LTT-MELISA® is clinically relevant for detecting and monitoring metal sensitivity

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Submitted: October 12, 2006 *Accepted: November 24, 2006*

Key words: **allergy; heavy metals; lymphocyte transformation test; MELISA®; metal sensitization**

Neuroendocrinol Lett 2006; **27**(Suppl 1):17–24 **PMID:** 17261998 NEL270706A10 **www.nel.edu**

Abstract

OBJECTIVES: Chronic low-level metal exposure may result in metal sensitization and undesirable side-effects. The main sources of metal exposure are from the environment or from corrosion of dental metal alloys. Affected patients are routinely diagnosed with the epicutaneous (patch) test. However, such testing may induce false-positive (irritative) reactions and may in itself sensitize or exacerbate symptoms. Alternatively, MELISA® (Memory Lymphocyte ImmunoStimulation Assay), an optimized lymphocyte transformation test (LTT), can be used. In this study we analyzed the overall frequency and distribution of metal sensitization among symptomatic, metal-exposed patients. In addition, we determined the reproducibility of the assay and assessed its clinical relevance for detecting and monitoring hypersensitivity to metals.

METHODS: To analyze the frequency and distribution of metal sensitization, blood from 700 consecutive patients was tested against a total of 26 metals in the validated LTT-MELISA®. For reproducibility testing, 391 single metal tests from 63 patients were performed in parallel. Finally, to assess clinical relevance, 14 patients with known metal exposure showing local (dry mouth, Oral Lichen Planus, Burning Mouth Syndrome, eczema) and/or systemic (chronic infections, fatigue, autoimmune disorders, central nervous system disturbances, depression) effects were tested in LTT-MELISA®. In 7 cases testing was repeated following removal of the allergy-causing metals or, in 2 additional cases, without therapeutic intervention.

RESULTS: Of the 700 patients tested, 74.6% responded to ≥ 1 metal in LTT-MELISA®, with a subgroup of 17.9% responding to ≥ 3 metals. Reactivity was most frequent to nickel (68.2%), followed by cadmium (23.7%), gold (17.8%), palladium (12.7%), inorganic mercury (11.4%), molybdenum (10.8%), beryllium (9.7%), titanium dioxide (4.2%), lead (3.7%), and platinum (3.4%). Reproducibility was 94.9%, with

To cite this article: Valentine-Thon E., Müller K., Guzzi G., Kreisel S., Ohnsorge P. and Sandkamp M. LTT-MELISA® is clinically relevant for detecting and monitoring metal sensitivity. **Neuro Endocrinol Lett** 2006; **27**(Suppl 1):17–24.

ORIGINAL ARTICLE

most discordant results in a low-positive range. Removal of the alloys or prostheses containing allergenic metals resulted in remarkable clinical improvement correlating with a significant reduction or complete normalization of specific lymphocyte reactivity. In contrast, both LTT-MELISA® reactivity and clinical symptoms remained unchanged in follow-up samples from the 2 patients who did not remove the source of metal exposure.
CONCLUSION: The optimized LTT-MELISA® test is a clinically useful and reliable tool for identifying and monitoring metal sensitization in symptomatic metal-exposed individuals.

Abbreviations & Units

BMS: – burning mouth syndrome
CFS: – chronic fatigue syndrome
CNS: – central nervous system
Cpm: – counts per minute
LTT: – lymphocyte transformation test
MELISA®: – memory lymphocyte immunostimulation assay
OLP: – oral lichen planus
SI: – Stimulation Index

