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Chapter 8

Palladium-based dental alloys are associated with oral disease and palladium induced immune responses

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8.1 Abstract

Objectives: The study was designed to explore possible associations between the presence of Au- and Pd-based dental alloys, oral lesions, systemic complaints, and specific *in vivo* and *in vitro* immune responses.

Methods: The investigated population consisted of three groups: 26 non-metal allergic volunteers, 25 metal allergic patients, and 20 oral disease patients. Medical histories were taken, oral examinations were carried out, and compositions of all dental alloys were determined. Next, Au and Pd skin tests and *in vitro* assays were performed, revealing cytokine production of peripheral blood mononuclear cells (Th1: IFN-γ; Th2: IL-5 and IL-13) and lymphocyte proliferation (LTT-MELISA®).

Results: Non-plaque-related gingivitis (NPG) was associated with the presence of Pd-based dental alloys, Pd-positive skin tests and *in vitro* assays. Collectively, participants with Pd-based dental alloys showed increased Pd skin test reactivity ($p<0.05$) and lymphoproliferation ($p<0.05$). In contrast, oral lichenoid lesions were associated with Au-based alloys ($p<0.05$), but this was not reflected by Au-specific immune reactivity.

Conclusions: Oral lesions and Pd-induced immune responses are associated with the presence of dental alloys. Still, most oral disease patients did not show positive skin test results or *in vitro* signs of specific immune reactivity, suggesting local toxic reactions or involvement of innate immune responses.

8.2 Introduction

Both gold (Au) and palladium (Pd) are well-known contact sensitizers primarily associated with dental cast alloys and oral disease, like oral lichenoid lesions and gingivitis (1, 2). For Au a positive correlation was found between number of dental Au restorations and positive patch test results (3). One study reported that Pd mono-sensitization was only found in patients with oral lesions and Pd containing dental alloys, whereas ACD patients frequently (70-90%) show concomitant reactivity to both nickel and Pd (4). In fact cross-reactivity between the two metals has been documented both *in vivo* and *in vitro* (5, 6). Of note, the prevalence of Pd allergy has been underestimated until the introduction of a more sensitive test allergen, i.e. sodium tetrachloropalladate (Na_2PdCl_4) (7). Although *in vitro* immune assays to substitute *in vivo* skin testing for diagnosing metal allergies are desirable, to date none have been found satisfactory, most probably due to the complex laboratory procedures involved and gaps in knowledge of the exact pathogenesis. Contact allergy to nickel is historically considered a Th1 mediated immune response but convincing evidence is piling up suggesting mixed Th1 and Th2 responses (8-10). For Au and Pd such evidence is scarce (11, 12).

Clinically, both Au and Pd allergies are mainly associated with oral disease like lichenoid lesions and gingivitis (2, 13-15). Also oral and systemic subjective complaints have been described, albeit attributed to dental alloys in general (16). Oral complaints reportedly vary from burning mouth, dry mouth, and metal taste whereas possibly related systemic complaints may comprise fatigue and joint- and muscle pain (17). Furthermore, an association between urine Pd concentrations and thyropathy has been reported (18).

A prerequisite for adverse reactions to dental alloys is the dissolution of metal ions mostly resulting from corrosion processes. As the corrosion properties of dental alloys depend on their composition and metallurgical structure, metal ion release may vary significantly between different alloys. Therefore, some Pd-based alloys are more prone to corrosion than others depending on the alloying components like silver (Ag), copper (Cu), and Au (19). On top of that the laboratory casting procedures and porcelain firing may alter the corrosion behaviour of especially Pd-based alloys (20). Especially Pd-Cu and Ag-free Pd alloys are associated with Pd ion release and possible subsequent adverse reactions (21).

The current study was designed to explore possible associations between the presence of various Au- and Pd-based dental alloys and oral lesions, and systemic

complaints, and both Au and Pd induced immune reactivity as detected by *in vivo* skin testing and *in vitro* immune responses, in three well defined patient groups. This *in vivo* study is unique since clinical features of adverse reactions and metal-induced immunologic responses are associated with specific dental cast alloys.

8.3 Materials and methods

The investigated population consisted of three groups, that are, a group of non-metal allergic volunteers (non-allergic), a group of patients with a history of metal induced allergic contact dermatitis (metal-ACD), and a group of patients with oral lesions attributed to their dental restorations (oral-disease). From all participants a medical history was taken, oral examinations were carried out, and compositions of all dental alloys were determined. Next, Au and Pd skin test and LTT-MELISA® (a commercially available lymphocyte proliferation test) were carried out. Furthermore, metal-induced immunologic reactivity was measured, in particular cytokine production of peripheral blood mononuclear cells (PBMCs) (Th1: IFN-γ; Th2: IL-5 and IL-13).

