Palladium allergy in relation to dentistry
Muris, J.

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Chapter 1

General introduction - a review of the literature

Joris Muris

A part of the introduction is submitted to *Dermatitis*
1.1 Introduction

Dental alloys in dental appliances are located intraorally for years to decades. During this time period patients are exposed to various different chemicals when the metal components begin to dissolve or corrode. To date, it is unclear what the biological consequences of such chemical byproducts might be. Hence, many objective and subjective complaints have been attributed to this prolonged exposure to heavy metals. This chapter is not intended to serve as a systematic review, but rather as a compilation of evidence that sheds light onto the complex aetiology of adverse reactions to dental alloys in general, and allergic reactions in particular. The term ‘adverse reaction’ is intended as a catchall phrase that includes allergies but is not limited to them; an allergy is just one type of adverse reaction that could occur as a result of prolonged exposure to dental alloys.

Dental applications

An enormous variety of dental applications is available to restore or replace decayed dental elements (teeth). These applications can be divided material-wise into metals, resin-based materials and ceramics; often a combination of these materials is used. Dental applications can be categorized as restorations (fillings), Fixed Dental Prosthesis (FDP), Removable Dental Prosthesis (RDP), dental implants and orthodontic appliances.

Fillings are initially, applied in a soft form intraorally (direct method). Setting/hardening of the material occurs due to a chemical reaction, which may be initiated by blue light (400 - 500 nm) not to be confused with ultraviolet light (300 - 400 nm). The quality, both mechanically and biologically, in terms of ‘biocompatibility’, of these restorations, mostly amalgam and composite (resin-based), is, to a large extent, operator dependent.

Fixed Dental Prostheses (FDPs) or (partial) dental crowns and dental bridges are applied to teeth that are severely decayed or to replace lost and/or missing teeth. These restorations are fabricated outside the mouth (indirect method), and then fixed with cement onto the element (tooth). These constructions can also be cemented or screwed to dental implants. Mostly, these constructions are made of alloys and are often veneered with porcelain. The veneers may complicate the diagnosis of adverse reactions because such restorations can be difficult to distinguish from natural teeth. There is a huge arsenal of dental alloys available, which are roughly divided in high-noble, noble and base alloys, and titanium (Ti) alloys (according to the American Dental Association’s revised classification system for fixed prosthodontics) (Table 1.1). High-noble (or gold [Au]-based) alloys largely
Introduction

consist of Au and are mostly alloyed with platinum (Pt), and/or palladium (Pd). The price of these materials is high and their use is therefore limited. Noble, predominantly Pd-based alloys, are usually a composition of Pd with Au, silver (Ag), copper (Cu) and/or gallium (Ga). This group of alloys is probably most popular, as they combine fair prices with presumed ‘biocompatibility’. Base alloys, like stainless steel and nickel-titanium (Ni-Ti) alloys, are mainly used in orthodontics. Still, nickel-chromium (Ni-Cr) and chromium-cobalt (Cr-Co) alloys are used for FDPs due to their low prices. Titanium and its alloys are considered ‘biocompatible’ and are mainly used for endosseous dental implants and their supra-structures.

Removable Dental Prostheses (RDPs) are appliances that replace multiple lost/missing teeth. Complete RDPs (or full/complete dentures) replace all teeth in one jaw and are mostly made of resin-based materials, i.e. polymethylmethacrylate (PMMA). Partial RDPs (or partial dentures) replace one or multiple missing teeth and often consist of a metal base or core structure that is finished with PMMA. They are attached to remaining teeth and/or implants by clamps, ‘click-systems’ or magnets. Such appliances are usually made of Cr-Co alloys (called Vitallium®) or are Ti-based to provide sufficient strength and stiffness. For parts of these constructions, such as mounting bars between implants, other alloys can be used. Dental implants are basically Ti screws anchored in the mandibular or maxillary bone (endosseous). On the implant a so-called abutment is placed which is usually made of Ti, but other alloys or zirconia (Zr) may be used. The abutment connects the implant with the supra-structure, like a crown/bridge or removable prosthesis, which in turn can be made of a different material.

Orthodontic appliances are used to move teeth to a more functional or aesthetic position within the jaw. Typically stainless steel (316L) is used in combination with flexible alloys like Ni-Ti. Active orthodontic appliances are usually in situ for approximately 2-3 years. However, to retain the treatment result, a retention wire is generally placed behind the frontal teeth which remains in situ for decades. These retainers are commonly made of stainless steel.

Epidemiology and demography of dental appliances

Obviously, the prevalence of dental prostheses use is strongly associated with the prevalence of tooth loss, which in turn is strongly associated with age. The most frequently lost elements are molars, maxillary premolars and upper front teeth. Muller et al. reviewed in 2007 the prevalence of tooth loss in Europe and concluded the following (1): (i) There is a documented decline in edentulism (condition of being toothless) but great differences still exist between countries and groups with various backgrounds. (ii) The mean number of lost teeth increases with age. (iii) A great
number of variables is associated with tooth loss, and there is no consensus whether
dental-disease-related or socio-behavioural factors are most important. And (iv) institutionalised elderly have in general more compromised oral health, including fewer teeth, than those living in their own homes. Zitzmann et al. reviewed in 2007 the prevalence of various types of dental prostheses in Europe (2). They concluded that (i) more than 50% of the adult (Western) European population has FDPs or RDPs. (ii) In countries of lower gross national product (GNP) restorations are less frequent but it was assumed that the need for treatment is high. (iii) Removable dental prostheses are most frequent in older age groups, while FDPs were the dominant type of restoration in the younger and wealthier population. (iv) Removable dental prostheses are more frequent in subjects living in rural areas, with a lower socio-economic and educational level.

In contrast to the high levels of sensitization to metals like, Ni, Au, Pd, Co, and Cr in the general population (3-25%) (3), adverse reactions to metal-based dental restorations are considered infrequent even in sensitized patients. A major problem is to determine the clinical relevance of allergy to metals in oral disease, therefore reliable figures on prevalence and incidence of oral disease as a result of metal allergy are difficult to obtain (4).

**Metals used in dentistry**

While metals such as gold and platinum were used more extensively in the early 20th century, their use has been gradually replaced with other metals and Pd in particular during the last decades (5). The choice of metals depends on the purpose (restoration, implant, orthodontics etc.) but it also varies significantly between countries depending on the culture, health care system, demand and level of income. For years, Au-based alloys were considered the benchmark of restorative dental alloys, but due to increasing gold prices, and recent advances in dental porcelains as well as consumer focus on aesthetic results, the demand has decreased. Metal-fused-to-porcelain crowns are still the most abundantly used type of dental crowns although zirconia-based (ceramic) crowns are gaining popularity in Western European countries. Overall, there seems to be an ever-increasing variety of products and alloys produced by the dental industry, and to date thousands of different alloys have been produced. The metal composition of dental work is complex and diverse. Still, it is important to know the composition. Such knowledge will help dentists ascertain which dental device needs to be replaced in cases of a clinically relevant allergy, since replacing dental devices may be invasive and expensive. The most important metals used for dental appliances are discussed (also see Table 1.1).
Table 1.1  Classification of dental alloys based on weight percentage according to the American Dental Association (ADA). Thousands of different dental alloys exist for which a great diversity of metals is used

<table>
<thead>
<tr>
<th>Classification</th>
<th>Percentage of noble metals</th>
<th>Subgroups</th>
<th>Most important components</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-noble</td>
<td>≥60% Au+Pt+Pd (≥40% Au)</td>
<td>Au-based alloys</td>
<td>Au-Pt, Au-Pd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pd-based alloys</td>
<td>Pd-Au (&gt;40Au)</td>
</tr>
<tr>
<td>Titanium (alloys)</td>
<td>&gt;80% Ti</td>
<td>Commercially pure Ti</td>
<td>Ti (&gt;99%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ti alloys</td>
<td></td>
</tr>
<tr>
<td>Noble</td>
<td>≥25% Au+Pt+Pd</td>
<td>Pd-based alloys</td>
<td>Pd-Au (&lt;40Au)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pd-Ag, Pd-Cu</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag-based alloys</td>
<td>Ag-Pd</td>
</tr>
<tr>
<td>Base-metal</td>
<td>≤25% Au+Pt+Pd</td>
<td>&gt;20% Cr</td>
<td>Ni-Cr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;20% Cr</td>
<td>Ni-Cr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cr-Co (e.g. Vitallium®)</td>
<td>Cr-Co</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stainless steel*</td>
<td>Co-Cr-Ni of Cr-Ni</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ti alloys</td>
<td>Ni-Ti*</td>
</tr>
</tbody>
</table>

*mostly applied in orthodontics; Noble metals: gold (Au), iridium (Ir), palladium (Pd), platinum (Pt), rhodium (Rh), ruthenium (Ru); Base metals: aluminum (Al), beryllium (Be), chromium (Cr), cobalt (Co), copper (Cu), gallium (Ga), indium (In), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), niobium (Nb), silver (Ag), tin (Sn), titanium (Ti), vanadium (V), zinc (Zn), zirconium (Zr)

Gold (Au) is a noble metal that, due to its soft and malleable properties needs to be alloyed with metals like copper, platinum, and/or palladium. From a dentist’s point of view gold alloys are still first choice due to their optimal mechanical properties. Gold is one of the least reactive metals. Still, sensitization to gold is frequently observed in patients tested with metal series (6-8), however, is rarely relevant for allergic contact dermatitis and its relevance in oral disease is still unclear (9). Nevertheless, sensitization to gold seems to be related to oral lichen lesions (10) and to dental gold (11).
Platinum (Pt) is an important strengthening component of gold alloys. Platinum is rarely causes allergic contact dermatitis but may play a role in IgE mediated allergy and adverse reactions to drugs.

Palladium (Pd) is a noble metal that is widely used in dentistry as a substitute for platinum and gold. Palladium is a hard metal that, like platinum, adds strength to alloys. It has a white appearance and is metallurgically compatible with gold and therefore useful in gold alloys. Dental alloys may consist up to 90 weight% (wt%) palladium (12-14). Despite the fact that it’s a noble metal, palladium is reactive in nature and is assumed to cross-react with nickel (15-18). Therefore, palladium is considered an important allergen that is associated with oral disease (19). Still, the relevance of positive patch test results (sensitization to palladium) is compromised, since it may be considered as cross-reaction to nickel (see below) and mono-reactivity to palladium is infrequent.

Cobalt (Co) is an important constituent of Vitallium®, an alloy trademark (60% Co, 20% chromium [Cr], and 5% molybdenum [Mo], and other metals) that is commonly used for metal-based RDPs. Similar to Ni-Cr alloys, Cr-Co alloys are also used for FDP, especially in countries with low GNP. Some alloys used in orthodontics may contain cobalt. There is an ongoing debate whether or not the cobalt allergy has clinical relevance in oral disease, as allergic reactions are usually related to consumer products and occupational exposure (3).

Chromium (Cr) is a part of stainless steel (18-25%) and abundantly used in orthodontics. Furthermore, it is a constituent of Ni-Cr alloys as mentioned above and Co-Cr-Mo (Vitallium®) alloys are typically used in fixed and removable prostheses in dentistry. Chromium easily oxidizes, resulting in a passivation layer, which prevents corrosion. Sensitization to chromium generally manifests in dermatitis from contact with leather products or occupational exposure (3).