Introduction

Metal sensitization is routinely diagnosed with the so-called epicutaneous or patch test. Patch testing, however, may yield false-negative or false-positive (irritative) results, demonstrates poor reproducibility, is evaluated subjectively, may in itself cause or exacerbate a sensitization, and is validated primarily for dermally-sensitizing allergens [2, 9, 19]. An alternative is provided by the *in vitro* lymphocyte transformation test (LTT), most optimally in the MELISA® (Memory Lymphocyte ImmunoStimulation Assay) modification as developed by Stejskal et al. [22] and validated for routine clinical analysis by Valentine-Thon et al. [28, 29]. The test itself cannot induce sensitization, is suitable for detecting both dermally and non-dermally sensitizing antigens (e.g. beryllium [Be] and titanium dioxide [TiO_2]), and yields objective and highly reproducible results. Due to the lack of an adequate "golden standard" for diagnosing a type IV hypersensitivity, however, the sensitivity and specificity of LTTs have remained difficult to assess [2, 4]. To approach this, the clinical relevance of the assay in cohorts and in individual patients can be evaluated. Test results should correlate with the presence of the specific immunizing metals, and both lymphocyte reactivity and clinical symptoms should decrease following cessation of exposure. While this has been described for several large cohorts [18, 23, 34], few studies have demonstrated this effect in single, well-characterized patients [25, 27, 29]. In this study, therefore, we first evaluate the overall technical utility and reproducibility of LTT-MELISA® in identifying metal sensitivity in symptomatic patients with suspicion of metal sensitization. Thereafter, we assess its specific clinical relevance in patients before and after therapeutic intervention as well as in patients who did not undergo any therapeutic intervention.

Material & methods

Patient samples

For frequency and distribution analysis, blood from 700 consecutive patients with clinical suspicion of metal sensitization was submitted to the former Laboratory Dr. M.Sandkamp, B.Köster, Dr. R.Hiller in Bremen, Germany (now Laboratory Center Bremen), accredited according to DIN EN ISO 15189 and DIN EN ISO/IEC 17025, for LTT-MELISA® testing. All samples were submitted in CPDA monovettes (Sarstedt AG & Co., Nümbrecht, Germany) or ACD Solution A vacutainer tubes (Becton Dickinson GmbH, Heidelberg, Germany) and transported by normal mail or by private courier to arrive in our laboratory within 24, occasionally up to 48, hours. Most samples were from practices in Germany, 116 were from practices in other European countries, and 1 each from practices in Australia, New Zealand, and the USA.

To determine reproducibility, 391 single metal tests from 63 randomly-selected patients were performed in replicate plates by the same or by two different technicians.

Finally, to assess clinical relevance, blood from 14 patients with known dental, orthopedic, or occupational metal exposure and presenting with local and/or systemic health problems was tested in LTT-MELISA® prior to dental metal replacement (n = 5) or both before and at various time-points following therapy (n = 7). In two patients, testing was performed both before and in follow-up samples without any dental metal removal.

LTT-MELISA® test

The LTT-MELISA® test was performed as previously described [22, 28]. The method has been accredited in Germany since 2001. Isolated lymphocytes were tested with various concentrations of up to 26 different metal salt solutions: aluminum (Al), Be, cadmium (Cd), calciumtitanate (CaTi), chromium (Cr), cobalt (Co), copper (Cu), ethylmercury (EtHg), gallium (Ga), gold (Au), indium (In), inorganic mercury ($HgCl_2$), iridium (Ir), lead (Pb), methylmercury (MeHg), molybdenum (Mo), nickel (Ni), palladium (Pd), phenylmercury (PhHg), platinum (Pt), ruthenium (Ru), silver (Ag), tin (Sn), titanium (Ti), TiO_2, and vanadium (V). Lymphocyte proliferation was measured on day 5 by ^{3}H-thymidine incorporation and, for quality control, by microscopic examination of the lymphoblast transformation (see Figure 1 in Müller and Valentine-Thon, this issue [13]). A Stimulation Index (SI) was calculated from the quotient of test counts per minute (cpm) and the average cpm from three negative

