Metal-Specific Lymphocytes: Biomarkers of Sensitivity in Man

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Introduction

During the last years there has been an increasing interest in the possibly harmful effects of dental amalgams. Silver amalgam fillings contain a least 50% inorganic mercury together with copper, tin, silver, zinc and palladium.

The toxic effects of mercury, a potent mitochondrial poison (1), are well documented and have been subject to many review articles. However, since mercury and other dental metals such as gold or silver also bind strongly to proteins *(figure 1),* they may function as haptens and elicit allergic and autoimmune reactions.

Since induction of allergic and autoimmune reactions is generally dependent on genetic haplotype, most of the research concerning immunotoxic potential of inorganic mercury was performed in animals, where inbred strains with well-defined genetics are available. There is no doubt that inorganic mercury and gold can induce autoimmunity in genetically susceptible rats or mice. However, it is unclear why experimentally induced autoimmunity in rodents is a transitional phenomenon while clinical autoimmune disease in man often persists.

Human studies have been hampered by the fact that with the exception of monozygous twins, subjects with identical genetic susceptibility/resistance to metal-induced side effects are not available.

One of the ways to study the possible role of metals in the pathogenesis of various degenerative diseases is the screening of exposed populations for biological markers of susceptibility. T-lymphocytes play a crucial role in the induction of all types of allergic and autoimmune reactions and are therefore obvious candidates. Following the contact with antigen, which may be a foreign protein (for example pollen) or low-molecular hapten presented on the surface of autologous cells, antigen-specific lymphocytes together with B-cells and macrophages induce inflammatory reactions and cell damage. The specificity to causative antigen is retained on the surface of memory cells which are located in lymph nodes and circulate in the blood. Upon re-exposure to the same or a chemically similar cross-reacting antigen, memory lymphocytes trigger rapid recall reactions. Through cytokine release, other cell types are activated and the resulting reaction is either protective for the body or, in case of allergy or autoimmunity, detrimental. The activation of memory cells can be performed outside the body, *in vitro,* and forms a basis of laboratory test called lymphocyte proliferation test (LTT or LST).

The lymphocyte proliferation test has been routinely used in clinical immunology for 30 years for evaluation of cellular immunity but also for the diagnosis of allergy to drugs, metals (2, 3) and low-molecular chemicals (4-7). This test is approved by insurance companies in several European countries, for example in Germany and in the Czech Republic. Metals, such as inorganic mercury or nickel, may induce both antigen-specific responses and antigenindependent, mitogenic responses when added to peripheral blood lymphocytes *in vitro* (2, 8, 9). To be able to use lymphocyte test as an indicator of metal-induced allergies, it was therefore necessary to modify it in such a way that only antigen-specific responses were operating. The test was tentatively called MELISA, an acronym for Memory Lymphocyte Immuno Stimulation Assay. The major methodological differences compared to standard assay are cultivation of lymphocytes in macrocultures instead of microcultures, double-depletion of adherent monocytes/macrophages and lowering of metal concentrations to optimal levels. The details are given elsewhere (7, 10). Usually, control cultures and positive cultures are also examined for the presence of activated lymphocytes, so called lymphoblasts. The smears are prepared in cytocentrifuge, stained and examined with light microscopy. With the same method, one can also study interaction of monocytes/macrophages with metal salts.

Results and comments

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With the help of MELISA, it is possible to screen a large number of individuals claiming to suffer from local or systemic side-effects triggered by components of amalgam and other metal restorative materials such as palladium-containing gold alloys or titanium constructions. In addition to other pigments based on metals, titanium dioxide (Ti02, E 171), a white coloring agent, is used in many dental materials such as composite fillings, dental cements and root fillings. An organic mercury preservative, phenylmercury (phenyl-Hg), is still used together with lead and arsenic as a component of root filling material, N2, though banned in Sweden since 1984. We also studied the reactivity of lymphocytes to thimerosal (merthiolate, thiomersal), an ethylmercury-based salt, widely used as a preservative in soft contact lenses, nose and eye drops and in vaccines. Last but not least, the reactivity to nickel, clinically the most common sensitizer, was also studied.

The study was performed by two Swedish and one German laboratory. The majority of patients (a total of 3 162 patients studied) were referred for testing by dentists or physicians. Participating laboratories agreed to strictly follow the laboratory protocol (7). The clinical data of patients are shown in *figure 2.*

The frequency of positive lymphocyte responses in 3 162 patients is shown in *figures 3a-c.*

In MELISA, nickel was the most common sensitizer, followed by inorganic mercury, phenylmercury, palladium, cadmium, and gold. The least sensitizing metals were silver and platinum. The results from the three laboratories were similar but not identical which may reflect differences in the referral of patients. The patients referred for testing in Uppsala were highly selected since the majority had clinically demonstrable metal intolerance in their case history. Patients usually reported intolerance to various types of metal exposure including earrings, jeans buttons, and intrauterine copper devices (IUD). They also suffered from worsening of their symptoms following dental treatment. However, routine laboratory findings were usually within normal range.

When the data were grouped separately by gender, it became apparent that females responded more frequently to nickel than males *(figures 4a-c).* Since nickel sensitization is dependent on the wearing of nickel-containing earrings, it follows that females would have a higher rate of sensitization to nickel than males (11, 12). This fact is well documented in the literature and positive patch tests to nickel are more common in females compared to males. Female lymphocytes responded to nickel more frequently than lymphocytes from males in Södertälje and Munich laboratories but the difference was less pronounced among the Uppsala patients.

Interestingly, lymphocyte responses induced by inorganic and organic mercury were sex-independent in all three laboratories, implying the major role of mercury from dental amalgam and/or other external sources as a cause of sensitization. It has been shown that dental amalgam is the main source of the exposure to inorganic mercury in man (13). Clear-cut positive responses (SI $\geq$5) were present in 21 percent of patients tested in Södertalje and 33 percent of patients tested in Uppsala. In the German population the reactivity to inorganic mercury was 14 percent.

