CHAPTER 1

Introduction

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P-phenylenediamine

Structure
P-phenylenediamine (PPD) is a low molecular weight chemical of 108 Dalton. Its chemical structure comprises two amino-groups (NH$_2$) attached to an aromatic benzene ring in a meta-position, and hence, it belongs to the aromatic amines (Figure 1). In addition to the common free base, it also exists in the dihydrochloride and sulphate form. PPD typically exists as a white to light purple powder, which darkens after (air) oxidation into red, brown and eventually black colours. Therefore, it is commonly used as an intermediate in dyeing processes.

![Figure 1 Para-phenylenediamine, PPD.](image)

Common synonyms: 1,4-phenylenediamine, 1,4-diaminobenzene, 1, benzenediamine, para-aminoaniline, para-aminobenzene.

Sources
PPD has been used as an ingredient in hair dyes since the 19th century and still is among the most commonly used dye precursors in permanent hair dyeing, together with the structurally related dye precursor p-toluenediamine (PTD). More recently, its use became popular in temporary ‘black henna’ tattoos to accelerate the darkening process and to achieve a long-lasting effect. Much less frequent - and mainly of past interest - sources of PPD and structurally related compounds are printing inks, leather, (under)garments, fur, industrial rubber products and tissue stains for microscopy purposes.

Adverse effects
Scientific interest in PPD within the scope of these sources primarily focuses on its extreme skin sensitizing potential after topical exposure and consequently the possibility to elicit an allergic contact dermatitis. However, besides this delayed type of allergy also cases of immediate type allergy to PPD are reported, although extremely rare. Far more common, the extreme swelling of the face and scalp actually resulting from a severe allergic contact dermatitis to PPD is mistaken for angioedema. A few case reports on oral PPD ingestion - in India and East Africa - report severe systemic toxicity with angioneurotic oedema resulting in respiratory distress and rhabdomyolysis leading to acute renal failure. It is very unlikely that the high doses of PPD needed to achieve these systemic toxicity, can be obtained by ingestion of hair dye.
since an enormous amount of hair dye formulation should be ingested. Indeed, the majority of reported cases were based on accidental or intentional ingestion of solid PPD.\textsuperscript{13,14} Since certain aromatic amines formerly used in hair dyes were found to be carcinogenic,\textsuperscript{15} PPD has also been subject of investigation in - especially urothelial - carcinogenicity studies. Epidemiological studies investigating the association between hair dye use and bladder cancer are contradictory and hence, the debate on carcinogenicity is still ongoing.\textsuperscript{16} However, based on experimental toxicokinetic and mutagenetic data, the Scientific Committee on Consumer Safety (SCCS) concluded that the use of PPD containing hair dyes does not entail a carcinogenic risk to consumers.\textsuperscript{17}

**Metabolism: oxidation versus N-acetylation**

Although the first studies on the immunogenicity of PPD date from 1859,\textsuperscript{18,19} PPD is still a popular subject of investigation. This is explained by the fact that PPD is unstable when it comes into contact with skin or when it is present in aqueous solution, thereby leading to formation of a wide range of PPD intermediates, whose exact allergenicity still is not fully understood. However, in general it is assumed that PPD itself is not immunogenic, since it needs to be (auto)oxidized before it can be bound to protein; a pre-requisite for chemicals to stimulate the immune system. It is proposed that auto-oxidation of PPD results in the formation of a $\textit{p}$-benzoquinonediimine ($\textit{p}$-BQDI), which, in contrast to PPD itself is a protein-reactive intermediate. $\textit{P}$-BQDI is susceptible to sequential oxido-conjugation reactions, ultimately leading to the formation of the trimer Bandrowski’s base (BB). Experiments using exaggerated oxygenation, suggested that next to $\textit{p}$-BQDI and BB, other di- and trimeric structures\textsuperscript{20} or even pentamers can be formed.\textsuperscript{21} Moreover, oxidation of PPD also results in formation of reactive oxygen species (ROS) and it has been found that oxidative stress from ROS may play a significant role in the sensitization phase of contact allergy to PPD.\textsuperscript{22}