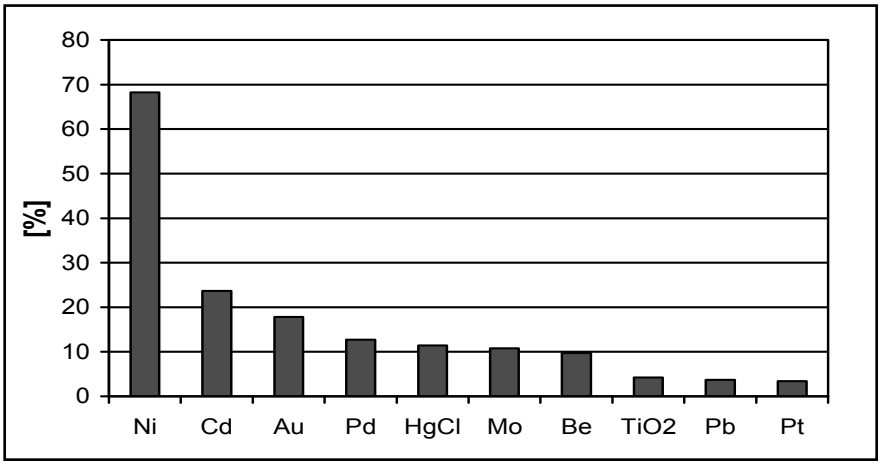

Figure 1. Frequency and distribution of metal reactivity in 700 symptomatic patients.
x axis: metals with the highest frequency of response; y axis: percent (%) of response.

controls. SI < 2 was considered negative, SI ≥ 2 but < 3 suggested a possible sensitization, SI ≥ 3 indicated positive sensitization. Among positive results, SI 3–5 was considered "weakly positive" and SI ≥ 10 "strongly positive". Valid results were those showing an average negative control (background proliferation) response of < 3000 cpm, a strong positive control (Pokeweed Mitogen [PWM, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany]) response of SI ≥ 30, and morphological confirmation.

Results and discussion

Frequency and distribution of metal reactivity

Among the 700 patients, Ni was found to be the most frequent allergen (68.2%), followed by Cd (23.7%), Au (17.8%), Pd (12.7%), $HgCl_2$ (11.4%), Mo (10.8%), Be (9.7%), TiO_2 (4.2%), Pb (3.7%), and Pt (3.4%) (Fig. 1). The reactivity to 0, 1, 2, 3, 4, or ≥ 5 metals was 25.4%, 38.1%, 18.4%, 8.0%, 4.6%, and 5.3%, respectively (data not shown). Frequencies of < 2.7% were found for Al, Ag, Co, Cr, Cu, EtHg, In, MeHg, PhHg, and Sn. Reaction frequencies to CaTi, Ga, Ir, Ru, Ti, and V (generally very low) were not considered, as too few patients were tested with these metals.

In nearly all studies of symptomatic, metal-exposed patients, reactivity to Ni has been found most frequently [12, 23, 28, 34]. High responses to Cd, Au, Pd, and $HgCl_2$ have likewise been reported [23, 25, 34,], whereas the relatively low response to TiO_2 in this study is compatible to that reported by others [12, 23] but in sharp contrast to our original report in 2003 involving 250 patients [28]. The high response to TiO_2 in the latter study (42%) was not due to mitogenic effects of TiO_2. The response was reproducible, correlated with TiO_2 exposure in individual patients, and decreased following removal/avoidance of dental or orthopedic Ti-containing alloys. Fifty-six of these patients are described in Müller and Valentine-Thon, this issue [13]. It appears in retrospect that the inadvertent inclusion of a large number of symptomatic Ti-exposed patients in that cohort of 250 patients biased the data [28]. At the same time, those results clearly demonstrated the ability of Ti to induce clinically-relevant hypersensitivity in certain individuals, as reported sporadically by others in the past [5, 10, 16, 20, 26, 27, 32, 33].

Reproducibility

Sixty-six tests were concordant positive and 305 concordant negative for an overall concordance rate of 94.9% (data not shown). Most (n = 18) of the 20 discordant results showed "weakly positive" values of SI 3.0 to 4.6. No positive discordant result was in the "strongly positive" range (SI > 10). These results confirm and extend earlier reports by our group [28, 29] as well as our recent report describing LTT-MELISA® as applied to detecting active Lyme borreliosis [30]. Stange et al. [21] likewise described high intralaboratory concordance (91.9%) for samples tested in the well-established Be-Lymphocyte Proliferation Test. Such data underline the technical reliability of validated *in vitro* lymphocyte proliferation tests for detecting sensitivity to metals and other allergens.