Volunteers and Patients

The participants of the non-allergic group (n=26) were recruited from personnel within the local university campus on the basis of a strictly negative history of metal allergies. Participants of the metal-ACD group (n=25) were selected from patients attending the Skin Allergy Unit of the Department of Dermatology from the VU University Medical Centre in Amsterdam, by taking appropriate medical histories. Patients were included if they responded positively to the question whether they had ever suffered from itching, redness and/or oozing and crusting as a result of direct prolonged skin contact to jewellery or metal objects and/or as a result of wearing inexpensive, base-metal containing piercings/earrings. The oral-disease group (n=20) consisted of patients referred to the adverse reaction unit of the Academic Centre for Dentistry Amsterdam (ACTA) with oral lesions attributed to their Pd and/or Au containing dental crowns or bridges. The study was approved by the medical ethical committee of the VU University Medical Centre and the research institute of the Academic Centre for Dentistry Amsterdam. All participants gave written informed consent.

Dental alloys

Samples of all metal-based dental appliances of all participants were taken to determine the composition using Scanning Electron Microscopy- Energy-Dispersive X-ray spectroscopy analysis (SEM-EDX), a method for qualitative and quantitative composition assessment of alloys (22). Dental alloys were first categorized in Au- and Pd-based alloys. Au-based alloys consist of at least 45 atom percentage (At%) Au, and not more than 20At% of Pd, referred to as Au-based. Subsequently, three subgroups were defined. The first group is free of Pd (Au-Pd^{Free}) (Au: 63-93At%), the second consists small amounts of Pd (Au-Pd^{low}) (Au: 70-83At%; Pd: 1-10At%), and finally a group of alloys with some Pd but less Au (Au-Pd^{med}) (Au: 45-64At%; Pd:2-20At%). The Pd-based alloys consist of 30-90At% Pd and are alloyed mainly with Au (<45At%), copper (Cu) (15-30At%), or silver (Ag) (15-55At%), or with miscellaneous metals but consist of high amounts of Pd (Pd:>75%At%) and referred to as Pd-Ag, Pd-Cu, Pd-Au^{med}, and Pd-Misc. respectively.

Oral lesions

Based on an oral examination the presence of oral lesions was investigated by the same clinician (JM) and categorized as: a. none; b. Oral Lichen Planus or Oral Lichenoid Lesions (OLP/OLL); c. gingival inflammation attributed to metal contact and not to bacterial plaque (NPG = Non-Plaque related Gingivitis). Concordant oral lesions were also recorded. The putative relation between oral lesions and exposure to dental crowns was investigated within the whole population (n=71)

Systemic complaints

The medical history was taken by the same clinician (JM). The choice of what systemic complaints to investigate was based on earlier studies by Vamnes and Helm (17, 18): a. thyropathy; b. chronic fatigue; c.joint & muscle pain; and d. chronic sinusitis. Chronic fatigue was subjectively considered as chronic in terms of abnormally often tired for a long period of time without obvious reasons. Due to the relative small sample sizes no conclusive data were to be expected regarding possible associations between these complaints and exposure to distinct dental restorations, but trends might become visible.

Skin testing and immunologic parameters

Epicutaneous patch testing or skin testing (ST) was performed with Au (2.0% $AuNa_3(S_2O_3)_2.2H_2O$) (Hermal, Hamburg, Germany) and with Pd (3.0% Na_2PdCl_4 pet.)

(Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). Van der Bend patch test chambers (Van der Bend BV, Brielle, the Netherlands) on Fixomull tape were used. Patches were removed at day 2 and readings were done at day 2 and 3 (48 and 72h) according to the recommendations of the International Contact Dermatitis Research Group (ICDRG). Additional readings were performed after 1 week (168h). Skin tests were regarded positive if at least at one time point a positive reaction (+, ++ or +++ reaction) to the test salt was recorded. Strongest reactions were used for further analyses. Doubtful reactions, including follicular and erythematous reactions, were recorded as (+/-) but regarded as negative test results.

Furthermore, a commercial lymphocyte proliferation, LTT-MELISA® (MEmory Lymphocyte Immuno Stimulation Assay), for Au and Pd was carried out. LTT-MELISA® is a patented modification of the LTT or LPT using a standardized (23, 24) and validated (25) protocol. Blood samples, that is 30 ml heparinized blood per patient, were transported by private courier to arrive in the test laboratory (Dept of Immunology, Laboratory Centre Bremen, Germany) ultimately within 48 hours after the blood was drawn. Metal specific proliferation was assessed by the stimulation index (SI; ratio of mean [^{3}H]-thymidine uptake in stimulated/non-stimulated cultures). Test results considered positive when SI>5 as defined by the LTT-MELISA® laboratory. Pokeweed mitogen and non-stimulated (blanco's) wells were used as positive and negative controls, respectively.