Nickel (Ni), like chromium, is a component of stainless steel alloys (8-14 wt%) and is widely used in orthodontics for brackets, headgear and other parts, such as orthodontic retention wires. In contrast to the active orthodontic appliances, retention wires remain in situ for decades or even a lifetime. It has been shown that these retention wires can release great amounts of nickel in experimental setups (20) and could also be responsible for extra-oral eczema even in the absence of local reactions (21). Other parts of orthodontic appliances are the super-elastic Ni-Ti arches, containing 47-50% nickel (22). These arches connect the brackets to each other and provide the force needed for tooth movement. Finally, nickel is used in Ni-Cr alloys for FDPs, especially for financial reasons. Allergies to nickel are very common, estimated to be 17% and 3% in the general female and male population, respectively (3). It is in fact the most frequently occurring delayed type or type IV
allergy, and is usually associated with exposure to jewellery, piercings, consumer products (3), and/or medical devices (23).

Titanium (Ti) The vast majority of dental implants are made of commercially pure titanium (>99wt%) or its alloys like Ti6Al4V (Ti with 6wt% aluminium, and 4 wt% vanadium). Abutments, used to connect implants to the supra-structures, are also mostly made of titanium or its alloys. In orthodontics, titanium is used in alloys with nickel to produce very flexible Ni-Ti wires. Titanium surfaces, even when alloyed, immediately oxidize when exposed to air. This oxidation creates a passive layer, making the metal resistant to corrosion. Still, this passive layer (10-20nm) can be easily affected by many influences such as mechanical forces, exposure to high concentrations of fluoride, and corrosion (24, 25). Titanium allergy has rarely been identified as an allergen in oral disease using patch testing (26, 27). However, in in vitro assays, such as lymphocyte proliferation or transformation assays, (LPT/LTT) sensitization to titanium was frequently diagnosed (4.2-42%), although the clinical relevance of these positive test results is unclear (28, 29). Still, of 56 patients who developed health problems after dental implant insertion, half showed increased titanium-induced lymphocyte proliferation. Titanium-positive patients who had their implants removed showed considerable health improvement (30).

1.2 Corrosion of dental alloys

In general
All types of materials can react to their environment in one way or another (31). Metal corrosion processes are complex and multi-faceted, and only the basic principles and mechanisms are discussed in Table 1.2. For the most part, different corrosion mechanisms occur simultaneously, e.g. pitting, crevice and galvanic corrosion. The composition, processing and treatment of alloys influences their structure, which could lead to increased ion release and possible negative biological effects (12, 32-35).
### Table 1.2 Basic mechanisms of corrosion, modified from (36)

<table>
<thead>
<tr>
<th>Corrosion type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniform/Generalized corrosion</td>
<td>Most common type of corrosion. The corrosive environment has the same access to all parts of the metal surface. The metal itself is metallurgically and compositionally uniform. The composition of the corrosive environment is important for the corrosion rate. High temperatures in highly acidic environments owing to specific food consumption can significantly accelerate the process.</td>
</tr>
<tr>
<td>Pitting corrosion</td>
<td>Localized corrosion of an otherwise resistant surface. For example, passive oxide layers from base metals like stainless steel, Ti alloys, and Cr-Co alloys produce pitting corrosion. The presence of a low pH and ions such as chlorides and sulphides enhance the local oxide layer breakdown. Basically, the process is driven by an oxygen gradient between the crevice surface (low oxygen) and the bulk surface of the alloy. Finite surface oxidation in the crevice consumes dissolved oxygen (or other oxidizing compounds) faster than diffusion from the bulk solution, resulting in a more active metal surface in the crevice. The situation results in an electrochemical cell where the crevice acts as anode (corrodes) and the bulk surface as cathode (passivates). When the destruction of the layer propagates pits may turn into crevices.</td>
</tr>
<tr>
<td>Crevice corrosion</td>
<td>Corrosion of an alloy is greater in the small sheltered volume of the crevice. Examples of crevices in the oral cavity are propagated pits, the subgingival area, a gap between restorative alloy and tooth, or close contacts between different parts of the restorative structure. Basically the process is similar to pitting corrosion and is driven by an oxygen gradient between the crevice surface (low oxygen) and the bulk surface of the alloy (see pitting corrosion). In the crevice, mostly unstable metal chlorides are formed that tend to hydrolyze, resulting in an increase of H⁺ ions (lower pH) which further accelerates the corrosion processes.</td>
</tr>
<tr>
<td>Stress corrosion</td>
<td>Is a type of corrosion caused by the combined factors of tensile stress, susceptible alloys, and a corrosive environment. Tensile stress is induced by externally applied functional loads, e.g. mastication loads or orthodontic appliances.</td>
</tr>
</tbody>
</table>
Table 1.2 (cont.) Basic mechanisms of corrosion, modified from (36)

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erosion/Fretting corrosion</td>
<td>The combination of corrosive fluid or slurry (saliva with enzymes and food particles) and (high) velocity in the oral environment results in erosion corrosion. Conjoint action of chemical (enzymes, oxidizing compounds, and proteins) and mechanical wear, e.g. during mastication, is called fretting corrosion. This may also be the result of two metal surfaces rubbing against one another.</td>
</tr>
<tr>
<td>Intergranular corrosion</td>
<td>Solid metals are crystalline materials made up of large numbers of crystal grains. At grain boundary areas the composition is slightly different from the inner parts of the grain. On top of that, at grain boundaries atoms are not in perfect alignment with each other, resulting in increased strain energy. These phenomena result in the formation of anode activity at grain boundary sites and cathode activity at the inner parts of the grains.</td>
</tr>
<tr>
<td>Galvanic/Dissimilar corrosion</td>
<td>When two dissimilar metals/alloys (with different electrochemical potentials) are placed in direct contact in the presence of an electrolyte (saliva or other body fluids), generating a chemical reaction. This results in an oxidation (dissolution) at the anode (noble metal) and reduction (passivation) at the cathode (noble metal). The electronic exchange occurs through the contact and the ionic exchange through the electrolyte. Some alloys, like stainless steel and several palladium-based alloys, are called ‘multiple-phase’ alloys. Within these alloys, different ‘phases’ (areas with dissimilar compositions) co-exist, resulting in galvanic corrosion on a micro-level.</td>
</tr>
</tbody>
</table>

Palladium-based alloys
Pd-Cu and Pd-Ag alloys have been associated with increased ion release, and with potentially decreased ‘biocompatibility’ properties (12, 13, 37-41). In the following table the most important basic characteristics in terms of corrosion resistance for Pd, Ag, and Cu are discussed, based on the aforementioned publications.

Palladium (Pd) is the least resistant of all platinum-group metals, as it is easily affected by sulphides. Furthermore, in liquid phase it tends to dissolve oxygen, and especially hydrogen, with possible porosities as a result. Palladium increases the melting point of alloys substantially, which may lead to decomposition of the investment material with formation of sulphides. Subsequently, as palladium is
vulnerable to sulphides, metal-sulphide compounds will be deposited along the crystallite boundaries, leading to intergranular corrosion (Table 1.2).

Silver (Ag) is, like palladium, vulnerable to sulphides, which may lead to development of porosities, especially when alloyed with palladium. Pd-Ag alloys have been developed out of cost-saving financial considerations. These alloys have the propensity to form multiple phases within the alloys (phase segregation), meaning that islands of various compositions of the alloying metals occur within the same alloy, resulting in galvanic corrosion on a micro level and decreased corrosion resistance overall (Table 1.2). It is therefore important to cool the alloy slowly to allow homogenization. In general, it can be stated that Pd-Ag alloys are difficult to process and production process failures may easily occur. It is therefore important to follow the manufacturer instructions carefully, which can be time-consuming. Copper is often added to these alloys to add hardness, since silver is relatively soft.

Copper (Cu) adds hardness and lowers the melting point of the alloy, which makes it an easier alloy to produce. However, copper is a base metal, and a copper content of >10wt% in Pd-based alloys should not be used, as it decreases the corrosion resistance dramatically. Still, these alloys are found regularly in clinical settings. They are referred to as Pd-Cu alloys. Furthermore, copper may form oxides as it melts, which may dissolve in liquid copper of up to 3%. During solidification, the Cu-oxides dissociate again, and will be deposited at the crystallite boundaries with increased inter-granular corrosion properties as a result (Table 1.2). Furthermore, copper oxides decrease the viscosity of the liquid, making the casting process more difficult, because the alloy cannot stay in the liquid phase for too long. In general, the production process of Pd-based alloys is critical to their corrosion resistance, although the aforementioned disadvantages can be overcome when the manufacturer’s guidelines are strictly followed. The problem is that not all dental laboratories adhere to the production guidelines, because doing so costs time and money, and isn’t always possible with basic equipment. Moreover, even when the guidelines are followed correctly, many alloys available on the market are still sub-par, e.g., containing copper contents of greater than 10wt%. On top of that, for financial reasons, casting spurs are sometimes re-used, which leads to the introduction of contaminants such as metal sulphides and oxides. Metallurgy is a complex science and small alterations may have considerable effects on the ‘biocompatibility’ properties, especially for Pd-based alloys. In scientific literature, many corrosion studies are done on manufacturer-processed alloys, but these studies obviously do not reflect the corrosion properties of the alloys cast by
dental laboratories. Moreover, thousands of dental cast alloys are available on the market, which have almost certainly not all been properly vetted.

Surface structure
Surface structure is also an important factor when considering ‘biocompatibility’ of dental alloys. It has been established that polished Ni- and Co-based alloys are more ‘biocompatible’ than their ‘as-cast’ equivalents (42). However, polished Ni-Cr alloys were recently found to release significantly higher amounts of metal ions, and nickel in particular, compared to their air-sandblasted (roughened) equivalents (43). Subsequently, oral epithelial cells (TR146) in direct and/or indirect (immersion solution) contact with polished Ni-Cr alloys showed lower density, lower metabolic activity, and higher cellular toxicity as well as pro-inflammatory cytokine release (IL-1α, IL-8, PGE2 and TNFα). It was found that direct cell-alloy contact had a negative influence on the ‘biocompatibility’ compared to cells in immersion solution (43). Due to corrosion, the composition of alloy surfaces may be altered relative to the core of that same alloy. It has been suggested that this might be a decisive factor for ion release and subsequent ‘biocompatibility’ concerns (44, 45). For example, it has been proposed that the surface of Pd-Cu and Ag-free Pd-Ga alloys undergo Pd-enrichment, making them more prone to palladium release than Pd-Ag alloys, which might form a AgCl surface film (12). Mechanical wear and tooth brushing, especially with abrasive toothpastes or professional polishing pastes, can also lead to breakdown of the passive layer (46, 47). It is generally accepted that metal ion release is not proportional to the alloy composition, although this may not hold true for multiple-phase alloys (48).