Clinical relevance of LTT-MELISA® testing

Five patients presenting with various diseases including chronic fatigue syndrome (CFS, **patient 1**), Oral Lichen Planus (OLP, **patient 2**), severe systemic dermatitis (**patient 3**), chronic oral ulcers (**patient 4**), and Burning Mouth Syndrome (BMS) (**patient 5**), showed strong sensitization to metals present in their dental restorations. The results correlated well with patch test, if performed (Table 1). In all 5 cases, clinical symptoms subsided completely (patients 1–4) or significantly (patient 5) following removal of the sensitizing metals.

These cases demonstrate, first, the ability of LTT-MELISA® to detect sensitivity to metals present in the patient's own dental restorations, and second, the often good (but not perfect) correlation between skin test results and *in vitro* lymphocyte reactivity. While the latter has been an issue of controversy in the past [4], modern LTTs are emerging as valid supplementary tools for detecting hypersensitivity to metals and other allergens [2, 8, 17]. These patients, in addition, confirm the

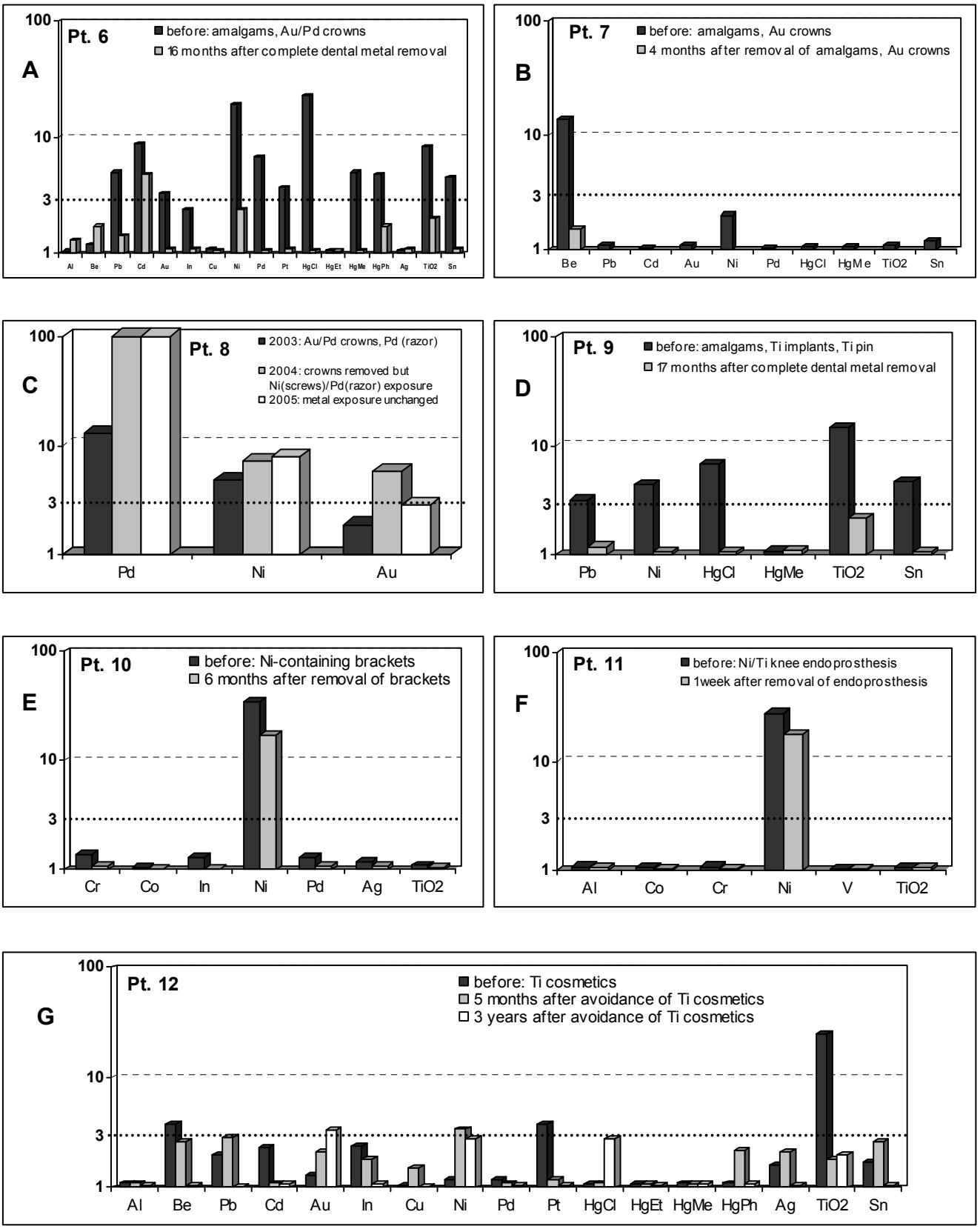

Figure 2. LTT-MELISA® reactivity in 7 patients before and after therapeutic intervention. See text for details.
x axis: metals tested; y axis: Stimulation Index (SI ≥ 3 indicates positive sensitization).

Table 1. LTT-MELISA® testing in 5 symptomatic patients prior to successful therapeutic intervention

Patient	Sex	Age (years)	Clinical symptoms	Metal exposure	Patch test	MELISA® reactivity (SI)	Therapy	Clinical effect of therapy