Finally, to measure Au and Pd induced IFN-γ (Th1) IL-5, and IL-13 (both Th2) cytokine productions, 90 ml of peripheral heparinized blood was taken directly before skin testing and subsequently transported to the laboratory within 2 hours. Culture media were supplemented with IL-12/IL-7 (0.1/0.03 ng/ml) for IFN-γ and with IL-4/IL-7 (3.0/0.1 ng/ml) for IL-5 and IL-13 production as described before (10, 26, 27). PBMC's in the three culture conditions were stimulated with/without maximum non-toxic doses and 3-fold dilutions of metal salts, i.e. 30 and 90 µg/ml Na_2PdCl_4 and 3 and 9 µg/ml $AuNa_3(S_2O_3)_2.2H_2O$, or with pokeweed mitogen as positive control. Metal induced cytokine increments (Δ) were assessed by subtracting the cytokine concentration of non-stimulated from stimulated culture supernatants. For practical reasons $\Delta pg.ml^{-1} < 1$ are set on 1. Cut-off values for Au- and Pd-induced cytokine responses were defined as the mean cytokine increment ($\Delta pg.ml^{-1}$) + 3 times the standard deviation (+3SD) of the non-allergic patient group (control-group). Of note, in the non-allergic group two volunteers showed, despite their negative history of contact allergy to metals, positive skin test results to Pd and were therefore excluded for the calculation of the cut-off values. For Au-induced immune responses no

positive skin test results were found. Still, few considerable outliers were found. Therefore, the highest (n=1) Au cytokine response for each variable (IL-5, IL-13 and IFN-γ) was excluded for the calculation of the cut-off value.

Blinding of the data

In order to prevent that the results of *in vitro* analyzes were affected by having knowledge of results of *in vivo* analyzes the data were collected separately. In other words skin test results were blinded to the investigators dealing with the *in vitro* assays and *vice versa*. To do so all data were collected by two independent investigators (see acknowledgements).

Statistics

IBM® SPSS® (IBM® SPSS® INC. SPAW Statistics, New York, United States, version 21.0) was used for statistical analyses. Differences in distributions of *in vitro* test results were calculated for statistical significance ($p<0.05$) using 2-sided Fisher's Exact Test. P-values smaller than ($p<0.05$) were considered statistically significant. When P-values below 0.05 were found Odds Ratios (ORs) were calculated, including 95% Confidence Intervals (95%CI)to convey information about the magnitude and precision of the associations.

8.4 Results

Comparison of patients groups

First, the clinical presentation of complaints and specific immune response were evaluated comparing the three selected patient groups, that are, non-allergic, metal-ACD and oral-disease patient groups (Table 8.1). Although the patients from the oral-disease group were suspected for adverse reactions to dental alloys, only half (10/20) of them reported a history of metal allergy. Evidently, oral lesions were most frequent in the oral-disease group and only non-plaque-related gingivitis (NPG) was found among all patient groups. Systemic complaints were not limited to the oral-disease group, as similar frequencies were observed in healthy and metal-allergic patients. Since systemic complaints notoriously increase with age and are thought to

Table 8.1 Summary of data according to patient group allocation. Exposure to gold (Au) and palladium (Pd) based dental alloys, positive skin tests and *in vitro* assays to Au and Pd, oral lesions and systemic complaints. **NOTE 1**: Systemic complaints increase with age and are thought to be related to the female sex, Therefore, to compare the groups for systemic complaints all male participants were excluded as well as patients under age of 39 to get groups that are equally distributed for age and gender. **NOTE 2**: The number of dental restorations obviously increase with age. Therefore to compare the groups for dental restorations all patients under age of 39 were excluded to get groups that are equally distributed for age. Grey areas represent selection criteria. Statistical significant differences are given (p<0.05) using 2-sided Fisher's Exact tests.