Individual factors
Unlike the skin, the mouth comprises an ideal environment for corrosion processes to occur. The constant presence of saliva, with corrosive compounds like hydrogen, chloride ions, sulphide compounds, dissolved oxygen and free radicals, enhances the corrosion of dental appliances, which in turn leads to metal exposure. Consumption of foods and beverages results in constant fluctuations in acidity (pH 1.5-8.0) and temperature (0-60°C), which also contributes to corrosion processes. The presence of proteins like serum albumin was also found to increase elemental release from dental alloys (49, 50). Serum albumin plays a fundamental role in the distribution of transition metals, including palladium, in the human body (51). Also, Pd ions bind strongly to lysozyme (52).
Individual general health aspects may also play a role in the corrosion processes. For example, it is well known that xerostomia, independent of its aetiology like Sjögren’s
Syndrome or as a side effect of many pharmaceutical drugs, decreases the saliva’s pH and its buffering capabilities (53). Hypertension has also been linked to decreased pH in unstimulated saliva (54). Nickel is particularly sensitive to pH fluctuations (48). Oral hygiene can also enhance corrosion. For example, fluoride ions, a key element in cavity prevention, are known to attack the passive oxide layers of titanium and Cr-Co alloys in vitro when concentrations rise above the range of 0.05-0.2% (25). Furthermore, it has been shown that tooth brushing also increases metal ion release, especially when abrasive toothpaste is used (46, 55). Recently, it was found that Streptococcus Mutans (a lactic acid-producing bacteria and the primary contributor to cavities), colonizes (within 24 hours) Ni-Cr alloys, significantly increasing their metal ion release and causing cytotoxic and pro-inflammatory cell responses (56).

Ion release and uptake

Although the mechanisms of corrosion are theoretically well known, due to individual, clinical, and alloy-production-process variables the exact in vivo corrosion mechanisms remain complex, and it is difficult to obtain reliable figures on in vivo metal ion release. The oral tissues do not absorb most of the released ions, as they are diluted by saliva. Still, as dental restorations often extend below the level of the gingiva within the gingival sulcus, micro-environments are formed where ion concentration can reach high levels due to the absence of saliva (35). Moreover, biologically adverse effects can be enhanced due to direct cell contact (43). It has been clearly shown that exposure to dental amalgam is associated with increased levels of mercury in blood, plasma, urine, and body organs as compared to people with no dental amalgams (57, 58) and that urinary mercury levels decreased after amalgam removal to levels similar to those of patients who never had an amalgam filling (59, 60).

Furthermore, some reports provide evidence for considerable absorption of released metal ions from high-noble or noble dental alloys. Significantly higher levels of gold and palladium were found in gingival tissues adjacent to dental cast restorations compared to control groups (61). Cristaudo et al. found significantly higher concentrations of palladium in saliva, blood serum, and urine in six patients with palladium containing dental restorations relative to negative control groups (62). Drasch et al. found that the palladium content of body fluids was correlated to the number of high-noble or noble dental alloys, but then calculated a maximum of 70µg Pd in one day’s saliva that did not correlate to the number of restorations. They then concluded that the composition, rather than the number of restorations, might be the critical factor for ion release with subsequent increased concentrations of palladium in body fluid. They found palladium concentrations in blood and serum were a factor
1000 higher than the ‘normal’ levels (0.05µg.ml⁻¹) (63). It has been calculated that exposure to palladium in the general population is mainly caused by dental restorations (64) and Pd-based dental alloys were shown to release up to 80ng.cm⁻¹ per day in artificial saliva (65-67). Likewise, for gold, the number of Au-based inlays (indirect fillings) is related to the concentration of gold in the blood, even after many years (63, 68). Furthermore, it has been reported that the gold concentration in blood positively correlates to patch test reactivity (69, 70).

In summation, the complex corrosion processes occurring in the oral cavity are difficult to quantify in vivo. Still, it is fair to say that substantial metal ion release will take place for all dental alloys, and some, including gold and palladium, will be at least partially absorbed by the body. Furthermore, corrosion is a continuous process that increases with time, especially in the case of crevice or pitting corrosion. It is well established that the release of nickel from dental casting alloys is most common.

1.3 Adverse reactions to dental alloys

Reporting systems
Norway, Sweden, and the United Kingdom have national reporting systems for adverse reactions to dental materials. In the U.S., such a system is executed by the Food and Drug Administration (FDA), although it is a part of MedWatch (71), and as such also records reports about the malfunction of dental devices. (72). An overview of the data obtained from the European reporting systems showed that patients with subjective and objective complaints attributed to their dental materials were 70-80% females and the most commonly affected age groups were 40-49 and 50-59 years of age for both men and women. Similar data was found in Tokushima, Japan (73) and in Amsterdam, Netherlands (own unpublished data). The vast majority of the reports concerned metals and amalgam (74). It is difficult to estimate the frequency of adverse reactions to dental materials because data widely vary. The prevalence is estimated in the range from 1:10,000 to 1:100 (74).

Clinical presentation
Although many complaints, both objective and subjective, are reported in the scientific literature (Table 1.3), there are no specific or pathognomonic manifestations of adverse reactions to dental alloys. Therefore, only the most important and most often described adverse reactions will be discussed.
Table 1.3  Manifestations possibly associated with allergic reactions from oral metal exposure in alphabetical order.

<table>
<thead>
<tr>
<th>Intra-oral</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Objective manifestations</strong></td>
<td></td>
</tr>
<tr>
<td>Angular cheilitis/Labial desquamation</td>
<td>Alopecia</td>
</tr>
<tr>
<td>Erythema</td>
<td>Exacerbation of pre-existing eczema</td>
</tr>
<tr>
<td>Gingival hyperplasia</td>
<td>Focal flaring of dermatitis</td>
</tr>
<tr>
<td>Non-plaque related gingivitis</td>
<td>Generalized or regional dermatitis</td>
</tr>
<tr>
<td>Peri-oral eczema</td>
<td>Generalized urticaria</td>
</tr>
<tr>
<td>Oral lichenoid reaction</td>
<td>Chronic sinusitis</td>
</tr>
<tr>
<td>Oral lichen planus</td>
<td>Thyropathy</td>
</tr>
<tr>
<td>Stomatitis</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Subjective manifestations</strong></td>
</tr>
<tr>
<td>Loss of taste</td>
<td>Chronic fatigue</td>
</tr>
<tr>
<td>Metallic taste</td>
<td>Concentration problems</td>
</tr>
<tr>
<td>Numbness</td>
<td>Joint &amp; muscle pain</td>
</tr>
<tr>
<td>Oral burning</td>
<td>Memory problems</td>
</tr>
<tr>
<td>Pain</td>
<td></td>
</tr>
<tr>
<td>Soreness at side of the tongue</td>
<td></td>
</tr>
<tr>
<td>Xerostomia</td>
<td></td>
</tr>
</tbody>
</table>

**Objective symptoms**

*Oral lichen planus (OLP) and Oral lichenoid lesions (OLL)*

In 2007, a review was published after the World Workshop of oral Medicine (75). The main findings are discussed here. Lichen planus is a chronic systemic disease of established (auto-)immune-mediated pathogenesis. It commonly involves the oral cavity, but it may involve other sites, such as skin, vaginal mucosa, glans penis, the scalp (alopecia), and the nails. Some cases have been described in which alopecia in patients with positive patch test results for nickel and palladium disappeared after the removal of the Pd and/or Ni-containing dental restorations (76). The author explained the pathogenesis by the high affinity of these metals to bind to the sulphur (-SS-) in hair follicles, and referred to this phenomenon as ‘internal contact dermatitis’. Oral lesions are mostly bilateral and symmetrical, characteristically with slender white lines (Wickham’s striae). The lesions may be reticular when a lacelike network of slightly raised gray-white lines is present; the plaque-like form is similar
to leukoplakia. In the case of erythematous/erosive OLP mostly the gingiva is affected. It is unlikely that sensitization to metals plays a role in the aetiology of OLP. Clinically and histopathologically, OLP may be indistinguishable from oral lichenoid lesions (OLL). OLL results from contact with dental materials, as a result of drug reactions or from graft versus host disease. Recently, Schlosser presented a list of drugs related to lichenoid lesions (77). Dental materials most commonly related to OLL are amalgam, gold, and palladium (9, 10, 78-80). The lesions are usually in close contact with the causative dental material(s). It is not fully clear whether or not these reactions result from a type IV allergy, as the value of patch testing has been debated (81-85). From this perspective, OLL may be a manifestation of irritant contact stomatitis (84). Still, the combination of a positive patch test to a component of the dental alloy and a strong topographic association between the lesion and restorative material are positively correlated, and the lesions generally disappear after the alloy is removed. (86, 87). Other allergens, such as perfumes, cinnamaldehyde (in cinnamon), carvone (in caraway and dill), and other food additives are also related to OLL (8, 85, 88). In the scientific literature dealing with adverse reactions to dental alloys, the distinction between OLP and OLL is often not made or not well-described.

Other local manifestations
A variety of symptoms and lesions have traditionally been associated with dental alloys; however, most studies report on small numbers, making it difficult to draw definitive conclusions. Most reported lesions/complaints attributed to metals are stomatitis and gingivitis/bleeding, and/or swelling of the gingiva, and are similar to inflammatory responses to bacteria and/or viruses.

Finally, it has been reported that referring dentists often overlook intra-oral lesions, since many more lesions have been reported by specialists in the field, such as those working in adverse reaction units (89). This could mean that oral lesions are being underreported by general dentists and dermatologists. Furthermore, this indicates that studies in the field of adverse reactions to dental materials executed without the collaboration of a medical specialist/dentist/oral surgeon should be interpreted with caution. In this context it is noteworthy to stress that dental metal-based restorations are often difficult to distinguish from natural teeth as they are mostly veneered with porcelain.
Subjective symptoms

General complaints
Several studies report on decreased health complaints after the removal of amalgam fillings (58, 89-94). The most reported complaints that improved after replacement were: pain from muscles and joints, memory and concentration problems, complaints about ear/nose/throat, and fatigue. However, treatment in terms of health promotion programs without removing the offending amalgams were also found to significantly reduce the symptoms (92). Furthermore, these complaints are also frequently observed in the general population, although the intensity of the complaints is lower (90, 91). Stejskal et al. (95) have studied the relation between dental alloys, various subjective complaints and lymphocyte transformation test results. They reported significantly increased palladium-, gold- and mercury-induced lymphocyte proliferation in 111 chronic fatigue-like patients compared to 116 controls. Of those 111 patients, 98 had their dental restorations removed, and 76% (n=83) reported long-term health improvement. Interestingly, during a follow-up, 73 patients who removed their dental alloys were retested, and showed dramatically reduced lymphocyte proliferation of the aforementioned metals. Of note, nickel-induced proliferation was not reduced. Several interesting cases have been described in more detail (96). It has been suggested that ongoing chronic inflammation with subsequent increased cytokine levels may affect the hypothalamic-pituitary-adrenal axis (HPA axis), triggering non-specific somatic and psychological symptoms (95). Indeed, there is a well-known connection between the central nervous system and the immune system (97-99), including a link to allergies. Although it is not possible to confirm that chronic stress may directly induce allergies, evidence from several studies suggests that in genetically susceptible patients, chronic stress may favour the appearance of allergic disease (100). Chronic stress modifies the Th1/Th2 balance, favouring the Th2 responses (100, 101). Furthermore, stress reduces saliva production with xerostomia as a possible result, which lowers the buffering capacity and the pH of saliva (53), thereby increasing the levels of ion release as discussed above. Additionally, lipopolysaccharide, a cell component of Gram-negative bacteria, can induce anhedonia and depression-like symptoms (102) in mice. This finding is of great interest, since nickel, palladium, and cobalt were found to cause similar immunologic effects as LPS (see below)(103, 104). This could imply that exposure to these metals can lead to depression-like symptoms, which are also often present in patients with complaints attributed to their dental alloys.