1	F	20	CFS, overweight, tremor in extremities, depression	3 amalgams	Ni +++ $HgCl_2$ +	Ni (14.6) +++ $HgCl_2$ (5.5) ++	Removal of all amalgams	After 3 months: all symptoms subsided, 10 kg weight loss
2	F	69	OLP for 4 years, steroid-resistant	4 amalgams, 15 Au crowns; amalgams and crowns in close contact (galvanic effect)	Ni + ++ Au + + Co ++ $HgCl_2$ -	Ni (37.1) +++ Au (17.5) +++ Co (2.3) +/- $HgCl_2$ (1.5) – TiO_2 (9.4) ++ EtHg (4.8) + Pd (4.2) + Al (3.9) + Pt (3.4) +	Removal of all amalgams, partial removal of gold crowns	After 18 months: OLP disappeared
3	F	56	Severe systemic dermatitis	5 amalgams, Co-Cr dental prosthesis	Ni + Cr ++	Ni (10.9) ++ Cr (5.2) ++	Removal of all amalgams and Co-Cr prosthesis	After 4 months: dermatitis cleared completely
4	F	36	Chronic oral ulcers (canker sores)	6 amalgams, 3 Au-ceramic crowns (galvanic effect)	Ni +++ Pd + Au -	Ni (54.5) +++ Pd (12.6) +++ Au (6.5) ++	Removal of all amalgams	After 6 months: full remission of oral ulcers; at 33-month follow-up, patient still disease-free
5	F	45	BMS, inflammation of gums, paradontitis	Amalgams, Au crowns, Ti implants	Ni ++ Au ++ Pd +	Ni (178) +++ Au (20.3) +++ Pd (4.0) +	Replacement of all dental metals with zirconium-oxide	Symptoms significantly improved

CFS = chronic fatigue syndrome; OLP = oral lichen planus; BMS = burning mouth syndrome

beneficial health effect of dental metal removal, reported frequently by others [6, 11, 13, 15, 18, 23, 24, 34].

The kinetics of reactivity in seven patients undergoing specific therapeutic intervention is shown in Figure 2A-G.