Lymphocyte responses to ethylmercury

The responses induced by thimerosal were virtually identical to responses to ethylmercury (ethyl-Hg). This reflects the fact that ethyl-Hg is the main allergenic epitope in thiomersal, ethyl-Hg thiosalicylate. Another organic mercurial, methylmercury (methyl-Hg), induced strong positive responses in 8 percent of the patients tested in Södertalje and in 9 percent of the Uppsala patients. This is the first time that the responsiveness to methyl-Hg was demonstrated in a larger population. One can only speculate about the clinical significance of these findings. Humans are exposed to methyl-Hg through consumption of polluted fish. However, some reports indicate the possibility of formation of methyl-Hg from inorganic Hg by bacteria in the oral cavity (14) and in the intestinal tract. The transformation of inorganic Hg to methyl-Hg by certain bacterial strains *in vitro* has been demonstrated previously (15, 16). Since methyl-Hg is highly neurotoxic, the presence of methyl-Hg-specific lymphocytes in some patients may indicate exposure and warrants further studies.

Phenylmercury: a bactericidal agent and neglected sensitizer?

Phenylmercury (phenyl-Hg) salts are used as preservatives in eye-drops and cosmetics and may induce

allergic reactions (17). In the oral cavity, phenyl-Hg has been identified as one of several toxic components of N2, a widely used root-filling material in many countries (18). The majority of patients tested had old root-fillings which may have contained N2. Eighteen percent, respectively 13 percent of patients tested in Sweden reacted to phenyl-Hg with positive responses with stimulation index (SI) equal to or more than 5. Phenyl-Hg has been previously used in patch testing but has been omitted in the last years in spite of continued usage of phenyl-Hg.

While studying possible immunological responses induced by mercury, one must consider that organic and inorganic mercurials *do not cross-react.* Even within the family of organic mercurials, phenyl-Hg does not cross-react with methyl- or ethyl-Hg. The situation is less clear when responses induced by methyl-Hg and ethyl-Hg are studied. Lymphocyte responses indicate that those two aliphatic mercurials may partly cross-react (7).

Gold and palladium - metal salts with strong sensitizing potential

The high prevalence of gold- and palladium-specific responses in all three laboratories is not surprising when considering the results of patch tests. In a recent study of 397 patients claiming intolerance to dental metal alloys, 23 percent demonstrated patch test positivity with gold sodium thiosulfate. The prevalence of positive tests to palladium was 8 percent and to nickel 22 percent (19). In a study of 832 cases, the frequency of gold-positive skin reactions was 8,6 percent (20).

The lymphocyte reactivity to gold thiosulfate *in vitro* varied from 18 to 19 percent in Swedish patients while the responsiveness in Germany was only 6 percent. The corresponding values for palladium, a frequent component of dental metal alloys, was 1 3 to 17 percent in Sweden and 3 percent in Germany. In a study of 52 patients with gold-positive patch test, 75 percent of patients were positive in lymphocyte proliferation tests (3).

Hypersensitivity to metals - a frequent phenomenon?

In an unselected Danish adult population (561 subjects) the prevalence of nickel-positive patch tests was 11 percent for women and 2 percent for men. The frequency of reactions to thimerosal ranks 3 percent in both sexes. The authors conclude that in general populations IgE-mediated and cell-mediated hypersensitivity is a common finding (21).

Inorganic mercury - allergen or mitogen?

The results of MELISA in 3 162 patients indicate that lymphocyte reactivity to low concentrations of inorganic mercury is rather common, 14-33 percent of patients tested had positive responses (SI≥5). On the average 719 patients, or 23 percent, of those tested had mercury-responsive lymphocytes in their blood. This number directly disputes the current opinion regarding the frequency of mercury-induced sensitization. The notion that mercury sensitization may be more common as previously expected is supported by recent results from Finland. Thus, 80 patients with oral lichenoid changes out of 118 tested displayed positive patch test reactions to metals of dental fillings. 76 reactions were found to various mercury compounds but no positive reactions to acrylates were seen (22).

One could still argue that mercury-induced lymphocyte responses might be mitogenic in nature. Some individuals could have a lower threshold for triggering of such responses with low concentrations of inorganic mercury which are usually non-mitogenic in normal population.

If mercury-induced responses were *mitogenic in nature,* the removal of the offending allergen in hypersensitive individuals would have no impact on mercury-induced lymphocyte reactivity. In other words, the low triggering threshold would not be affected by amalgam removal. In contrast, if the mercury-induced lymphocyte proliferation was *truly allergenic in nature,* the replacement of metallic dental alloy (amalgam) by metal-free materials would result in diminished exposure to allergen *in vivo* and consequently in reduced levels of mercury-induced lymphocyte reactivity. To delineate between these alternatives the following study was performed.

The impact of dental metal replacement on lymphocyte reactivity *in vitro* - a prospective longitudinal study

The design of the study is shown in *figure 5.* A total of 105 patients (100 females and 5 males) were tested by MELISA during 1991-1993. The majority of the patients suffered from symptoms resembling Chronic Fatigue Syndrome (CFS) and from metal intolerance *(figure 6).*

The results of MELISA at the start of the study are shown in *figure 7.*

The results are in agreement with findings in larger patient materials *(figures 3a—c)* and can thus be considered as representative. At the end of the study, a total of 86 patients had completed replacement of amalgams and other metallic restorations with composites and metal-free ceramics.

The patients were repeatedly contacted by letter or telephone and asked to comment upon their current health status as compared to their health before admission to the study. The length of time that patients have been metal-free is shown in *figure 8.*

At the end of 1997, over 50 percent of the patients had been metal-free for more than two years.