It may be concluded that there is some evidence for improvement of systemic complaints as a result of dental alloy removal, although several other factors are
important, especially psychosomatic factors (58, 105). However, it remains an intriguing quest to determine the cause-effect relationship of specific complaints.

Local complaints
The most reported subjective local complaints are burning mouth/tongue, metal taste/taste disturbance, and/or dry mouth (90, 91, 106). However, there is little evidence for true associations with the allergy to dental alloys, in particular for burning sensations/burning mouth syndrome (BMS) (107). The exact aetiology of BMS remains imprecise and is likely multifactorial, including neuropsychiatric, endocrine, immunologic, nutritional, infectious, and iatrogenic causes (108). In the context of immunologic aetiology also food allergens can be involved (8). Many drugs also induce xerostomia and/or taste disturbance, and may present further confounding variables (109, 110), so it is not clear to what extent irritant reactions are involved. Furthermore, metal taste is primarily a sign of exposure due to corrosion. The lack of evidence for an association with allergy does not exclude an association with exposure. Moreover, burning sensation and xerostomia are probably related, since in case of xerostomia the mucin layer, with its important barrier and protection function, is absent, resulting in increased susceptibility to irritation/burning sensation from otherwise harmless food components and/or additives.

Another important issue to address is the possible influence of menopause on oral health. The female population within the age range of 40-60 is the largest patient group afflicted by oral disease attributed to dental materials. Periodontal disease, burning mouth syndrome and xerostomia are common manifestations in post-menopausal women (111). The density of important immune-regulating cells was found to be drastically reduced in gingival tissues of healthy subjects older than 40 relative to those under 40, a finding which contributes to the predisposition for oral disease in the older population (112).

Structure of the oral mucosa
Structurally, the skin and the oral mucosae are similar as they are both stratified squamous keratinized epithelia; however, there are certain differences. Within the granular and keratinized layers of these tissues lipids, like (acyl)ceramides from membrane-coating granules, are deposited intercellularly to form an effective (water) barrier (113). The oral keratinized epithelium appears to have 25-50% less (acyl)ceramides than the epidermis, which might explain the relatively greater permeability of this epithelium compared to the epidermis (114). Not surprisingly, the non-keratinized part of the oral mucosa, that is, the lining mucosa (floor of the mouth, the buccal mucosa, and lateral parts of the tongue), is even more permeable,
with the floor of the mouth being most permeable (113). Besides the absence of stratum corneum, this epithelium lacks (acyl)ceramides. Still, the non-keratinized epithelium contains membrane-coating granules which form an amorphous intercellular barrier material that, while less efficient as a water barrier, does limit the penetration of larger molecules, such as toxins, enzymes, and pharmaceuticals (113). The oral cavity also contains a unique, so-called junctional epithelium (JE) that is not visible intra-orally. It forms the transition from the bottom of the gingival sulcus to the cemento-enamel junction of the tooth. The JE maintains a tight seal against the mineralized tooth surface (enamel) with hemi-desmosomes, called the ‘epithelial attachment’. It tapers off in the apical direction, and consists of 15-30 cell layers coronally and only 1-3 cell layers at the cement-enamel junction (115). It is a stratified squamous non-keratinized epithelium that is made up of 2 strata only: a basal layer and supra-basal layer; it lacks membrane-coating granules and is therefore highly permeable. As the cells are interconnected by a few desmosomes only, the intercellular spaces are relatively wide, allowing for fluid secretion and transmigration of leucocytes. These leucocytes form the basis for the crevicular fluid, which comprises the first line of peripheral host defence against the bacteria in this area. In a situation of inflammation, the epithelial attachment may be lost or the JE may even get disrupted due to either increased fluid flow or bacterial products and leucocytes passing through (115). The JE has been shown to be permeable to a variety of materials ranging from carbon particles (116) to proteins (117), especially when the tissue is inflamed.

All oral epithelia in vivo, except the JE, are covered by a layer of saliva. Not only does saliva have an important cleansing function, it also contains mucins that bind covalently to the epithelium surface, serving to concentrate secretory immunoglobulin A (IgAs) and lysozymes, which in turn limits the attachment of micro-organisms. Immunologically important differences also exist between the skin and the oral mucosa. Most importantly, in the oral mucosae different subtypes of immune cells reside that primarily react in a tolerogenic manner upon contact with antigens (118), like lipopolysaccharide (LPS), a cell membrane component of Gram-negative bacteria. This explains why patients who are allergic to nickel may still tolerate nickel-containing orthodontic appliances, and why oral disease is seen relatively infrequently despite the continuous attack by pathogenic bacteria and other exogenous antigens. In addition, oral exposure to metals like Ni and Co, e.g. wearing orthodontic braces, previous to cutaneous exposure leads to immunologic tolerance (119, 120).
1.4 Immunology

Brief overview of the immune system

A brief overview of the basic immunologic mechanisms is now provided based on current textbooks (Contact Dermatitis, 5th edition by Johansen, Frosch, and Lepoittevin; Basic and Clinical Immunology, 2nd Edition by Peakman and Vergani; and Janeway’s Immunobiology, 7th edition by Murphy, Travers, and Walport). The immune system is truly a fascinatingly complex system of cells and their communication in which two distinct parts exist separately yet are closely linked: the innate and the adaptive immune system. The combination of the two makes it possible for the body to recognize self and non-self, and to recognize and eliminate pathogens and damaged or changed self-proteins. The functionality of the immune system is accomplished by cell-to-cell contact in combination with a large number of small soluble mediators: cytokines, chemokines and cell-membrane-bound molecules. Cytokines are immunologic messengers in the broadest sense of the word, and have complex functionality. Different cytokines may have redundant and even opposing effects on their respective functions. They can act on different cells (pleiotropy) and have autocrine, paracrine, and endocrine functions. Often they provide up- or down-regulation of specific cell functions by changing their cell surface molecules. Chemokines have chemotactic functions and can attract specific cells according to a concentration gradient.

Innate immunity

The innate immune system refers to non-specific mechanisms that serve as the first line of defence. It exists prior to antigen exposure and has neither specificity nor antigen-specific memory. It prevents exogenous attacks from invasion and defends the body with non-specific inflammatory responses. It comprises: anatomical barriers (epithelia), physiological barriers (temperature, pH), molecules (complement system, antimicrobial peptides, and cytokines), and pro-inflammatory cells (basophils, mast cells, and eosinophils), phagocytic cells (neutrophils, monocytes, macrophages, and dendritic cells), and natural killer cells (NK cells).

The innate immune system recognizes highly conserved substrates of pathogenic germ lines via pattern-recognition receptors (PRRs), which may be membrane-bound (e.g. Toll-like receptors; TLRs), cytoplasmatic (NOD-like receptors) or secreted (complement system and cytokines). Functionally they can be divided into signalling PRRs, which may change the cell function or enhance maturation, and endocytic PRRs, which promote attachment, engulfment and destruction of micro-organisms. Recently, TLRs have been found to be important in allergies to metals like nickel,
palladium and cobalt (103, 104). There are basically 10 different TLRs in humans. These TLRs recognize so-called Pathogen Associated Molecular Patterns (PAMPs). These PAMPs are small molecular motifs conserved within a class of microbes. Lipopolysaccharide (LPS), a cell membrane component of Gram-negative bacteria, is one such PAMP that is recognized by TLR-4.

Antigen presenting cell (APC)  
Antigen presenting cells (APCs) are able to uptake, process, and subsequently present small fragments (epitopes/peptides) of foreign/pathogenic/self-molecules. These epitopes are presented on the cell surface in the context of major histocompatibility complex (MHC) molecules, class I or II. MHC class I molecules are mainly associated with intra-cellular or endogenous antigens (e.g. viruses) and MHC class II with extra-cellular or exogenous antigens (e.g. bacteria). MHC class II molecules are normally only expressed on APCs; whereas MHC class I molecules occur on almost all nucleated cells. Dendritic cells (DCs) are the most potent APCs and are so-called ‘professional’ APCs. There are many subtypes of DCs, each equipped with specific features relevant to their location in the body. The Langerhans cell (LC) is typically found in the epidermis. They reside in the skin and mucosae and form a network with their characteristic dendrites with which they continuously sense their environment. Not only do they present foreign antigens, they also process and present self-proteins. However, full activation only occurs when an additional danger signal is present; such a signal is generally sent by the innate immune system via signalling PRRs such as TLRs. Upon full activation, the DCs migrate towards local lymph nodes to present the epitopes to naïve B and especially T lymphocytes (B- and T-cells). Part of the maturation process is up-regulation of surface molecules CD80/CD86, which are important for later T-cell activation. Therefore, DCs play a crucial role in linking the innate and the adaptive immune systems and act as a switch to activate the adaptive immune system.

Adaptive immunity  
This part of the immune system is characterized by specificity and antigen-specific memory. Cell types involved are B- and T-lymphocytes (B- and T-cells). Key molecules are antibodies or immunoglobulins (Ig’s), T- and B-cell receptors (TCRs and BCRs = surface immunoglobulin), and MHC molecules. Memory B- and T-cells are clones from the effector cells; they travel through the body and rest in lymph nodes. Throughout the body these memory cells may be activated to jump-start an immune response. They only develop after antigen encounter and have the same properties as their effector clones.
**Humoral immunity**

Immunoglobulins are glycoproteins produced by B-cells, plasma B-cells in particular, and have at least two binding sites (Fc: binds to cells and Fab: binds to antigens). They recognize and bind to specific epitopes on whole antigens, and can neutralize toxins and activate immune effectors such as complement, mast cells, phagocytes and others. Immunoglobulins (Igs) are displayed by B-cells or circulate as soluble proteins (sIgs). Different parts (epitopes) of one antigen can be recognized by different antibodies. A single B-cell and its progeny produce a single antibody that recognizes a single epitope of an antigen (mono-clonal antibody). Different B-cell clones may recognize the same antigen via different epitopes (poly-clonal response). There are five major groups of antibodies: IgG, IgA, IgM, IgD, and IgE. IgG is most abundant and has four subtypes. IgA has a secretory variant (sIgA), which is important in external surface defence and is secreted via mucosal fluids, e.g. saliva. IgM is a low-affinity antibody important for the initial immune response. Later, IgM-producing plasma cells switch to the production and secretion of other Igs, but retain the Fab domain, and thus antigen specificity. IgE is the main antibody involved in type I allergy (hay fever, peanuts etc.) and immune response to parasites. IgD is only expressed on naive or virgin B-cells and disappears after activation.

In their naive state as they reside in lymph nodes (germinal centers), B-cells bear two types of surface immunoglobulins (IgM and IgD with low affinity) specific for one epitope of an antigen. Upon antigen binding (presented by DCs or T-cells), B-cells start optimizing their affinity to the epitope (somatic hypermutation), choose a specific class of antibody (IgG, IgE, IgA or IgM (class switching, which may be regulated by (helper) T-cells), lose their IgD surface immunoglobulin, and present the antigen in the context of MHC class II on their surface to T-cells. Only those with the highest affinity and best capacity to present the antigen will survive this process and will then be activated by an antigen/epitope- specific T-cell.