Patient 6 (Fig. 2A): Female, 51 years old, presented with central nervous system (CNS) disturbances, concentration difficulties, loss of memory, and coordination problems. She also suffered from fatigue, depression, muscle weakness, and chronic infections. She has been exposed to amalgam fillings and Au/Pd crowns but also to pentachlorophenol and lindan in her home. All skin tests in standard and metal series were negative. In LTT-MELISA® she showed strong multiple metal sensitization, especially to $HgCl_2$ and Ni. She underwent complete amalgam and Au/Pd crown removal, followed by metal elimination therapy with glutathione, sodiumthiosulfate (10%), and thiopronine. She also changed her place of residence. After 16 months, significant improvement of all symptoms occurred. At the same time, LTT-MELISA® to most metals became negative. In this case, LTT-MELISA® initially showed strong sensitization especially to $HgCl_2$ and Ni, despite negative patch test results; clinical relevance of this sensitivity was confirmed by its significant reduction upon removal of the relevant dental metals. Such cases demonstrate a major advantage of LTTs compared to skin testing in their ability to detect systemically- as well as dermally-induced sensitizations [2, 8, 13, 24, 31]. In addition, because heavy metals (particularly $HgCl_2$) and pentachlorophenol are both detoxified by the glutathione system, complete recovery of this patient required not only removal of dental metals and avoidance of pentachlorophenol but also metal elimination therapy [29].

Patient 7 (Fig. 2B): Male, 70 years old, with amalgam fillings and Au crowns, presented with seronegative oligoarthritis. LTT-MELISA® was strongly positive to Be but negative to all other metals including Ni. Four months after complete dental metal replacement and metal elimination therapy, significant improvement of clinical symptoms and normalization of Be-specific reactivity occurred. As this patient reported no occupational exposure to Be, exposure was most likely due to Be-contaminated dental metals [1, 29].

Patient 8 (Fig. 2C): Female, 77 years old, presented early 2003 with stomatitis and extremely dry mouth. She had Pd-containing Au crowns, and her lymphocytes reacted to Pd and Ni. One year after removal of crowns, her symptoms did not improve. Her metal-induced reactivity also persisted, and she became positive to Au as well. The patient reported that she had had two Ni-containing screws inserted into her kneecap in December 2003 and, in addition, used an electric razor daily. The rotating head of the razor was subsequently

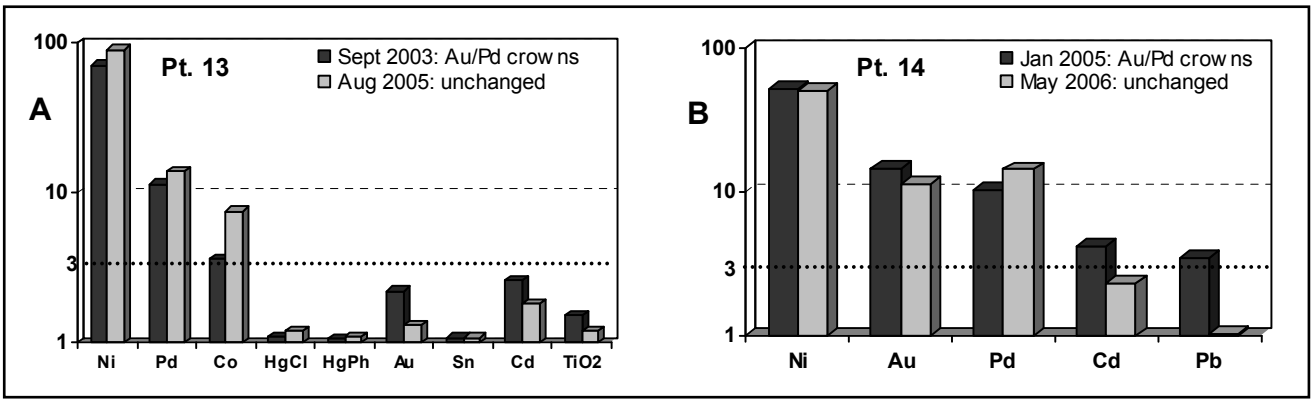

Figure 3. Follow-up LTT-MELISA® testing in 2 patients without therapeutic intervention. See text for details. x axis: metals tested; y axis: Stimulation Index (SI ≥ 3 indicates positive sensitization).