A total of 78 percent of the patients reported improvement in health status as compared to the period prior to metal removal *(figure 9).*

Results of the follow-up MELISA, as compared to the initial MELISA, in 54 patients who agreed to repeated testing, is shown in *figure 10.*

It can be seen that the number of positive lymphocyte responses to inorganic mercury, as well as to other metals present in dental metal alloys and/or root filling materials (palladium, gold, cadmium, lead, phenyl-Hg, titanium) diminished significantly. In contrast, the reactivity to nickel, a ubiquitous metal which is avoided as a component of dental metal alloys in Sweden, did not change significantly.

Discussion

This study demonstrates that a significant number of patients with dental metal intolerance have mercury-specific lymphocytes in the blood. Among other metals, nickel was the most frequent sensitizer, followed by inorganic mercury, phenylmercury, gold, cadmium, and palladium.

From an immunological point of view, the binding of mercury or other metals with strong affinity to SH-groups (i.e. gold, cadmium, and lead) to autologous cell proteins may change their antigenicity and make such proteins *foreign* and therefore vulnerable to the attack of immunocompetent cells *(figure 1).*

The binding of radioactive Hg may easily be demonstrated by autoradiography (Seo A, unpublished). Ongoing metal-induced chronic inflammation and resulting increase in cytokines may affect the hypothalamic-pituitary-adrenal (HPA) axis and trigger a myriad of physical and psychiatric complaints (23). The symptoms include profound tiredness, muscle-skeletal pain, sleep disturbances, gastrointestinal and neurological problems as well as other recognized features of Chronic Fatigue Syndrome and fibromyalgia (24-26). Following the removal of offending agents, which is established clinical practice in allergology, the activity of the immune system is down-regulated and the patient recovers. The clinical improvement can be monitored by decreased metal-specific responses *in vitro.* In fact, Japanese researchers have recently shown that a low metal diet and/or dental metal removal was successful in 70 percent of patients with moderate to severe atopic dermatitis who showed positive patch tests to metals (27). Following metal avoidance, systemic skin symptoms disappeared in the majority of patients. The protocol of allergen avoidance together with replacement of dental metals in sensitive patients is routinely used in the treatment of psoriasis in Kyoto, Japan (28) and in Neukirchen, Germany (29). In the Kyoto Clinics, the sensitization to dental metals is routinely diagnosed by lymphocyte proliferation tests (28).

Conclusions

The results presented above indicate that lymphocyte sensitization to heavy and transition metals is a common finding in patients with suspected metal intolerance. Since memory lymphocytes circulate through the entire body, they may, upon contact with tissue-bound metal, initiate an inflammatory

process. Chronic inflammation stimulated by the release of metal ions from dental metal implants may affect the HPA axis and trigger a myriad of non specific symptoms classified as CFS, fibromyalgia, Multiple Chemical Sensitivity (MCS) and others. Thus, in addition to the investigation of possible infectious agents such as bacteria and viruses, the metal etiology of above-mentioned chronical diseases should be taken in account.

The majority of allergic reactions to metals belong to the delayed-type hypersensitivity, It is generally accepted that these reactions are located in the skin, oral mucosa, or the gastrointestinal tract. The possible role of metal-specific lymphocytes in organ-specific and systemic autoimmunity remains a challenge for further studies. Metal-specific lymphocytes may be used as markers of sensitization in such studies.

Recommendations for future studies

Identification and treatment of patients at risk for metal-induced side effects

1. First selection may be made through inquiry.
2. Second selection may be made by a lymphocyte proliferation test, e.g. MELISA.
3. Subjects with definite positive responses to mercury or other metals present in their dental restorations should be referred for medical and odontologic evaluation. If the results of *in vitro* tests and/or patch tests are considered clinically relevant, the patient should be recommended to replace offending metal alloys by biocompatible materials at an individual basis.
4. The beneficial effect of dental metal removal on health status in sensitive patients can be monitored by a lymphocyte proliferation test and by the decrease of inflammatory markers in serum. The proper markers have yet to be determined.

Addendum

A similar protocol based on *in vitro* diagnostics and allergen avoidance is currently used in USA. The factories involved in the production of beryllium-containing products have started to screen beryllium-exposed individuals with the help of lymphocyte proliferation tests (LTT). The presence of beryllium-specific lymphocytes in the blood is taken as an evidence of beryllium sensitization. If symptom-free individuals have positive tests, they are considered latently sensitized and at risk for development of beryllium disease. These subjects are routinely relocated to a beryllium-free environment. According to Newman, about 50 percent of LTT-positive symptom-free subjects will develop beryllium disease if beryllium exposure continues (30).