**Cellular immunity**

The cellular branch of the adaptive immune system is provided by T-cells. After their genesis in the bone marrow they further mature in the thymus (hence the name T-cell) where they are equipped with an antigen-specific T-Cell Receptor (TCR) but are not yet activated (naive T-cell). Although many subsets of T-cells exist, they all carry a collection of molecules that transduce activation signals, referred to as a `cluster of differentiation 3 (CD3) complex’—these are specific T-cell markers. There are two major and distinct subsets of T-cells found in the blood and lymph nodes, characterized by two accessory molecules: CD4 and CD8, 66% and 33% respectively. CD8 T-cells are typically associated with killing target cells, and are
therefore called cytotoxic T-cells (Tc-cells). They only recognize antigens in the context of MHC class I molecules (via CD8). CD4 T-cells only recognize antigens in the context of MHC class II (via CD4). These CD4 T-cells differentiate into several subsets upon activation by antigen presenting cells (APC). Whether or not an APC presents the antigen in the context of a MHC class I or II molecule depends on the kind of antigen. Generally, extracellular antigens will be presented on MHC class II molecules (exogenous pathway) and intracellular antigens, e.g. virus-derived, are presented on MHC class I molecules (endogenous pathway). Either way, the antigen needs to be processed intracellularly to get the right size (epitope) and needs to be associated with the MHC molecules.

**Figure 1.1** Polarization of T-cells in specific subsets is dictated by the cytokine microenvironment (next to black arrows) produced by the antigen-presenting cell. Different subsets activate different transcription factors (see arrows) that lead to specific ‘effector’ cytokines typical for each subset (leftmost cytokines). Courtesy of dr. B.M.E. von Blomberg (*Contact Dermatitis* 5th edition)
Table 1.4  Th1, Th2, and Treg subsets with their phenotype immune responses, cytokine profiles and inducing cytokines (differentiation), transcription factors, and shared cytokines with other subsets resulting in functional overlap.

<table>
<thead>
<tr>
<th>T-cell subset</th>
<th>Phenotype immune response</th>
<th>Cytokine profile</th>
<th>Cytokines involved with differentiation</th>
<th>Transcription factors involved</th>
<th>Shared cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>Intracellular pathogens</td>
<td>IFN-γ, TNF-β</td>
<td>IL-12, IL-18, IL-27, IFN-γ</td>
<td>T-bet: IFN-γ suppress Th2, Th17</td>
<td>IL-2, IL-3</td>
</tr>
<tr>
<td></td>
<td>Contact allergy</td>
<td>(IL-10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG2 production</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th2</td>
<td>Extracellular pathogens</td>
<td>IL-4, IL-5, IL-13, IL-4</td>
<td>GATA-3:IL-4</td>
<td></td>
<td>IL-2, IL-3</td>
</tr>
<tr>
<td></td>
<td>IgE and IgG1 production</td>
<td>IL-9, IL-24, IL-25, IL-13</td>
<td>TSLP, IL-33 (produced by keratinocytes)</td>
<td>Suppress Th1, Th17</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>Parasites</td>
<td>Contact allergy</td>
<td>(IL-10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nTreg/iTreg</td>
<td>Abrogation of response</td>
<td>IL-10</td>
<td>TGF-β</td>
<td>FOXP3</td>
<td>IL-2, IL-10</td>
</tr>
<tr>
<td></td>
<td>Oral tolerance</td>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cancer control</td>
<td>IL-2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

nTreg: natural T-regulatory cell, iTreg: inducible T-regulatory cell

T-cell recognition

T-cell recognition occurs in 3 steps accompanied by 3 signals to the T-cell. Upon TCR recognition of the MHC-antigen complex, CD3 activates the T-cell (signal 1) resulting in up-regulation of co-stimulatory molecules (CD28). If CD28 then binds to its ligand (CD80/CD86) (up-regulated on the APC as a part of the maturation process), survival of the T-cell is established (signal 2). Upon ligation of CD28 with CD80/CD86 the APC will polarize the T-cell into a specific subset (Th1/Tc1, Th2/Tc2, Treg, etc...) (signal 3) with specific cytokine release to dictate the type of immune response (Figure 1.1). In this way full activation and differentiation will only occur when there is an appropriate TCR-MHC interaction by fully maturated APC. The absence of any of the 3 signals will lead to anergy or apoptosis (programmed cell death) of the T-cell; these mechanisms prevent undesired immune responses. The nature of signal 3 is not yet fully understood, but distinct cytokine profiles released from differently polarized DCs play a crucial role. Table 1.4 summarizes the Th1, Th2, and Treg
Table 1.5  Summary of the most relevant cytokines involved in allergic reactions and their producers and functions.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>T helper profile</th>
<th>Also produced by</th>
<th>Activities</th>
<th>Towards T-cell profile</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>Th1</td>
<td>CTLs, NK-cell, MΦ</td>
<td>Anti-viral activity + APCs + MΦ + NK-cells + ICAM-1 endothelial</td>
<td>Th1(secondary) Anti Th2</td>
<td>Suppresses IL-4 mediated activity Vital to cellular immune response</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2</td>
<td>PMNs, CTLs</td>
<td>+ B-cell (MHC II, CD80/86), class switch IgM→IgE, IgG4 + T-cell + VCAM-1(T-cell and PMN cells recruitment; but not Neutrophils) - Th1, - IL1, IL-6, TNF-a, + IL-1ra (inactivated IL-1) and IL-10</td>
<td>Th2 Anti Th1</td>
<td>In an environment with allergic inflammation more allergic responses to bystander antigens develop</td>
</tr>
<tr>
<td>IL-5</td>
<td>Th2</td>
<td>Mastcells, NKTcells, Eosinophils</td>
<td>+ Eosinophils + IgA producing B-cells Chemotactic for eosinophils Eosinophils secretion Class switch to IgA</td>
<td></td>
<td>Less important in asthma</td>
</tr>
</tbody>
</table>
Table 1.5 (cont.) Summary of most relevant cytokines involved in allergic reactions and their producers and functions.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>T helper profile</th>
<th>Also produced by</th>
<th>Activities</th>
<th>Towards T-cell profile</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-13</td>
<td>Th2</td>
<td>Similar to IL-4</td>
<td>Similar to IL-4</td>
<td>Does NOT induce Th2 profile</td>
<td>IL-13 is more widely produced than IL-4, detected earlier in allergic inflamed tissue</td>
</tr>
<tr>
<td>IL-10</td>
<td>Treg</td>
<td>Monocytes, B-cells</td>
<td>Th1 (IFN-γ) and Th2 (IL-4 IL-5), MHC II and CD80/CD86 on APC, IL-1α, IL-1β, IL-6, IL-8, IL-12, TNF-α</td>
<td>- Th activation by APCs, - Th activation through (CD28), Constitutively expressed in lungs</td>
<td></td>
</tr>
</tbody>
</table>

mΦ: Mononuclear phagocyte; MΦ: Macrophage; + Stimulate activity or up-regulation; - Inhibition activity or down-regulation; PMN: polymorphonuclear

Dendritic cell polarization

The cytokine profile of the micro-environment generated by the APC during T-cell activation (Figure 1.1) is dictated by the type of antigen, the concentration of antigen, and the presence of co-stimulatory/inhibitory molecules. These factors polarize the DC that will ultimately determine the outcome of the functional T-cell subset. In this way PRRs, like TLRs, bridge the innate and the adaptive immune system (122-124). TLR-4 activation in APCs traditionally leads to pro-inflammatory cytokine production (IL-1, IL-6, TNF-α) and Th1 directing cytokines IL-12 and IL-18, whereas blocking this activation leads to Th2 differentiation (in a murine model) (125), hence DCs are imprinted to produce primarily Th1 inducing cytokines (IL-12) upon receiving danger signals (126). However, this is not an absolute rule, as the dose of antigen (e.g. LPS) plays an important role (122). For example, low doses (0.1 µg) of inhaled LPS induced Th2 responses, whereas a higher dose (100µg) resulted in a Th1 response in a murine allergic sensitization model (127). Other reports suggest that the purity of the ligand may also influence the polarization into Th1 or Th2 responses (128, 129). Furthermore, it was demonstrated that repeated stimulation of TLR-2 and TLR-4 induced tolerance of both receptors with reduced cytokine production (130, 131).
Oral mucosa

The oral mucosal DCs have a unique repertoire of receptors that induce tolerance rather than inflammation. They express high affinity receptors for IgE that upon ligation lead to IL-10 and TGF-β production, which is necessary for the induction of Tregs (118). Also, oral DC activation by TLR-4 (by LPS) will induce Tregs expressing FOXP3, IL-10, and TGF-β (132). Oral DCs express constitutively more B7.H co-inhibitory molecules and thereby contribute to immune-silencing (132); their expression is up-regulated by ligation of TLR-4 (118). B7.H inhibits T-cell activation through ligation with CD28 (signal 2). Finally, in a murine model, oral DCs bind and process topically applied ovalbumin, which leads to the production of IFN-γ and IL-10-producing T-cells (133). Human serum albumin is an important carrier protein for metals like nickel and palladium.

The clinical outcome of these tolerogenic properties of the oral mucosae is observed in patients who had orthodontic treatment or oral exposure to nickel prior to ear-piercing, resulting in decreased levels of sensitization to nickel compared to ear-pierced patients without previous orthodontic treatment (119, 120). In a guinea pig study it was shown that oral tolerance resulted from antigen-specific immunosuppression, and was induced more effectively after direct contact with the oral mucosa (using an ointment) than via feeding (134). In a murine study, tolerance to nickel was effectively achieved by intra-gastric feeding of nickel (135). Even nickel-releasing cages and drinking nipples were sufficient to induce tolerance for this metal in mice (135).

Another important factor that influences immune response is age. From studying skin, it is known that the number of DCs decrease with age, possibly in response to UV exposure. Even though UV exposure is not likely to occur in the oral cavity, a considerable decrease in DCs was still reported in subjects older than 40 (112), which might be why oral diseases are more frequently observed in the elderly. The induction of oral tolerance was found to be less effective in older guinea pigs compared to younger animals (134).

All in all, there are many factors that influence the final host defence (T-cell polarization), and the exact mechanisms are not fully understood. It has been suggested that during the initial contact with antigens, the majority of emerging effector/memory T-cells belong to a less polarized subset, that is Th0 cells, that have a mixed cytokine profile (IL-2, IFN-γ, IL-4, IL-10). A more pronounced differentiation will occur upon repeated stimulation (136). T-cell subdivision is based on the cytokine profile alone; there are no clear-cut surface markers (CDs) for the different subsets. This subdivision is not absolute, being based on the predominance of certain cytokines produced; moreover, the different subsets of T-cells may co-exist. In this
In the context it is important to stress that humans are not mice, because much of the current knowledge is based on murine models. In contrast to murine studies, categorically distinct Th cytokines profiles are seldom apparent in human cells, although there remains an inverse relationship between the tendency of T-cells to produce IFN-γ as opposed to IL-4/IL-5 or IL-17 (121). Moreover, molecular mechanisms may differ in important ways. For example, nickel was found to activate TLR-4 in humans, but not in mice (104). Finally, the central nervous system also plays a role in the immune responses as discussed above, and could therefore influence the predominance of a certain T-cell subset; e.g., chronic stress skews Th1 responses towards Th2 responses (100, 101).