	Non-allergic (**n=26**)	Metal-ACD (**n=25**)	Oral-disease (**n=20**)
Metal allergy history	0% (n=0)	**100%** (n=25)	**50%** (n=10)
Oral lesions	**12%** (n=3)	**28%** (n=7)	**100%** (n=20)
Oral Lichen Planus /Oral Lichnoid Lesions (OLP/OLL)	0% (n=0)	0% (n=0)	**50%** (n=10)
Non-plaque related gingivitis (NPG)	**12%** (n=3)	**28%** (n=7)	**65%** (n=13)
NOTE 1: age and gender corrected n=39	n=5	n=16	n=18
Systemic complaints	**40%** (n=2)	**50%** (n=8)	**72%** (n=13)
Thyropathy	**40%** (n=2)	**6%** (n=1)	**11%** (n=2)
Chronic fatigue	0% (n=0)	**13%** (n=2)	**22%** (n=4)
Joint & muscle pain	0% (n=0)	**50%** (n=8)	**50%** (n=9)
Chronic sinusitis	0% (n=0)	**6%** (n=1)	**11%** (n=2)
NOTE 2: age corrected n=49	n=12	n=17	n=20
Exposure to Au OR Pd crowns	**75%** (n=9)	**88**% (n=15)	**100%** (n=20)
Patients with Au-based crowns (≥1)	**67%** (n=8)	**59%** (n=10)	**70%** (n=14)
Patients with Pd-based crowns (≥1)	**50%** (n=6)	**71%** (n=12)	**75%** (n=15)
Gold induced immune responses	n=26	n=25	n=20
Positive skin test results (+, ++, +++)	0% (n=0)	0% (n=0)	0% (n=0)
Positive LTT-MELISA® (SI>5)	0% (n=0)	**4%** (n=1)	0% (n=0)
IL-5 > 22.1 $\Delta pg.ml^{-1}$ *	**8%** (n=2)	**28%** (n=7)	**25%** (n=5)
IL-13 > 3.4 $\Delta pg.ml^{-1}$ *	**8%** (n=2)	**24%** (n=6)	**15%** (n=3)
IFN-γ > 148.1 $\Delta pg.ml^{-1}$ *	**8%** (n=2)	**12%** (n=3)	**20%** (n=4)
Palladium induced immune responses	n=26	n=25	n=20
Positive skin test results (+, ++, +++)	**8%** (n=2)†‡	**68%** (n=17)†±	**35%** (n=7)‡±
Positive LTT-MELISA® (SI>5)	**4%** (n=1)†	**32%** (n=8)†	**20%** (n=4)
IL-5 > 26.2 $\Delta pg.ml^{-1}$ *	**4%** (n=1)†	**52%** (n=13)†±	**10%** (n=2)±
IL-13 > 4.8 $\Delta pg.ml^{-1}$ *	**8%*** (n=2)†	**56**% (n=14)†±	**25%** (n=5)±
IFN-γ > 816.6 $\Delta pg.ml^{-1}$ *	**8%** (n=2)	**8%** (n=2)	**15%** (n=3)

† Significant difference (p<0.05) between non-allergic and metal-ACD groups ‡ Significant difference (p<0.05) between non-allergic and oral-disease groups ± Significant difference (p<0.05) between metal-ACD and oral-disease groups * Mean increment +3*SD of controls

be associated with the female gender the groups were corrected for both parameters. When comparing homogenous groups by focusing on female participants above the age of 38, systemic complaints were not associated with a particular patient group. Nevertheless, a trend was observed for an increased frequency of joint & muscle pain in both the metal-ACD and oral-disease groups.
The presence of dental crowns obviously increases with age, but it is less likely that gender may play a role in the presence of dental restorations. Therefore, to compare exposure data in terms of presence of dental alloys between the patient groups, it was chosen to correct for age only, that is inclusion of participants older than 38. Pd-based alloys were found more frequently in the oral-disease group compared to the non-allergic group, although this was not significant.

Table 8.2 Chi-square tests for palladium (Pd) induced positive test results *in vivo* (skin test) versus *in vitro*, that are, LTT-MELISA® (cut-off SI>5), Pd induced IL-5 (cut-off 26.2 $\Delta pg.ml^{-1}$), IL-13 (cut-off 4.8 $\Delta pg.ml^{-1}$), and IFN-γ (cut-off 816.6 $\Delta pg.ml^{-1}$). It can be concluded that although positive skin test results outnumbered those of *in vitro* results, the latter followed the skin test results to a large extent.

Patients from all groups n=71	Pd-induced LTT-MELISA® positive n=13	Pd-induced IL-5 positive n=16	Pd-induced IL-13 positive n=21	Pd-induced IFN-γ positive n=7
Pd Skin Test positive n=26	n=13/13 $p<0.001$	n=13/16 $p<0.001$	n=16/21 $p<0.001$	n=6/7 $p=0.008$

As to the immune responsiveness, no positive skin test results to Au were observed, still *in vitro* immune responses were found in all three patient groups. As expected, more positive Pd (17/25) skin test results were obtained in the metal-ACD group as compared to both non-allergic and oral-disease groups ($p<0.05$). As expected oral-disease patients also showed more frequently positive skin test results to Pd (7/20) than non-allergic individuals (2/26) ($p<0.05$), since 50% (10/20) reported contact allergy to metals. Moreover, also 2 out of the 10 oral-disease patients without a metal allergic history reacted positive with Pd skin testing. Palladium induced LTT-MELISA® responses supported the skin test results, although they were less frequent in all groups, and significance was only reached between the non-allergic and metal-