found to contain Pd (175 mg/kg). No Pd was found in her blood (< 0.2 µg/ml) or stool (< 10 µg/kg). Despite her history of metal sensitivity, she was fitted with a Ti bridge in early 2005. A subsequent third LTT-MELISA® in mid-2005 showed still very strong responses to Pd (SI = 119.5), which could be caused by her razor [7], higher responses to Ni than previously (SI = 7.9), perhaps due to the screws, and now lower responses to Au (SI = 2.9), probably due to Au crown replacement. The patient's symptoms remained unchanged. This case demonstrates that a thorough and ongoing anamnesis of the patient is required to identify all, often obscure, sources of metal exposure [7].

Patient 9 (Fig. 2D): Female, 46 years old, presented with acute polyarthritis 10 days (!) after implantation of a Ti pin. She had amalgam fillings as well as Ti implants. Multiple metal reactivities were found at the lymphocyte level, in particular to TiO_2 (SI = 14.8) and $HgCl_2$ (SI = 6.8). Seventeen months after complete dental metal removal as well as chelation with 2,3-dimercapto-1-propane sulfonate (DMPS) and 2,3-dimercaptosuccinic acid (DMSA), significant clinical improvement and normalization of all metal reactivities was observed. This case strongly suggests that the patient developed sensitivity not only to $HgCl_2$ due to Hg-containing amalgams but also to Ti due to chronic exposure to Ti-containing implants. The insertion of a Ti pin possibly provided a booster effect leading to the acute clinical symptoms. Ti sensitization has been reported in the past [5, 10, 16, 20, 26, 27, 32, 33] as well as in an extensive study by Müller and Valentine-Thon [13].

Patient 10 (Fig. 2E): Female, 13 years old, developed severe acne, fatigue, and headaches after implantation of Ni-containing orthodontic brackets. Her lymphocytes reacted strongly to Ni (SI = 34.2) only, while the patch test to Ni was negative. The patient also had high interleukin-2 (IL-2) in blood (127 pg/ml, normal value < 15 pg/ml). Six months after removal of brackets, acne and other symptoms significantly improved, *in vitro* reactivity to Ni significantly decreased (SI = 17.1), and the IL-2 level returned to normal. Further therapy, including Ni-free diet, is ongoing. Ni-containing brackets have been reported to induce Ni sensitization with local and/or systemic effects [3, 31]. Furthermore, in this issue, Muris and Feilzer describe the improvement of chronic fatigue in a Ni allergic patient after the removal of a Ni-containing orthodontic wire [14]. The fact that the patch test to Ni is sometimes negative despite clinical Ni allergy might be due to alternative exposure to Ni via mucosal membranes, rather than via dermis of the skin. Such cases underline the relevance of *in vitro* tests to detect such clinically-relevant sensitization.

Patient 11 (Fig. 2F): Female, 65 years old, presented at the end of 2004 with chronic excruciating pain in her knee six months after implantation of a knee endoprosthesis. As she was patch test positive for Ni, she was assured that her implant was a Ni-free Ti alloy. Because of her pain, she contacted the laboratory for testing of possible Ti allergy. In LTT-MELISA® tests of two consecutive blood samples, she was found to have no sensitization to the common components of Ti alloys but showed a strong sensitization to Ni (SI = 25.8 in the first test, 27.8 in the second test, average result depicted in Fig. 2F). Her orthopedic clinic assured her that this had no clinical relevance as her implant did not contain Ni and advised her to continue her palliative treatment of physiotherapy and pain medication. After 10 more months of chronic pain, the patient insisted on removal of the implant, one portion of which was subsequently found to contain no Ti but primarily Co, Cr, and Mo (> 99%) and a small amount of Ni (0.124%), the other portion containing primarily Ti (96%), V (4%), and spurious amounts of other metals including Ni (0.014%). The clinic admitted its regrettable mistake and replaced both parts with a "pure" Ti prosthesis. One week later (!) the patient was free of pain, and her Ni reactivity had dropped to SI = 17.8. Further monitoring is planned. This case dramatically demonstrates the risk of implanting metal alloys in metal-sensitive patients. Prophylactic testing may be advisable, preferably with *in vitro* proliferation tests [8].

