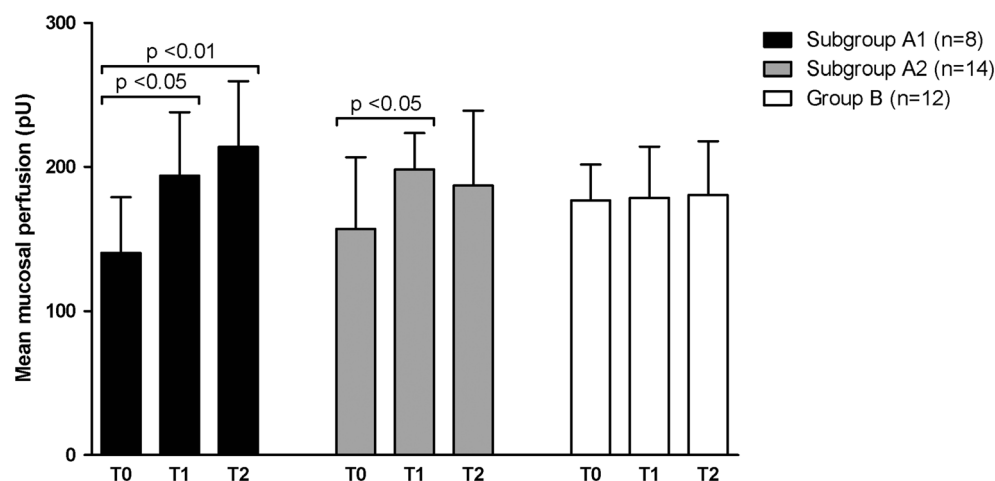
of the innate immune response, especially involving the activation of TLR4 and its downstream signaling. This hypothesis is in agreement with the results obtained by other works [8], which demonstrated that Ni is able to induce an inflammatory response via the activation of TLR4. The Ni-TLR4 complex activates an intracellular signaling cascade that culminates with the NF- κ B- and IRF3-dependent transcription of type I INF and pro-inflammatory molecules. These pro-inflammatory molecules could be responsible not only for the early vascular changes (edema and hyperemia observed at T_2 after the omPT) acting on endothelial cells but also for late manifestations (aphthous stomatitis) occurring at T_3 after omPT, depending on a cell-mediated adaptive immune response.

In conclusion, omPT properly supported by LDPI may be actually useful for diagnostic purposes in allergic contact mucositis to Ni. This also applies to those symptomatic Ni-sensitive patients who do not have the typical aphthous stomatitis after 24–48 h from Ni omPT and could risk to miss the diagnosis.

A possible limit of our study can be the absence of a clinical double-blind placebo-controlled Ni challenge test to prove the causative role of dietary Ni in provocation and maintenance of symptoms. On the other hand, the hypothesis of Ni dependence of symptoms was sufficiently assessed by the entry questionnaire administered by a medical doctor and an expert dietician. Moreover, a rigorous double-blind placebo-controlled Ni challenge test is quite difficult to achieve since, being Ni a ubiquitous element worldwide, a total elimination phase is impossible.

On the basis of limitations due to the small sample size of our study, further investigations with a larger population are still needed to integrate clinical and diagnostic knowledge of Ni allergy, a continually expanding and impactful field. However, most part of gastroenterologists and clinical

Fig. 4 Flow-chart summarizing the diagnostic workup. Delayed mucosal lesions after omPT were visible only in some of the patients with Ni-related symptoms. On the other hand, all patients presenting Ni-relating symptoms showed an increased mean mucosa perfusion after omPT, despite of control population. LDPI laser Doppler perfusion imaging, Ni nickel, omPT oral mucosa patch test, Pts patients



nutritionists should be aware of Ni allergic contact mucositis and should consider it in differential diagnosis among other IBS-like conditions.

Acknowledgments

Guarantor of Article Antonio Picarelli is the author who is acting as the submission's guarantor.

Compliance with Ethical Standards All procedures followed in this study were in accordance with the ethical standards of the institutional committee responsible for human experimentation. Furthermore, a written informed consent was obtained from each patient studied.

Submission Declaration The authors declare that the work described has not been published previously, that it is not under consideration for publication elsewhere, and that its publication is approved by all authors.

Conflicts of Interest The authors declare that they have no conflict of interest.

Source of Funding No institutional, private, or corporate financial support for the work was received.

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