Table 8.3 Associations between presence (≥1) of Au- and Pd-based dental crowns and local lesions in all patients regardless the original group allocation (non-allergic, metal-ACD, and oral-disease). Patients with a specific type of dental alloy (≥1) are compared to patients without that specific type of dental alloy. Au-based subtypes are 1. Au-based but free from Pd (Au-PdFree) (Au: 63-93At%); 2. Au-based but with small amounts of Pd (Au-Pd^{low}) (Au: 70-83At%; Pd: 1-10At%); 3. Au-based alloys with some Pd but less Au (Au-Pd^{med}) (Au: 45-64At%; Pd:2-20At%). Pd-based subtypes were defined based on their alloying metal, that is: 1. Pd-Ag with silver (Ag) (15-55At%); 2. Pd-Cu with copper (Cu) (15-30At%); 3. Pd-Au^{med} with gold (Au) (<45At%); and 4. Pd-Misc. with miscellaneous metals (Pd:>75%At%). Statistical analyses to calculate *P*-values were done using 2-sided Fisher Exact Tests and Odds Ratios (OR) were calculated including 95% Confidence Intervals (95%CI) were appropriate. Significance was set on $p<0.05$ (grey cells).

Patients from all groups with n=71	OLP/OLL* n=10	NPG** n=23
Au-based dental alloys (≥1)		
Au-PdFree n=18	***p*=0.002 (n=7)**	*p*=0.249 (n=8)
Odds Ratio	OR=10.6	
95%CI	2.4 – 47.6	
Au-Pd^{low} n=20	***p*=0.025 (n=6)**	*p*=0.055 (n=10)
Odds Ratio	OR=5.0	OR=2.9
95%CI	1.2 – 20.4	1.0 – 8.6
Au-Pd^{med} n=17	*p*=0.433 (n=1)	***p*=0.015 (n=10)**
Odds Ratio		OR=4.5
95%CI		1.4 – 14.2
Pd-based dental alloys (≥1)		
Pd-Ag n=17	*p*=0.694 (n=3)	***p*<0.001** (n=13)
Odds Ratio		OR=14.3
95%CI		3.8 – 53.2
Pd-Cu n=16	*p*=0.684 (n=3)	***p*<0.001** (n=12)
Odds Ratio		OR=12.0
95%CI		3.2 – 44.5
Pd-Au^{med} n=5	***p*=0.018** (n=3)	*p*=1.000 (n=1)
Odds Ratio	OR=12.6	
95%CI	1.8 – 89.2	
Pd-Misc. n=13	*p*=1.000 (n=2)	***p*=0.021** (n=8)
Odds Ratio		OR=4.6
95%CI		1.3 – 16.2

* Oral Lichen Planus/Oral Lichnoid Lesions (OLP/OLL)
** Non-Plaque related Gingivitis (NPG)

Table 8.4 Associations between presence of palladium (≥1) (Pd) based dental alloys and systemic complaints in all patients regardless the original group allocation (non-allergic, metal-ACD, and oral-disease), but corrected for age (patients younger than 39 excluded). Patients with a specific type of dental alloy (≥1) are compared to patients without that specific type of dental alloy. Pd-based subtypes were defined based on their alloying metal, that is: 1 Pd-Ag with silver (Ag) (15-55At%).; 2. Pd-Cu with copper (Cu) (15-30At%); 3. Pd-Au^{med} with gold (Au) (<45At%); and 4. Pd-Misc. with miscellaneous metals (Pd:>75%At%). Calculations for systemic complaints were corrected for age. Statistical analyses to calculate *P*-values were done using 2-sided Fisher Exact Tests and Odds Ratios were calculated including 95% Confidence Intervals (95%CI) were appropriate. Significance was set on $p<0.05$ (grey cells).

Patients from all groups (corrected for age) n=49	Thyropathy n=3	Chronic fatigue n=7	Joint & muscle pain n=18	Chronic sinusitis n=5
Pd-based dental alloys (≥1)				
Pd-Ag n=17	*p*=0.542 (n=0)	*p*=0.397 (n=1)	*p*=0.122 (n=9)	*p*=1.000 (n=2)
Pd-Cu n=16 Odds Ratio 95%CI	*p*=0.245 (n=2)	***p*=0.030** (n=5) OR=7.0 1.2 – 41.7	*p*=1.000 (n=6)	***p*=0.034** (n=4) OR=10.1 1.1 – 105.3
Pd-Au^{med} n=5	*p*=1.000 (n=0)	*p*=1.000 (n=0)	*p*=0.342 (n=3)	*p*=0.430 (n=1)
Pd-Misc. n=13	*p*=0.555 (n=0)	*p*=1.000 (n=2)	*p*=0.508 (n=6)	*p*=1.000 (n=1)

ACD groups (*P*=0.011). Essentially similar information was obtained with other Pd *in vitro* tests. In general it can be concluded that for Pd the *in vitro* responses followed the skin test results, although less often positive results were obtained (Table 8.2).