Figures and tables

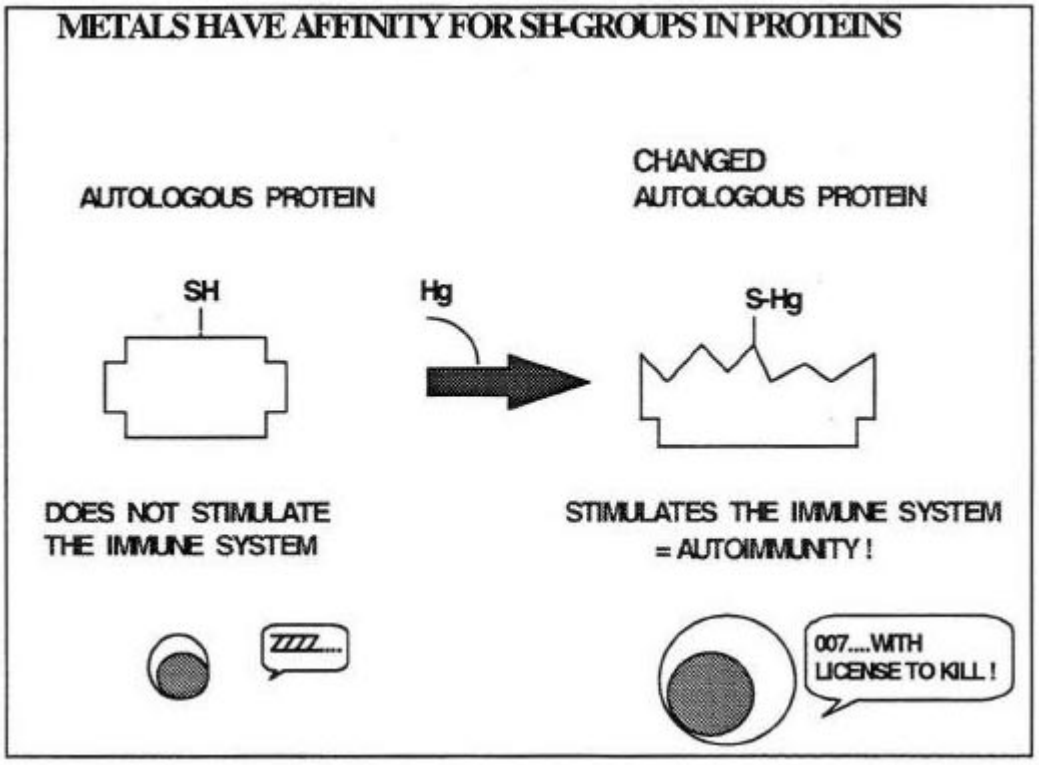

Figure 1. Metals have strong affinity to SH-groups in proteins and alter their structure.

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Total number of patients: 3162

Södertalje		**Females**	**Males**
No. of patients	930	700 *(75%)*	230 *(25%)*
Age, mean (years)	*47,5*	48,3	45,2
Age, median (years)	48,0	48,0	46,0
Munich		**Females**	**Males**
No. of patients	1120	771 (69%)	349 31%
Age, mean (years)	39,7	41,4	*37,5*
Age, median (years)	38,0	38,0	37,0
Uppsala		**Females**	**Males**
No. of patients	1112	799(72%)	313(28%)
Age, mean (years)	49,0	49,9	46,7
Age, median (years)	49,0	51,0	47,5

Table 1. Clinical data of patients included in the study.

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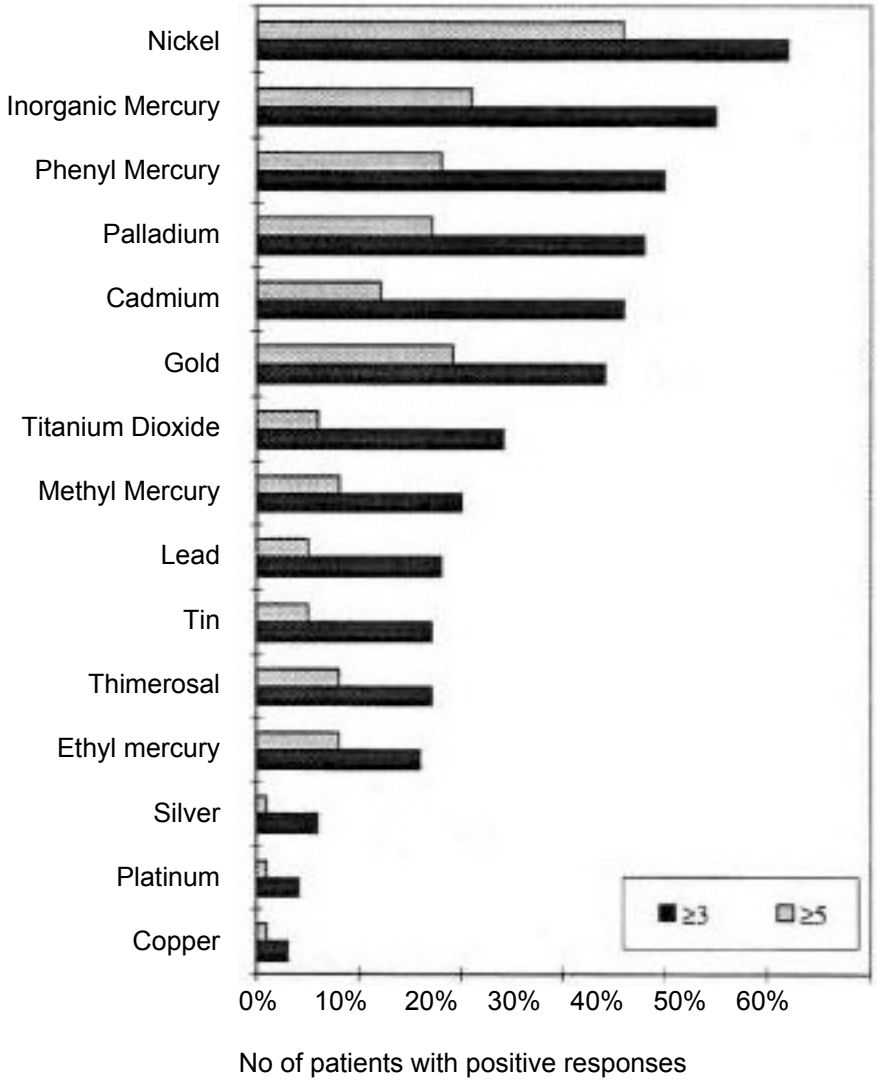

Figure 3a. Prevalence of positive lymphocyte responses in Södertälje (n=930).