**Allergic Contact Dermatitis (ACD)**

There are four basic possible hypersensitivity reactions that can occur according to the Gell-Coombs classification. Hypersensitivity is defined as an exaggerated reaction to a stimulus, whereas allergy is the disease resulting from hypersensitivity due to either specific T- or B-cells. Allergy in relation to dental materials is considered to be a type I (IgE-mediated) and especially type IV (T-cell mediated) hypersensitivity reaction. A prerequisite for these types of allergic reactions is a sequential occurrence of facts: exposure to the allergen, penetration or absorption of the allergen, and sensitization to the allergen. Another typical phenomenon of allergies is that a small amount of allergen can be sufficient to cause a response. Importantly, allergic reactions are normal immune responses—the body responds as it would to a pathogen. The difference is that allergic antigens (allergens) only generate immune responses in some patients, and that allergens *per se* are not pathogenic.

**Metals**

Metal ions released from jewellery or dental alloys due to corrosion are allergens that need to bind to protein carriers to become allergenic. They are called haptens due to their limited size and their need to bind to carrier molecules. In contrast to other haptens, metal ions form non-covalent coordination protein-metal chelate complexes with nucleophilic residues of some amino acids: cysteine, lysine, methionine, tyrosine, and especially histidine. This means that metal ions may change from one carrier protein to another with equal or higher affinity (137). Human serum albumin (HSA) is believed to be an important carrier molecule because it easily binds to nickel and other metal ions (51). However, nickel complexes with HSA-derived peptides appear not to contribute to the antigen determinant (138). Still, HSA is abundantly available both *in vivo* and in cell cultures and it is assumed to play an important role (138). Ni-HSA complexes also have the potential to stimulate nickel-reactive T-cells
in vitro (139). Nevertheless, other molecules may be involved, such as apotransferrin, a scavenger for free iron ions, which reportedly binds to nickel ten times more efficiently than HSA, and could stimulate Ni-specific T-cells (137). In addition, various intracellular proteins, such as tubulins and chaperone/heat shock proteins from human B-cells, were found to bind to nickel (140). Finally, the most important recent finding in this context is the direct activation of TLR-4 in humans by nickel, palladium, and cobalt (103, 104).

Metal-protein complexes are taken up by APC and presented as a metal-epitope on their MHC class I and/or II molecules, which may subsequently be recognized by specific T-cell (CD4+ or CD8+) (141). Ni-protein-MHC conjugates can, however, be recognized by Ni-specific T-cell independent of APC processing in two different ways. These reactions are either dependent (16) or independent on the peptide-epitope (142). In the latter case, nickel was found to bind to the MHC molecule directly. Moreover, Ni-free cryptic self-peptides could result from metal-induced alterations of protein processing, with subsequent T-cell activation (143). Although mostly Ni-complexes were studied, it is likely that similar interactions would be observed in palladium, since two independent groups have proven its cross-reactivity with nickel on a T-cell recognition level (16, 17).

Sensitization phase
The ability of metals to easily induce sensitization depends primarily on the induction of the innate immune system. In general, danger signals are required, and the allergenicity of allergens roughly correlates with their toxicity. In mice, for example, additional adjuvants are necessary to induce sensitization because their TLR-4 does not recognize metals (104). As a result of Ni-contacts in humans, however, an unspecific local pro-inflammatory immune response will be generated by local keratinocytes and DCs. Released cytokines and chemokines facilitate the recruitment of other immune cells primarily from the innate branch, including DC precursor cells from the blood, which play an important role in the sensitization to haptens (144, 145). The next crucial step is the uptake, processing and presentation of metal-protein complexes on MHC class I and II molecules by DCs, which will, upon full activation, migrate towards the local lymph nodes to present their antigen to CD4+ and CD8+ naive T-cells. Primed T-cells will undergo clonal expansion, resulting in both specific CD8+ and CD4+ with either effector or memory functions. The effector cells in particular will express specific receptors leading them to the skin (skin-homing). So taken together, antigen specific T-cells can be found in the lymph nodes (central memory T-cells), in the blood and in the skin (peripheral effector and
effector memory T-cell). The whole sensitization mechanism may take up to 15 days in humans.

**Elicitation phase**

Re-exposure of sensitized patients will lead to typical allergic contact dermatitis (ACD) with 24-72 hours. In contrast to the sensitization phase, this is a rather fast response, induced by circulating effector memory T-cells, after local activation by APCs. Still, compared to IgE mediated hypersensitivity, that may occur within minutes, hence immediate type hypersensitivity, ACD is considered a delayed response and is therefore also called a delayed-type hypersensitivity. Recently, it has been demonstrated that B-cells may play a significant role in the early recruitment of effector memory T-cells, via IgM-antigen conjugates and subsequent complement activation via C5a (146-148). Although the exact role of different subsets of T-cells is not yet fully understood, not only CD4+ Th-cells (149-151), but also CD8+ cytotoxic T-cells (Tc1-cells) are important effectors in ACD (152, 153) and that they are recruited early after challenge with antigen. In a recent study, elicitation was induced using an oral mucosal nickel patch test in Ni-sensitive and non-sensitive patients, and local and systemic lymphocyte typing was performed by immunohistochemistry (154). In Ni-sensitive patients, CD4+ circulating T-cells were significantly increased, especially in the intermediate layers of the mucosa of the contact site, whereas only a moderate increase of CD8+ T-cells was observed in the capillaries (154). CD8+ T-cell activation and activity induces recruitment of leucocytes, contributing to the clinical appearance of ACD. CD4+ subsets within this group of leucocytes regulate the immune response. CD4+ T-cells are thought to play a crucial role in the regulation of specific CD8+ T-cells. Induction of ACD in CD4-deficient mice is possible, and leads to enhanced responses; by contrast, CD8 deficient mice cannot develop ACD (155, 156). The exact role of Treg cells in ACD is yet not clear; however, their influence is considered important in the regulation and abrogation of ACD (156).

Although the sensitization and elicitation phases are mechanistically distinct, in real life antigens may be present in the skin for days after exposure, leading to the recruitment of specific T-cells after the first exposure (primary ACD). Strong haptens are much more efficient in inducing sensitization because of their ability to activate the innate immune system. Since metals like nickel, palladium and cobalt can activate TLR-4, they are considered potent sensitizers.
Irritant Contact Dermatitis (ICD)
Irritant contact dermatitis is a non-specific inflammatory dermatitis brought about by the activation of the innate immune system induced by the pro-inflammatory properties of chemicals (157). As such, there is no specific T-cell activation in the local lymph nodes or at the contact sites. Still, ICD and ACD are closely related, since activation of the innate immune system, e.g. by TLRs, creates the conditions for ACD as discussed above. Clinically, ICD and ACD may be difficult to distinguish, although there are important differences. One of them is that ICD is limited to the site of contact, whereas in ACD lesions may extend beyond the contact site (157). ICD patients may also experience a burning or prickling sensation, while in ACD itching is the more common complaint. Still, no clinical presentation is pathognomonic for ICD and it may mimic ACD (157). It is unclear to what extent irritant reactions play a role in oral disease with regard to dental alloy exposure. Subjective or sensorial irritation has also been identified as a clinical entity. These individuals experience sensory discomfort, like stinging, burning, or itching, in the absence of clinical or histological evidence of skin lesions. The threshold for this reaction varies between subjects, and is independent of the susceptibility to other types of irritation. The patho-physiology is mainly unknown, although a thinner and more permeable stratum corneum as well as changes in the nervous system are thought to contribute (158). Oral complaints, such as pain or burning, are likely to be associated with the individual irritability threshold.

Systemic Contact Dermatitis (SCD)
Systemic contact dermatitis (SCD) is defined by Veien (159) as dermatitis that may occur in contact-sensitized persons when they are exposed to the hapten systemically, i.e. orally, per rectum, intra-vesically, transcutaneously, intravenously, or by inhalation. Recurrent vesicular dermatitis or vesicular eruptions of the fingers and dermatitis in the axillae or antecubital fossae are reported most frequently (159). SCD is reminiscent to flare-up reactions and can be mechanistically explained by the retention of antigen-specific memory T-cells at the site of initial sensitization or earlier ACD (160-163). These specific T-cells are thought to be re-activated by circulating antigens. The majority of SCD studies have focused on nickel. Oral administration of 0.22-5.6mg may induce aggravation of nickel-Ni induced ACD. Because the daily uptake of nickel is high (0.22-1mg) (164), and because the nickel released from dental appliances may be substantial, it is plausible that oral exposure to nickel from dental appliances plays an additional role in SCD. In fact, one report describes a 56-year-old woman with severe hand dermatitis occurring after she
received orthodontic appliances. She was known to be allergic to nickel and palladium. The removal of the orthodontic devices alone was not sufficient; her nickel-and-palladium crowns also had to be removed for her condition to improve significantly. She fully recovered only after also disposing of her nickel-containing cookware (165). Another notable case report describes a nickel and cobalt allergic female with persistent peri-oral and peri-orbital eczema that appeared after she gave birth to her second child. Her orthodontic retention wires, which she had had for years, were responsible for the eczema, as it diminished within weeks after their removal and no flare-up reactions occurred thereafter (21).

Palladium exposure from dental alloys has also been found to cause systemic dermatitis (166-168). The most recent report describes a 54-year-old Taiwanese woman who suffered from full-body annular erythema for 15 years; her condition was alleviated almost immediately after one palladium-containing dental inlay was removed. No flare-up reactions occurred for 2 years following (167). Strikingly, no local oral lesions were visible in all three cases described above. A Japanese retrospective study reported that in patients suspected of having an allergy to metal in dental alloys, pustulosis palmaris et plantaris/dyshydrosic eczema and contact dermatitis was frequently found (±30%). Also in these cases mostly no intraoral signs of contact allergy were visible (73).

1.5 Palladium allergy

In general

Palladium was discovered by William Hyde Wollaston in 1803 and named after the asteroid Pallas. Soon, it became clear that this metal had very interesting chemical properties. It has a great ability to absorb hydrogen (up to 900 times its own volume) and is therefore used as a catalyst in many (de)hydrogenation reactions. Today, palladium chemistry is still of great interest; in 2010 the Nobel Prize in chemistry was awarded to Richard F. Heck, Ei-ichi Negishi and Akira Suzuki for palladium-catalyzed cross-coupling in organic synthesis. Palladium is widely used in chemical, electronic, and especially automotive industries as a catalyst (64), which taken together accounts for approximately 88.8% of the total palladium demand worldwide in 2013. Still, human exposure to palladium is mainly through contact with jewellery and dental appliances, which accounts for 4.0% and 5.3% of the total demand, respectively, or about 15.9 tons of palladium for the dental industry worldwide in 2013 (Johnson & Matthey: www.platinum.matthey.com).
Table 1.6  Palladium demand in tonnes for dental industry in various areas of the world.