Comparison according to exposure

Next, all patients were regrouped considering the presence of distinct dental alloys (see materials and methods) regardless the original group allocation. Gold- and Pd-based alloys were scored positive when at least one dental crown was present.

Associations between the presence of specific dental alloys and both local lesions (Table 8.3) and systemic complaints (Table 8.4) were investigated. Table 8.3 points out that OLP/OLL is associated with the presence of Au-based dental alloys or Pd-based alloys with considerable amounts of Au (Pd-AU^{med}), whereas NPG is clearly associated with presence of Pd-based dental alloys. Interestingly, presence of Au-based alloys with considerable amounts of Pd (Au-Pd^{med}) was also associated with NGP. For the systemic complaints (Table 8.4) again correction for age was carried

Table 8.5 Associations between presence (≥1) of palladium (Pd) based dental alloys, presence of oral lesions or systemic complaints and Pd-specific immune reactivity within the non-allergic and oral-disease group. For clarity only the significant and near significant associations are presented. Grey cells indicate that a significant association (p<0.05) (2-sided Fisher Exact) was also found when all participants were included for analyses (n=71). Pd exposure was subdivided for distinct alloy subtypes: 1. Pd-Ag with silver (Ag) (15-55At%); 2. Pd-Cu with copper (Cu) (15-30At%); 3. Pd-Au^{med} with gold (Au) (<45At%); and 4. Pd-Misc. with miscellaneous metals (Pd:>75%At%).

Non-allergic and Oral-disease n=46	Positive[1] Pd skin test n=9	Positive[2] Pd induced LTT-MELISA® n=5	Positive[3] Pd induced IL-5 n=3	Positive[3] Pd induced IL-13 n=7	Positive[3] Pd induced IFN-γ n=5
Pd-Ag n=10	***p*=0.015 / n=5**	***p*=0.006 / n=4**	***p*=0.008 / n=3**	***p*=0.031 / n=4**	*p*=0.061 / n=5
Odds Ratio	OR=8.0	OR=23.3	NA	OR=7.3	OR=7.3
95%CI	1.6 - 40.3	2.2 – 246.2		1.3 - 41.4	1.0 – 52.0
Pd-Cu n=10	*p*=0.087 / n=4	***p*=0.006 / n=4**	-	-	*p*=0.061 / n=5
Odds Ratio	OR=4.1	OR=23.3			OR=7.3
95%CI	0.9 – 20.0	2.2 – 246.2			1.0 – 52.0
Pd-Au^{med} n=4	-	-	-	-	-
Pd-Misc.n=9	-	***p*=0.044** / n=3	-	-	-
Odds Ratio		OR=8.8			
95%CI		1.2 – 63.9			
OLP/OLL n=10	-	-	-	-	-
NPG n=16	***p*=0.047 / n=6**	***p*=0.043 / n=4**	***p*=0.037 / n=3**	***p*=0.040 /** n=5	***p*=0.043 /** n=4
Odds Ratio	OR=5.4	OR=9.7	NA	OR=6.4	OR=9.7
95%CI	1.1 – 25.8	1.0 – 95.7		1.1 – 37.8	.1.0 – 95.7

[1] Skin test is considered positive when skin reactivity is marked as +, ++, or, +++
[2] LTT-MELISA® is considered positive when Stimulation Index is above 5 (SI>5)
[3] Cut-off values defined as mean+3SD of the non-allergic group (Table 8.1)
[age] Corrected for age (age<39 excluded) only done for calculations of associations concerning systemic complaints
NA Not Available due to empty cell in 2x2 table

out. Of note, no gender correction was performed as the number and type of dental alloys are assumed not to be gender related. It was found that Pd-Cu alloys in particular were associated with systemic complaints (chronic fatigue and chronic sinusitis). For Au-based alloys no associations were found (data not shown).

Immune responses

Both *in vivo* and *in vitro* immune response to Au and Pd were investigated. For these analyses the metal-ACD group was excluded since immune responsiveness might be

Table 8.5 (cont) Calculations for systemic complaints are corrected for age, i.e. patients under the age of 39 were excluded. Statistical analyses to calculate *P*-values were done using 2-sided Fisher Exact Tests and Odds Ratios were calculated including 95% Confidence Intervals (95%CI) were appropriate. Significance was set on $p<0.05$.