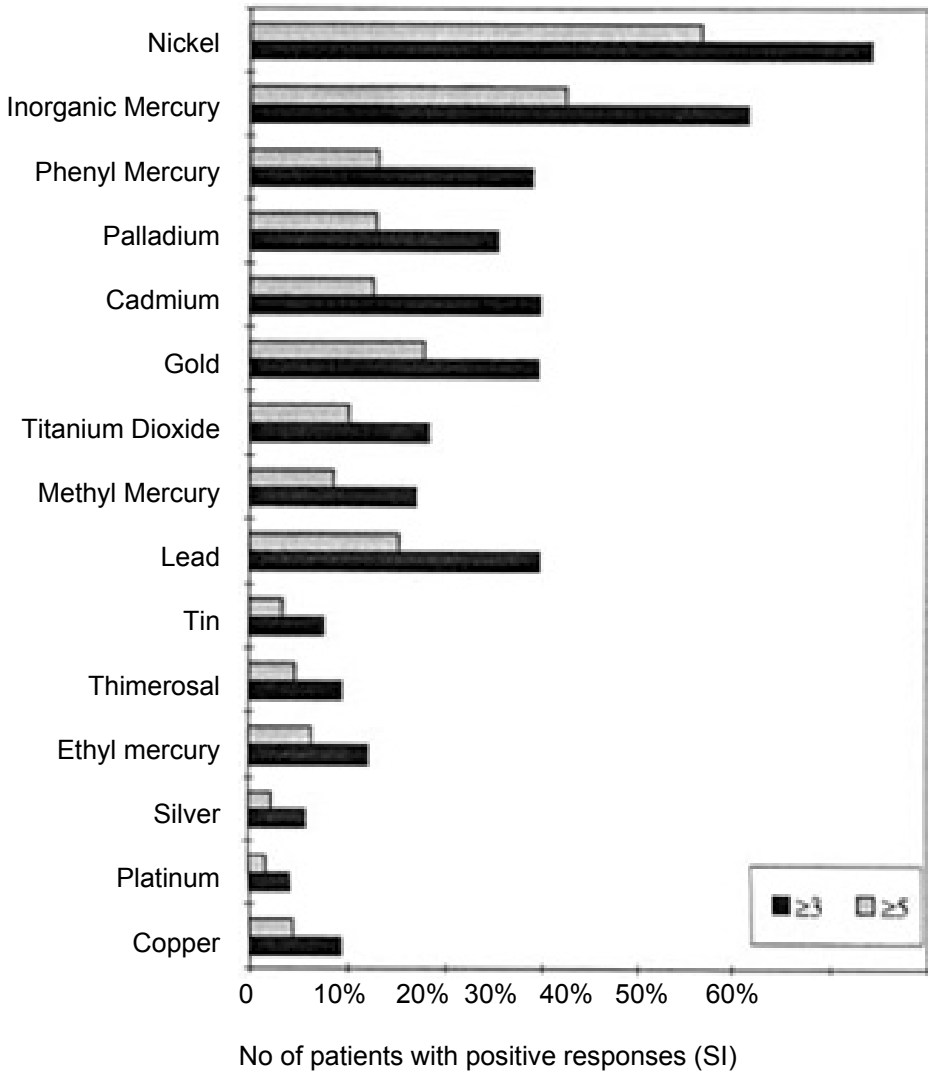

Figure 3b. Prevalence of positive lymphocyte responses in Uppsala (n=1112)

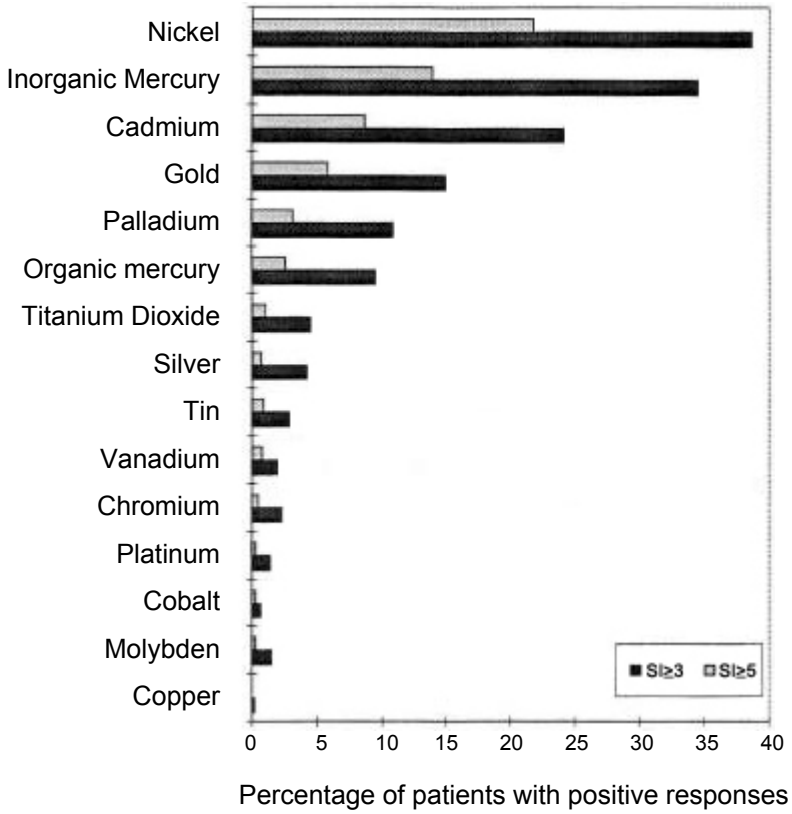

Figure 3c. Prevalence of positive lymphocyte responses in Munich (n= 1120).