<table>
<thead>
<tr>
<th></th>
<th>2004</th>
<th>2009</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>2.5</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Japan</td>
<td>16.2</td>
<td>9.2</td>
<td>6.4</td>
</tr>
<tr>
<td>U.S.</td>
<td>7.3</td>
<td>8.1</td>
<td>6.7</td>
</tr>
<tr>
<td>China</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rest of the world</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>26.4</td>
<td>19.8</td>
<td>15.9</td>
</tr>
</tbody>
</table>

Source: www.platinum.matthey.com

Current literature

Palladium and its compounds have a very low to moderate acute oral toxicity: about 200 to >4000 mg.kg\(^{-1}\) body weight depending on the solubility of the palladium compound used (169-171). However, intravenous administration shows much higher toxicity (6 mg.kg\(^{-1}\) body weight) (170).

The first report on the palladium allergy (1955) describes a 35-year-old housewife who suffered from contact dermatitis on her left fourth finger on which she wore a 90wt% Pd-containing wedding ring (172). In 1969, a contact allergy to palladium was reported by a chemist working with noble metal salts, including Na\(_{22}\)PdCl\(_{4}\) (173). Although palladium has been used for dental alloys for almost a century (38), its wide-scale use started in the 70s due to increasing gold prices (5). Shortly thereafter, palladium allergies emerged in the literature more frequently (5). The first report on palladium allergies from dental alloys was documented by two Dutch researchers, van Ketel and Nieboer (174). Japan has long been the largest palladium-consuming region for dental applications, followed by North America and then Europe, although Japan’s demand has decreased substantially in the last years (Table 1.6). Interestingly, palladium allergy prevalence seems to be distributed similarly, that is, 7-24% in Japan (73, 175), 8.5-13.3% in the US (6, 8, 176), and 4.9 (Germany)-11.7% (Spain) (177, 178) in Western Europe. In Europe much more data is available, and there are considerable variations between Northern and Southern European countries (19). Several extensive studies (including between 542 and 4446 patients) described the difference in prevalence between gender in dermatitis patients: 17.1% vs. 3.1% in Spain 14.8% vs. 2.5% in Turkey, 14.9% vs. 3.2% in Minnesota USA, and 6.7% vs. 2.3% in Italy for women and men, respectively (179-182).

Most reports on the palladium allergy are related to dental alloys and oral disease (14, 167, 168, 174, 183-193). Still, few describe clinically relevant allergic contact
dermatitis to palladium (172, 173, 194, 195). Several authors have described Pd-induced sarcoidal-type allergic contact granulomas due to body piercings (185, 196-201). Some have discussed the relevant systemic allergic contact dermatitis to dental Pd (73, 166-168, 202). Notably, a recent report described allergic contact gastritis due to Pd-containing dental bridge (189). It must be stated that patients who are allergic to palladium rarely exhibit a reaction to skin exposure to the metal (19, 203). Despite the numerous case reports describing adverse reactions to palladium-containing dental alloys, the clinical relevance of positive patch tests to palladium is still unclear, or at least difficult to assess. One of the reasons is that the clinical picture of palladium-induced allergic contact stomatitis is ambiguous. Furthermore, it’s possible that no oral lesions are present in the case of systemic contact allergy to dental materials, as pointed out in several case reports; systemic complaints or lesions could be atypical, e.g. gastritis or alopecia. Palladium sensitization, as measured by positive patch tests, is frequently found in the absence of clinical relevance, both intra- and extra-orally. Also, the ignorance of the presence of dental alloys and/or their composition with palladium allergic patients complicates the evaluation of the clinical relevance considerably. Palladium allergies have been estimated to be overall equally prevalent in dermatitis and oral disease patients at 7-8% (range <1 up to 24% worldwide) (19, 73). However, this figure is based on studies that have evaluated either dermatitis or oral disease patients. Therefore, inter-regional, inter-individual, and inter-laboratory variations, as well as test materials used, the number of patients, and the period of testing could skew these observations. Moreover, some investigators marked a 2+ reaction as positive, while others scored a 1+ reaction as positive, and patch test readings were done at various different time points and frequencies. Finally, because palladium is not included in standard patch test series but is rather part of specific ‘metal’, ‘oral disease’, or ‘dental’ screening series, it is not always clear what specific patients have been tested. Studies that compare the prevalence of dermatitis and oral disease patients are scarce, but they do indicate a higher prevalence among patients with oral disease relative to those with dermatitis. One study reported that among 106 palladium-sensitized patients, 55.7% suffered from oral disease and 29.2% from skin dermatitis (181). An older study retrospectively comparing patients with intra-oral complaints (n=397) to patients suffering from eczema (n=112), showed that especially gold and palladium sensitivity was significantly increased in the dental patients group: 23% vs. 6% for gold, and 8% vs. <1% for palladium (106). Another important issue to address in this context is the cross-reactivity between nickel and palladium.
Cross-reactivity to nickel and concomitant reactivity to other metals

The relevance of a positive patch test reaction to palladium is likely compromised by potential cross-reactions to nickel, even though exclusive positive reactions to palladium are also reported continuously and appear to be more prevalent in the last years (19). The simultaneous positive reactions of nickel and palladium are explained by (i) sensitization to both metals, and (ii) contamination of the palladium patch test material with traces of nickel (despite the fact that several studies have disproved this theory) (204), and (iii) by the fact that nickel and palladium have similar chemistry and electron arrangements, which could cause cross-reactivity at the T-cell level (16, 17). It has also been shown that nickel and palladium form similar complexes with sulphur ligands (205), which may explain why both metals form similar metal-protein complexes as suggested by Santucci (206). Hindsén et al. (15) provided in vivo evidence for cross-reactivity to nickel and palladium by systemic administration. They produced flare-up reactions on sites previously patch tested with nickel and palladium after oral exposure to nickel. In this study, contamination was excluded by chemical analysis.

Other metals often produce positive patch test results in palladium-sensitized patients. In Spain, researchers found concomitant reactivity to nickel (97%), cobalt (36%), and chromium (13%) (207). These figures are similar to findings in Austria (208). In the U.S., the instance of co-sensitization to nickel was considerably less (57.0%), and was strikingly only slightly higher than that for gold (48.2%) (181). In the latter report, co-sensitization to cobalt and chromium was measured at 37.6% and 10.2%, respectively.

1.6 Sensitization tests

Allergies are a clinical manifestation of an elicitation phase resulting from repeated allergen contact after previous sensitization, as discussed above. Therefore, the name ‘allergy test’ is actually incorrect, at least for in vitro assays. Basically, a sensitization test confirms whether a patient is sensitized to a particular allergen, and thus could suffer from allergy after re-exposure. In other words, a patient is sensitized when allergen-specific (memory) T-cells are present, but can be without allergy when there is no actual or insufficient exposure to the allergen. Thus, allergic reactions can only occur in sensitized patients in the case of re-exposure. Therefore, a sensitization test can be positive without an allergy being present. This is one of the reasons why a positive patch test for palladium does not necessarily mean that there is an ongoing allergic reaction; it is rather the combination of the test results and the clinical picture that results in a diagnosis of allergy. This brings us to one of
the major difficulties of diagnosing a palladium allergy. As previously reported, the clinical presentation of palladium allergies associated with oral exposure is not uniform, and local or oral manifestations of this allergy may be absent even when there is a clinically relevant allergy systemically, which compromises the diagnosis. Moreover, exposure to palladium is easily overlooked as the composition of dental alloys is often unknown and dental crowns may be difficult to distinguish from natural teeth. Taken with the fact that palladium cross-reacts with nickel, diagnosing a clinically relevant palladium allergy is clearly quite difficult.

*Patch-test or skin test*

The patch test was developed by German dermatologist Josef Jadassohn (1863-1936), and was first presented in 1895. It is an epicutaneous test in which, next to other allergens, metal salts are applied onto the back of the patient for two days. Before application the allergens are mixed with a neutral medium called the vehicle, usually petrolatum and sometimes water. Over the course of the two days the allergens dissolve in sweat, penetrate the skin, bind to proteins, and are presented by APCs to specific T-cells, mostly effector memory T-cells. The idea is that all T-cells, including the antigen-specific ones, will pass this test area during these two days. Indeed, when allergen-specific T-cells are present, an inflammatory reaction will occur. In principle this is an experimentally-induced allergy, which theoretically can only occur in sensitized patients, i.e. in the presence of allergen-specific (memory) T-cells. The severity of the inflammation is crucial for the strength of the reaction so that a distinction can be made between the various degrees of positivity ( +/−, +, ++, and +++). Since many allergens are also irritants, distinguishing between allergic and irritant reactions in patch testing can be difficult. For metal patch testing, readings are usually performed at days 2, 3/4, and 6/7. The interpretation of the strength of the reaction is carried out by the dermatologist or allergologist and requires knowledge and adequate training. It is clear that, although the basic idea of the test is smart, its performance relies on many factors such as the state of the skin, permeability, solubility, and concentration or dose of the allergen, duration of the application, the clinician, patient’s medication and compliance, etc.. The reproducibility of the patch test has been questioned based on simultaneous application of duplicate patch tests with well-known haptens on both sides of the upper back that failed to obtain double-positive or double-negative results in multiple cases (209). Discordant reactions were found in 5%, with nickel being most frequent. It may be argued whether this rate is high or low. In rare cases patch testing may lead to sensitization—for example, when test concentrations are too
high. For all its potential flaws, patch testing is still the first test used to diagnose sensitization/allergy to metal.

Due to the aforementioned drawbacks, scientists have been searching for in vitro or better ex vivo alternatives for decades. Two basic alternative tests have been researched. The Lymphocyte Proliferation Test (LPT), which measures the amount of lymphocyte proliferation after antigen stimulation in Peripheral Blood Mononuclear Cells (PBMCs). Since varying results were obtained, a more specific test relies on the cytokines released by antigen-stimulated specific PBMCs. The strength of the patch test is that it is simple to perform and that it evaluates the endpoint of elicitation, which irrefutably confirms, in the case of a clearly positive result, that the patient is sensitized and that elicitation will occur upon further exposure. However, in the case of a negative or doubtful test result, drawing straightforward conclusions can be difficult due to the aforementioned drawbacks. This weakness explains why the patch test has relatively good specificity (absence of false-positive test results) but lacks sensitivity (presence of false-negative test results). Only the two assays used in the investigations described in this thesis will be briefly described. Exact protocols are described in the relevant chapters.

*Lymphocyte proliferation test (LPT)*

Historically, this test was called the lymphocyte transformation test (LTT) as it evaluated, upon exposure to an antigen, the morphological transformation of lymphocytes into lymphoblasts as a marker for activation. In the late 70s, people began to call it the lymphocyte proliferation test because the proliferation of antigen-specific lymphocytes was measured instead of the transformation. The first publication on LTT dates from 1964 in the context of histocompatibility (210). In 1967 the first publication appeared in the context of hypersensitivity to drugs (211). One year later the test was used to detect sensitization to gold (212). In 1970 the first report of confounding errors when using nickel acetate was published (213). The first positive results detecting nickel sensitization date from 1975 (214). In 1997, the first results on palladium emerged, which were in fact, not too good relative to patch testing (215). Although many modifications of the test have been suggested to enhance its reliability, it basically works as follows: Patient’s PBMCs are isolated from anti-coagulated blood by Ficoll-gradient centrifugation and cultured in a medium supplemented with serum. Several dilutions of metal salt solutions are then added to the wells. The concentrations used are determined according to standard procedures to reveal non-toxic and non-mitogenic reactions. The wells are then incubated for 4-7 days at 37°C with 5-10% CO₂. At least 1 negative control (lymphocytes in 10% medium without antigens) and 1 positive control (lymphocytes in 10% medium with
pokeweed mitogen PWM or phytohemagglutinin PHA) are performed. Generally, experiments are executed in triplicate. Then the cells are radio-labeled with $[^{3}H]$-thymidine for 5-18 h, which will be incorporated into newly synthesized DNA in proportion to the lymphocyte proliferation. The radioactivity (counts per minute [cpm]) is measured in a liquid scintillation counter. A stimulation index (SI) is then calculated as a measure for the test outcome: $SI = (cpm \text{ in test well})/(cpm \text{ in negative control})$.