Non-allergic and Oral-disease n=46	Positive[1] Pd skin test n=9	Positive[2] Pd induced LTT-MELISA® n=5	Positive[3] Pd induced IL-5 n=3	Positive[3] Pd induced IL-13 n=7	Positive[3] Pd induced IFN-γ n=5
AGE CORRECTED (exclusion of patients under 39)					
Non-allergic and Oral-disease n=32	Positive[1] Pd skin test n=8	Positive[2] Pd induced LTT-MELISA® n=5	Positive[3] Pd induced IL-5 n=3	Positive[3] Pd induced IL-13 n=7	Positive[3] Pd induced IFN-γ n=4
Thyropathy n=2	-	-	-	***p*=0.042** / n=2 NA	-
Chronic fatigue n=5	-	-	-	-	*p*=0.105 / n=2 OR = 8.3 0.8 – 82.9
Joint & muscle pain n=10	***p*=0.005** / n=6 OR = 15 2.2 – 103.0	***p*=0.024 /** n=4 OR=14.0 1.3 – 150.0	-	-	*p*=0.079 / n=3 OR=9.0 0.8 – 101.2
Chronic Sinusitis n=4	-	-	-	-	-

[1] Skin test is considered positive when skin reactivity is marked as +, ++, or, +++
[2] LTT-MELISA® is considered positive when Stimulation Index is above 5 (SI>5)
[3] Cut-off values defined as mean+3SD of the non-allergic group (Table 8.1)
[age] Corrected for age (age<39 excluded) only done for calculations of associations concerning systemic complaints
NA Not Available due to empty cell in 2x2 table

based on skin sensitization rather than on mucosal exposure. Of note, statistical analyses for systemic complaints were corrected for age. The results are summarized in Table 8.5. In particular Pd-Ag and to some extend Pd-Cu alloys were associated with Pd-induced immune responses both *in vivo* and *in vitro* (IL-5 and IL-13). Significant associations between the presence of Pd-Ag dental alloys and most Pd induced immune responses were also found when the metal-ACD group was included for analyses (Table 8.5). OLP/OLL was not associated with any of the investigated immune responses, whereas Pd skin testing as well as all Pd-induced *in vitro* assays were strongly associated with NPG. From the systemic complaints only for joint & muscle pain multiple associations with Pd-induced immune responses were found.

8.5 Discussion

Unexpectedly no positive skin test results to Au were found, whereas Au allergy has been suggested to be frequent (about 15-20%) in patients with oral disease (2, 28), especially in association with OLP/OLL (13, 15). Still, OLP/OLL was clearly associated with exposure to Au (Table 8.3), which is in line with findings from Svedman *et al.* (29). Apparently, other mechanisms than classical Au-induced T-cell reactivity are involved in the pathogenesis of OLP/OLL. This point of view is supported by the finding that OLP/OLL frequently diminishes after removal of dental alloys also in absence of allergy (30).

Based on the metal allergic anamnesis more Pd positive skin test reactions were expected in oral-disease patients (7/20) as compared to non-allergic controls (2/26) (p=0.029). Still, of the oral-disease group 29% (2/7) of the Pd allergic patients did not report ACD to metals and were both mono-sensitized to Pd (in the context of nickel (Ni) and Pd cross-reactivity). On top of that, significantly more Pd mono-sensitized patients were found in the oral-disease group (4/7 mono-sensitized) as compared to the metal-ACD group (1/17)(P=0.014) (data not shown). These results are in line with the findings of Cristaudo *et al*. (4). Possibly Pd exposure (mainly oral) may rather induce Pd-specific clones, whereas Ni-exposure (mainly skin) may favour development of Ni-specific clones that cross-react with Pd.

In general ROC-analyses are used to calculate cut-off values for diagnostic tests. For Pd these analyses were carried out in an earlier study with the same patient groups (non-allergic and metal-ACD) (12). In that study positive controls (n=16 for Pd) were defined more strictly and based on a history of metal-ACD in combination with positive Pd skin test results on at least 2 out of 3 readings. Negative controls (n=21) were not allowed for any reactivity to Pd (+/-, follicular, or irritant reactions) at any reading. For Pd those cut-off values were essentially similar to those obtained in the current study (Mean+3*SD of the non-allergic volunteers), and both methods resulted in the same associations concerning Pd induced IL-5 and IL-13 cytokine production. Of note, using the ROC-analyses cut-offs, associations were generally even more pronounced. In the earlier study also nickel was investigated to compare the results to Pd, since in contrast to Pd, Ni is a well investigated allergen in terms of immune responses. The Ni induced *in vitro* assays performed similar, although more spreading of the data was observed probably due to the inevitable daily exposure to nickel (12). The non-parametric Spearman correlation between nickel and palladium was rho=0.735 (p<0.001; 2-tailed) and rho=0.731 (p<0.001) for IL-5 and IL-13,

respectively. Detailed information on these patients is available on the online repository of that article. For Au ROC-analyses were not possible due to the lack of positive skin test results. Therefore, to use a consistent methodology for both Au and Pd in this study the mean+3SD in the non-allergic group was used to determine cut-off values for all *in vitro* assays.