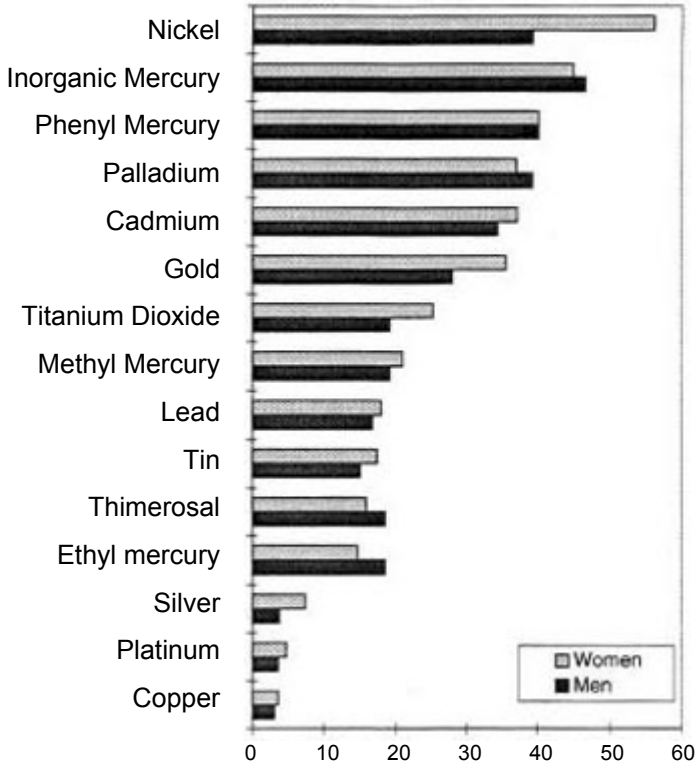

Figure 4a. Metal: reactivity for men and women in Södertalje (n=930).

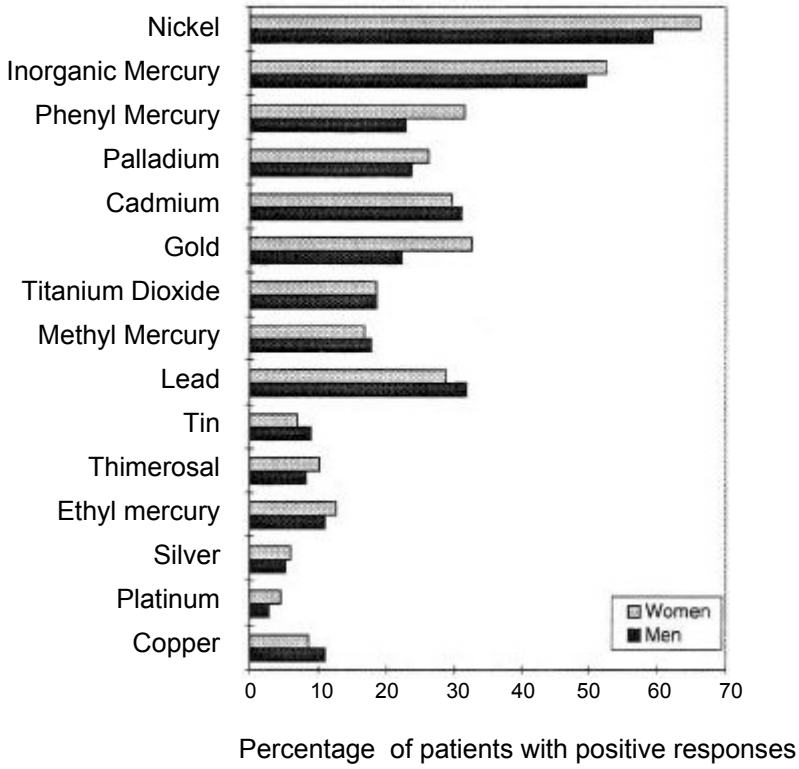

Figure 4b. Metal reactivity for men and women in Uppsala (n= 1112)

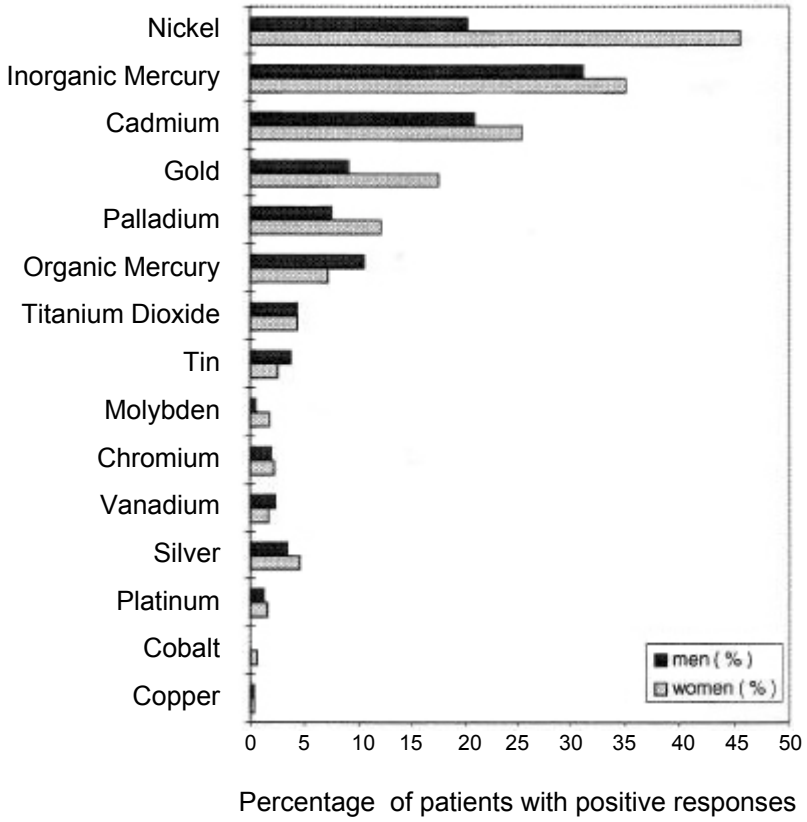

Figure 4c. Metal reactivity (SI ≥3) for men and women in Munich (n=1 120).