In general, a SI < 2 is considered negative, SI $\geq 2$ but < 3 may be interpreted as a 'possible sensitization', and SI $\geq 3$ may be presumed to be positive, i.e. evidence of sensitization. This so-called cut-off value is often rather arbitrary and some authors interpret SI>3 positive whilst others prefer to use SI>5. Ideally, the cut-off value is determined using a gold standard, that is, a test method that is considered 100% accurate. In general, the patch test is used as gold standard, however, as noted above this test is not 100% accurate, like many diagnostic tests. This explains why the usefulness of LPT is still debated. One can imagine that when the LPT is positive and the patch test is negative it might be difficult to determine which test result should be considered correct.

**Scheme 1.1** Procedure of the LPT and lymphocyte specific cytokine production test. Courtesy of prof. dr. T. Rustomeyer

**Lymphocyte specific cytokine production**

This test is often performed in parallel with LPT, as the same conditions are needed. Before adding radio-labelled $[^{3}H]$-thymidine, the concentration of cytokines produced, e.g. IFN-γ as Th1 marker, and IL-4, IL-5 and/or IL-13 as Th2 markers, are
measured in the supernatants using commercially available Enzym-Linked ImmunoSorbent Assays (ELISA). The unit of measurement may vary from a stimulation index, as described for LPT to actual production or increment $\Delta \text{pg.ml}^{-1}$, that is, pg.ml$^{-1}$ with antigen stimulation minus pg.ml$^{-1}$ without antigen (negative control) stimulation. This test is, in principle, more specific, as it evaluates the immune responses more downstream. As discussed previously, different subsets of T-cells can be produced upon sensitization and subsequent elicitation. Theoretically, a positive LPT test only confirms the presence of allergen-specific T-cells without differentiating between subsets. To give an extreme example, it could be possible that a positive LPT is based on the presence of T-regulatory cells, which are responsible for immuno-tolerance. This might explain 'false' positive test results with LPT, when a negative patch test result was also found. The cytokine production test measures the cytokine production of allergen-specific T-cells and therefore gives information about the subset of T-cells that are involved. Importantly, cut-off values need to be determined to differentiate between negative and positive results. There are two basic approaches used. First, a more mathematical approach, in which the cut-off is set on a value calculated by the mean increment in non-allergic negative control subjects added with 3 times the Standard Deviation (Mean+3SD). A more clinical approach (and more frequently used in medical practice) is based on comparing the test results to a gold standard using Receiver Operating Characteristic (ROC) analyses. Both are used in this thesis and will be described in detail in the relevant chapters.

Difficulties with in vitro assays

The main problem with in vitro data is the difference in technical procedures, which makes it almost impossible to compare studies, at least for the absolute data obtained. Obviously the metal concentration used is of great importance, and investigators may find different sub-toxic concentrations to work with, which could make comparison difficult. Furthermore, the solubility of metal salts may differ substantially and may lead to different cations and anions when different salts are used, which may lead to different immunologic potencies. In addition, the number of lymphocytes used may differ substantially, which would significantly affect the results. It has been reported that the usage of $1\times10^6$ or at least $1\times10^5$ cells may be of great importance (29) in LPT.

As mentioned above, the number of lymphocytes plays an important role in sensitization testing. Therefore, an informative calculation is presented here to provide guidance about the numbers of T-cells involved in various test methods. In general, humans have about 6 litres of blood that contains about $1\times10^{10}$
lymphocytes. The frequency of antigen-specific T-cells can vary considerably depending on the presence of exposure to the allergen (in case of actual allergy) and is estimated to be maximally $1:10^4$. Routinely, about 30 ml of blood is drawn for *in vitro* assays as discussed here, meaning that about $5 \times 10^7$ lymphocytes are ‘caught’ with one venepuncture. Of these, less than 5000 are antigen-specific. When evaluating $1 \times 10^5$ cells/well, containing maximally 10 specific T-cells, there is a risk of some wells showing allergen-reactivity while others do not (‘limiting dilution’). Therefore, multiple cultures should be performed per test condition. In contrast, when patch testing is performed, the antigen or allergen is applied to the skin of the patient for 2 days. It is assumed that in this time period all recirculating lymphocytes ($0.5-1 \times 10^{10}$ T-cells, that is, max. $1 \times 10^6$ antigen specific T-cells) have passed the skin to which the allergen is applied and have therefore been exposed to the antigen. N.B. that these are maximum numbers. So in the case of a sensitized patient who is not suffering from a relevant allergy, the number of recirculating specific T-cells might be considerably lower, perhaps a tenth of the maximum, or $1 \times 10^5$ for patch testing and 1 per well in case of an *in vitro* assay.

Bearing these numbers in mind, results may differ significantly when blood is drawn before or two days after patch testing. Therefore, patients with ongoing allergies will have many more recirculating T-cells than sensitized patients without current exposure and subsequent symptoms. However, it should be noted that with recurrent exposure compartmentalization occurs, meaning that specific T-cells gather in the lesions, which may lower the actual number in the blood. Still, it is unlikely that the number of recirculating specific T-cells is lower in patients with exposure relative to patients without. This could explain ‘false’ negative *in vitro* results (compared to patch testing). In fact, it is possible that sensitized patients without allergic symptoms have a positive patch test result (all T-cells are challenged within two days) and score negative in *in vitro* assays due to the low frequency of circulating allergen-specific T-cells. Interestingly, this may be considered an important strength of *in vitro* assays, since patch test results can be positive even in the absence of a relevant allergy, compromising the diagnosis. On the other hand, LPT is more likely to be positive in patients with current exposure and related disease (elevated specific T-cells in the circulation). However, in the case of high levels of exposure—for example, people come into contact with nickel daily through food, cookware, money, jewellery and piercing—positive LPT results could be obtained due to the proliferation of allergen specific T-regs. In that case, the patch test results will be negative and the LPT should be considered a false positive. One could say that the relevance of a positive LPT is only confirmed when the test result becomes negative or readouts decrease considerably after removal of the allergen.
Another issue to address is that different variables are often measured. One study investigates Th1 but not Th2 responses, while another study does both. In the first study nothing can be concluded about the possible Th2 responses, which could have been even higher than the Th1 responses. Furthermore, some researchers may investigate IL-4 as a marker for Th2, while others used IL-5 or IL-13. Researchers must also use precaution drawing conclusions about the human population from animal based studies. This is especially true in murine models, which often use strong sensitizers in an ideal setting (naive mice are sensitized and subsequently re-exposed). This may be ideal for investigating mechanistic pathways, but it is not transferrable to human diagnostic testing.

There is a clear need for internationally standardized in vitro assays that can be used by investigators worldwide. In this respect, the establishment of a standardized and validated LPT or LTT like LTT-MELISA® is encouraging (29, 95). This means that the test protocol is standardized and cut-off values have been determined and considered reliable based on extensive investigations. However, due to the absence of a true gold standard and the many variations associated with this test, the patch test is still considered the best way to diagnose allergic contact allergy to metals.
1.7 Aim and outline of the thesis

The presented work is the result of clinical findings which raised questions about the reliability of the patch test to diagnose palladium allergies. At the adverse reaction unit of the Academic Centre for Dentistry Amsterdam (ACTA), patients were often suspected of being allergic to metals, and palladium in particular, possibly resulting from exposure to their dental alloys. This suspicion was based on a combination of (i) the clinical picture, including e.g., oral lichenoid lesions (OLL) and gingivitis, which was not related to dental plaque, (ii) the presence of Pd-based dental alloys, and (iii) the presence of general complaints, like chronic fatigue and joint & muscle pain. In these cases, patients were referred for patch testing at the department of dermatology of the VU medical centre. However, negative test results were often obtained. Test results for palladium in particular were regularly negative, even in the presence of reasonable clinical evidence of that allergy. Therefore, the reliability of patch testing was questioned and alternative test possibilities were sought. The lack of patch test reliability could be due to (i) a suboptimal test allergen and/or (ii) the test method itself could be ineffective in diagnosing metal allergies associated with exposure to dental alloys due to different underlying mechanisms.

Often, in cases of negative patch test results, a commercially available *in vitro* assay, such as a lymphocyte proliferation test (LTT-MELISA®), was also used. However, this test’s accuracy is debatable, and its usefulness was not considered scientifically proven. Since patch testing is the current best way to test for metal allergies, a combination of a negative patch test and a positive LTT-MELISA® was scientifically insufficient to merit invasive treatment. Still, it once again raised the question of whether or not patch testing is a reliable way to diagnose patients with a palladium allergy related to dental alloys.

Clinically, palladium allergies are mainly related to oral diseases, and possibly associated with systemic lesions and/or subjective complaints. Therefore, the clinical picture is murky and may not provide a clear overview of allergies to this metal. Palladium’s relevance in allergic contact dermatitis is thought to be low, possibly compromising the diagnosis. Therefore, more knowledge about the clinical presentation of palladium allergies associated with oral exposure to palladium-based dental alloys is needed.

Based on these clinical findings and subsequent questions, several objectives were formulated, resulting in the investigations presented in this thesis.

**Chapter 1** gives a broad introduction to the subject in general, taking into account the different audiences, which are: oral care experts, dermatologists, allergists and
immunologists. It addresses basic dental information, including the corrosion of metals, epidemiology, immunology, and of course palladium allergy.

**Chapters 2 and 3** deal with the development of an improved test salt for the diagnosis of allergy to palladium.

**Chapter 4** describes a cross-sectional multi-centre study that evaluated the prevalence of palladium sensitization in different parts of Europe using both palladium and nickel test allergens.

**Chapter 5** describes an exploratory, cross-sectional study that evaluated the reactivity of Na₂PdCl₄ in LTT-MELISA® and compared the results to both PdCl₂ and nickel.

In **chapter 6**, various *in vitro* assays are calibrated against clearly defined positive and negative controls. Thus, it was inventoried when a test result should be interpreted as negative or positive. These *in vitro* assays were performed on peripheral blood mononuclear cells of patients. Through the use of different blood tests, it is possible to speculate on the type of immune response that is involved in allergies to palladium and nickel.

**Chapter 7** describes a method to qualify and quantify the composition of dental alloys which are present in the mouth without actually damaging them.

The research in **chapter 8** describes the test results of both *in vivo* and *in vitro* methods in patients with oral disorders attributed to the presence of specific dental alloys. It was also investigated whether or not the presence of certain types of alloys is associated with specific T-cell responses.

**Chapter 9** is a continuation of chapter 5. Here, possible relations between (i) presence of dental alloys, (ii), patch test results to palladium and nickel, and (iii) the clinical picture were evaluated.

**Chapter 10** provides a summary and the clinical applicability of the presented work.
1.8 References


Chapter 1


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