Patient 12 (Fig. 2G): Female, 56 year-old entertainer, presented with eczema and bone and join pain. She had only amalgam fillings but massive occupational exposure to TiO_2 via daily cosmetics (up to 854 mg/kg in some products). She exhibited strong reactivity to TiO_2 (SI = 24.9) and weak reactivity to Be and Pt (both SI = 3.8).

Five months after avoiding TiO_2-containing cosmetics, in addition to metal elimination therapy, significant clinical improvement and normalization of lymphocyte reactivity was observed. The patient continued avoiding TiO_2, and three years later, lymphocyte reactivity remained in a normal range, and the patient was still in good health.

This case demonstrates not only the potential risk of developing hypersensitization to TiO_2 in cosmetics but also the stability of negative LTT-MELISA® reactivity during consequent exposure avoidance.

On the other hand, strong lymphocyte reactivity to metals persisted in follow-up samples from 2 patients with multiple Pd-containing Au crowns who did not undergo dental replacement (Fig. 3).

Patient 13 (Fig. 3A) suffered from BMS, severe chronic dry coughing, and multiple allergies. She responded strongly to Ni (SI = 70.9), less strongly to Pd (SI = 11.4), and weakly to Co (SI = 3.6) in 2003 and nearly identically 2 years later. Lymphocyte responses to $HgCl_2$, PhHg, Au, Sn, Cd, and TiO_2 remained negative. Similarly, **Patient 14 (Fig. 3B)**, who complained of Quincke's edema of the tongue, vulvitis, and eczema of the hands, responded strongly to Ni (SI = 52.2), less strongly to Au (SI = 14.6) and Pd (SI = 10.3), and weakly to Cd (SI = 4.2) and Pb (SI = 3.5) in 2005. Her metal-induced lymphocyte responses were similar one year later, with only an insignificant decrease in reactivity to Cd and Pb. Interestingly, skin tests with this patient were positive for Ni, Pd, and Co as well as for Au in the form of sodium thiosulfate aurate but not for Au in the form of Au Degunorm discs obtained from the dentist. In both cases clinical symptoms, as well as lymphocyte reactivity, remained unchanged.

Summary and conclusion

The results presented here demonstrate a high prevalence of sensitization to metals in symptomatic metal-exposed patients. Excellent reproducibility as well as a higher sensitivity of LTT-MELISA® compared to skin testing for detecting clinically-relevant metal sensitization has been shown, in particular in systemically-sensitized individuals. There has been a good correlation between LTT-MELISA® reactivity and current metal exposure. Significant and long-term decrease in lymphocyte reactivity was obtained relatively soon after removal/avoidance of metal-specific exposure. There was a direct correlation between this decrease in reactivity and clinical improvement. Finally, if metal exposure persists, metal-induced lymphocyte reactivity and clinical symptoms remain unchanged. These results support the clinical relevance of LTT-MELISA® for detecting and monitoring metal sensitization in symptomatic metal-exposed patients.

Acknowledgement

The authors are grateful to Bianca Schüttpelz, Ursel Köster, and Gudrun Emmermacher for their excellent and dedicated technical assistance. The Pd analysis of the razor head of patient 8 performed by the LADR GmbH Medizinisches Versorgungszentrum in Geesthacht (Germany), the metal analyses of the knee endoprosthesis of patient 11 performed by the GKSS Research Center in Geesthacht, and the TiO_2 analyses of the cosmetics of patient 12 performed by the former Laboratory Dr. Schiwara in Bremen (Germany), are gratefully acknowledged.

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