Only few patients had positive test results with Pd induced IFN-γ production even in those with positive skin tests. This is due to the high cut-off value resulting from the mean+3SD method. Indeed, using the ROC-analyses a much lower cut-off value was obtained (>9.0 *vs.*>816.6Δpg.ml^{-1}). This is obviously due to the large spread in results in the non-allergic group. Interestingly, the obtained association for IFN-γ found in this study could not be calculated with the cut-off based on ROC-analyses. The role of IFN-γ in metal induced ACD has been a matter of debate for almost two decades (8, 9, 11, 31). Perhaps IFN-γ plays a role during the sensitization phase or with high dose exposres whereas Th2 cytokines like IL-4, IL-5 and IL-13 are more important during the elicitation phase and in (low dose) chronic disease. Either way, IFN-γ was found to be a poor predictor for Ni and Pd allergy. Still, the few results with high levels of Pd induced IFN-γ production were found in patients with Pd-Ag, Pd-Cu dental alloys, NPG, chronic fatigue, and joint & muscle pain.

Interestingly, despite the absence of positive Au-induced skin tests, several positive Au-induced *in vitro* immune responses were found suggesting better sensitivity for these *in vitro* assays as most of them were found in the metal-ACD and oral disease groups (Table 8.1). However, for Pd rather the contrary conclusion may be drawn, since positive Pd skin tests outnumbered the positive *in vitro* test results. Still, all Pd-induced *in vitro* assays were clearly associated with Pd skin testing (table 8.2), the presence of NPG and Pd-Ag dental alloys (Table 8.5). Therefore these *in vitro* assays could be of diagnostic value in patients for whom skin testing is contra-indicated or for metal allergens that lack sensitivity using skin tests.

A clear association was found between exposure to Pd-based dental alloys and the presence of NPG (Table 8.3). Although still significant, the association between NPG and positive Pd skin test results was less obvious (P=0.047; Table 8.5). In fact, only 38% (6/16; metal-ACD group excluded) of the patients with NPG showed positive Pd skin tests (Table 8.5). Possibly this relates to a recent finding that not only Ni (32) but also Pd is able to activate Toll-Like Receptor 4 (TLR-4) with subsequent non-specific inflammatory innate immune responses (33). In that perspective NPG should be interpreted as a sign of exposure, rather than as a sign of allergy.

Especially Pd-Cu and Pd-Ag alloys were found to be related to NPG (Table 8.4) and sensitization to Pd (Table 8.5). Pd-Cu alloys are known to leach considerable amounts of metal ions, including Pd, explaining the associations with immune responses and adverse reactions. Although, Pd-Ag alloys are believed to be more safe in this respect (20, 21, 34, 35) our *in vivo* results can't support these *in vitro* findings.

Finally, since there is a growing concern about metal induced auto-immune diseases (36) systemic complaints, like joint & muscle pain and chronic fatigue, which may be associated with auto-immune disease were also investigated. Within the clear limitations of this study, like small sample sizes and determination of complaints by interview, the results provide direction for further research. Exposure to Pd-Cu alloys was associated with chronic fatigue (Table 8.4). Interestingly, joint & muscle pain was clearly associated with positive test results in Pd induced skin testing and LTT-MELISA® whereas, a trend was found for IFN-γ production. Of course, larger studies are required to verify whether the latter results are coincidental or not.

8.6 Conclusions

The results of the present study show that oral lesions associated with dental alloys may result from concerted innate and T-cell mediated immune reactivity. OLP/OLL was strongly associated with Au exposure and NPG was closely related to Pd exposure. Interestingly, OLP/OLL was not related to Au-induced immune responses, whereas NPG was confirmed by positive Pd skin testing and Pd induced IL-5 and IL-13 production, albeit only in about 40% of the cases. Still, oral lesions in the absence of T cell-mediated allergy are not harmless and need further attention. For example, constant triggering of the innate immune system as a result of protracted Pd exposure may augment destructive inflammatory processes and may enhance sensitization to other (self) antigens. It is clear that other mechanisms than classic T-cell mediated allergy may play an important role in the development of adverse reactions to dental materials. Finally, skin testing remains the test of choice to diagnose oral disease as a result of Pd allergy. Still, in particular cases Au and Pd *in vitro* sensitization assays and LTT-MELISA® in particular may be helpful.

8.7 References

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