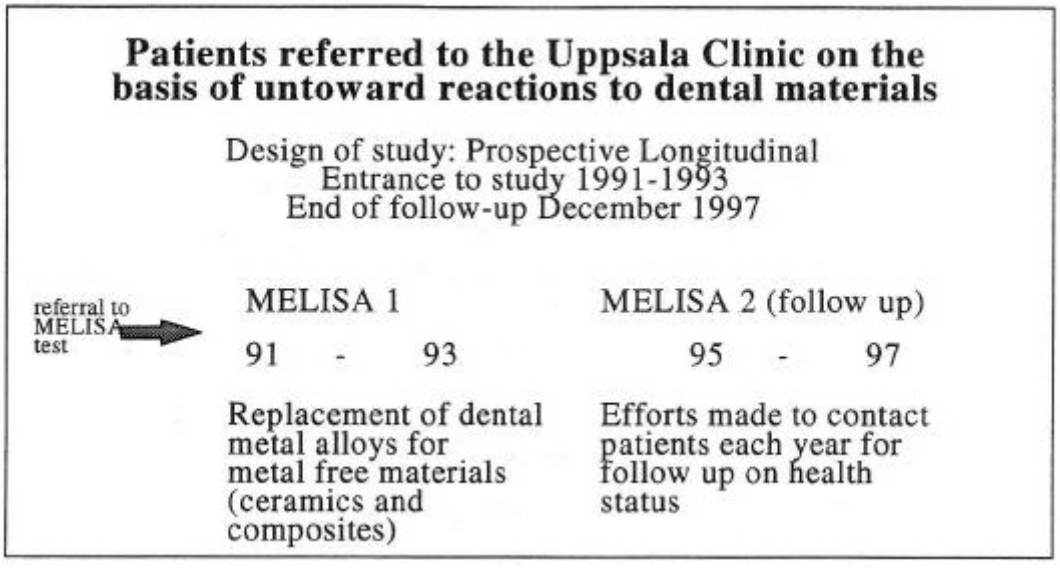

Figure 5.

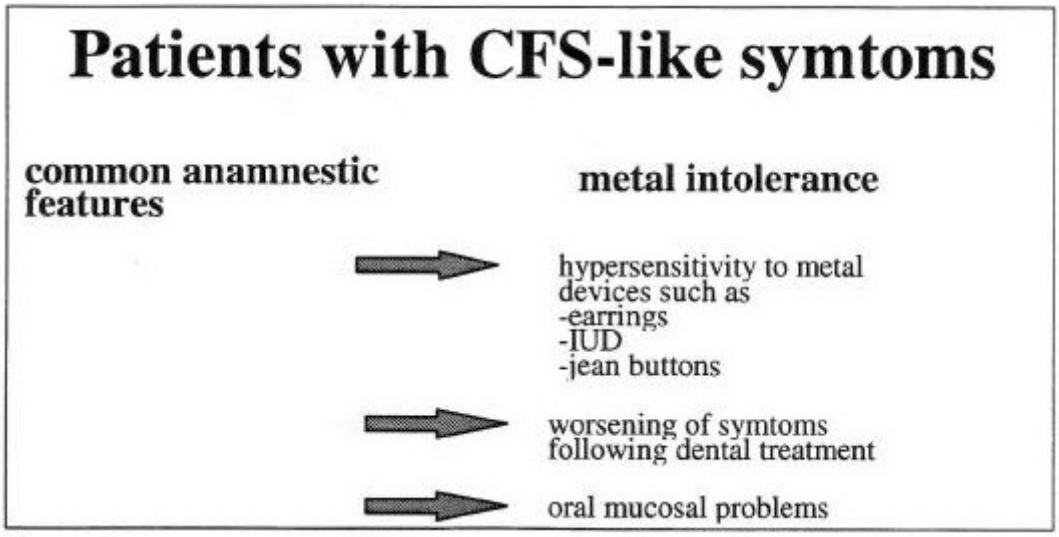

Figure 6.

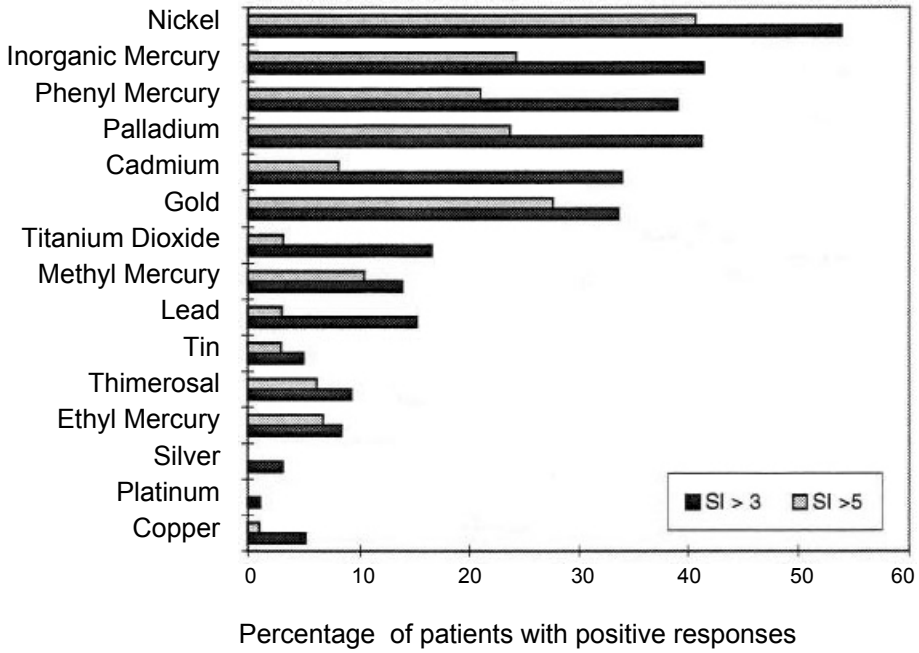

Figure 7. Prevalence of positive lymphocyte responses at the beginning of study (n=105).

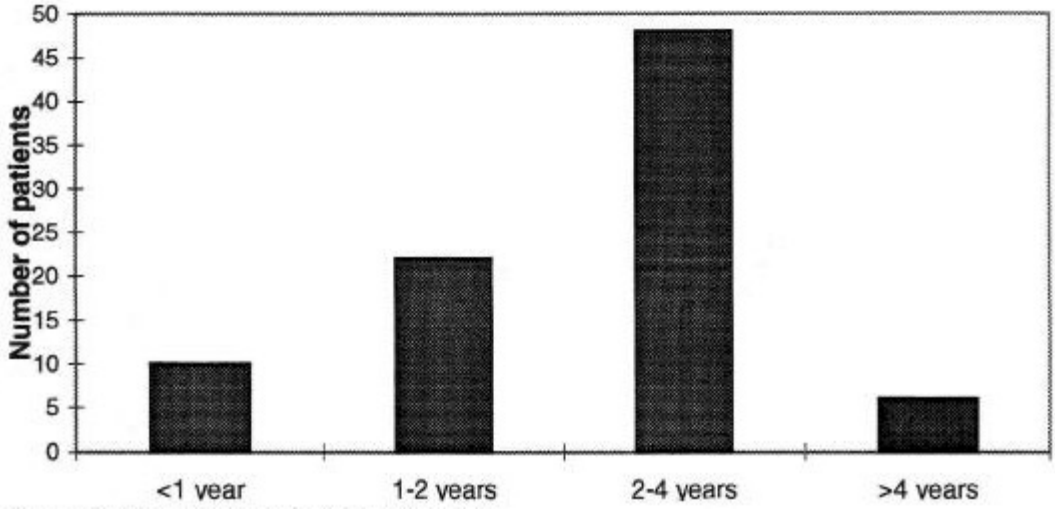

Figure 8. Metal-free period for 86 patients.

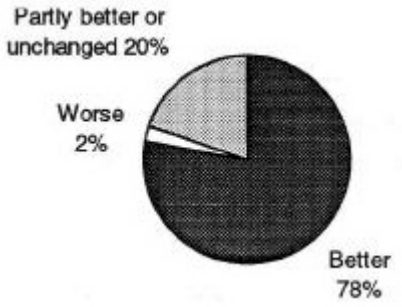

Figure 9. Health in 86 patients after dental metal replacement.

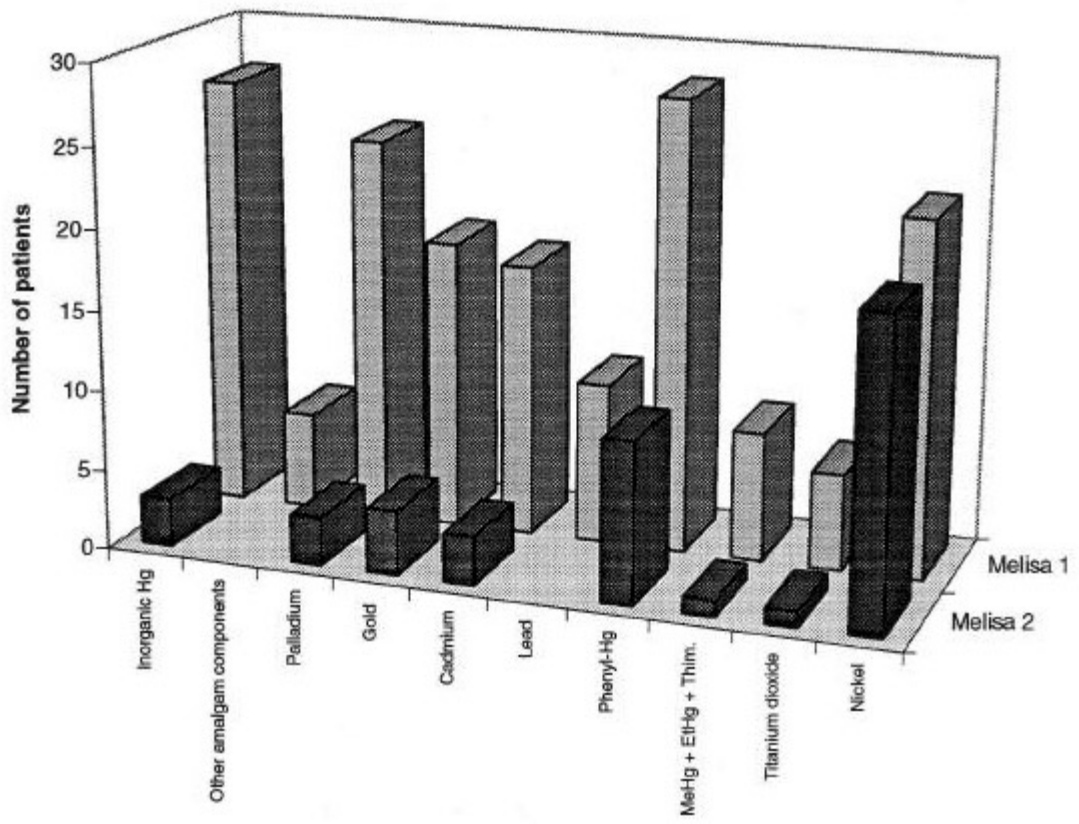

Figure 10. Residual Lymphocyte stimulation in 54 patients following dental metal replacement.

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