In addition to the non-enzymatic auto-oxidation, PPD is also known to be enzymatically converted in to monoacetyl-PPD (MAPPD) and diacetyl-PPD (DAPPD), by the $N$-acetyl transferase 1 (NAT1) enzyme which is abundantly present in epidermal keratinocytes.\textsuperscript{23} Importantly, in contrast to PPD which is considered an extremely potent skin sensitizer based on the Local Lymph Node Assay (LLNA),\textsuperscript{24} MAPPD and DAPPD were negative in the LLNA and are therefore considered non-sensitizing compounds.\textsuperscript{20} Thus, $N$-acetylation of PPD can be considered a detoxification pathway. In fact, patch tests with MAPPD and DAPPD in PPD-sensitized subjects were negative in (MAPPD) or very less capable of (DAPPD) eliciting allergic responses.\textsuperscript{25}
Allergic contact dermatitis

General

Allergic contact dermatitis is an inflammatory skin manifestation of exposure to an allergen to which an individual has been sensitized. The main symptoms are itch, erythema and oedema sometimes accompanied by papules, vesicles and bullae. In order to sensitize, allergens first need to penetrate the stratum corneum skin barrier to reach the viable epidermis. Penetration is dependent on several physicochemical factors, including molecular size, lipophilicity and polarity of the chemical. Furthermore, exogenous factors impairing the skin barrier such as exposure to detergents and irradiation, as well as endogenous factors like pre-existing inflammatory skin conditions and atopy, are, amongst others, known to affect the skin barrier.

Since most of the contact allergens are of low molecular weight - called haptens - they first have to be bound to resident proteins, in order to become ‘visual’ for the immune system. This process is called haptenation. The hapten-protein complex will be processed by the epidermal or dermal antigen presenting cells (APC) which then become activated. Upon activation, APCs migrate to regional lymph nodes where they may interact and activate allergen-specific naïve T cells. Clonally expanded progeny of these allergen-specific T cells, which then are turned into effector- and memory T cells, will recirculate and migrate into the periphery of the skin. At this stage, the individual is sensitized, i.e. capable of producing an allergic reaction on re-exposure to this particular antigen. This sensitization or induction phase takes at minimum 4 days to several weeks. On re-exposure to the initial antigen, the above mentioned process will recur, but now memory T cells are able to immediately release cytokines and chemokines, resulting in the clinical skin features of allergic contact dermatitis. This process evolves within 1 - 4 days and is called the elicitation, or effector phase.

PPD-specific penetration and haptenation

Studying the penetration and haptenation of PPD is challenging, because PPD is susceptible to oxidation, subsequent conjugation as well as N-acetylation. Moreover, the vast majority of data is derived from in vitro experiments, and / or animal skin or skin equivalents (reviewed in Chapter 2) and true in vivo evidence is lacking.

Epidemiological data

In the past, hair dyes were primarily used by woman of a certain age, to cover their grey hair. However, since the last decades an increasing number of men are dyeing their hair and people tend to start dying their hair at a younger age. In fact, in a recent descriptive study examining the demographics of all the children referred for patch testing in Denmark during 2003-2011, PPD was found to be among the five most common sensitizers in children (3.5% sensitization),
with the youngest being 4 years old. According to a large European multicentre study published in 2009, hair dye was still the most common cause of sensitization to PPD, with a weighted average of 41.8% caused by consumer hair dyeing. However, more and more data (reviewed by de Groot) suggest that PPD sensitization - especially in children and young adults - might be increasingly attributable to the application of temporary henna tattoos, containing widely varying, but generally very high concentrations of PPD. Prevalence figures of contact sensitization to PPD largely depend on the selected population, with in general the highest prevalence in subjects with confirmed hair cosmetic sensitization, followed by confirmed cosmetic dermatitis, unselected eczema patients and the general population. The most recent review on PPD sensitization among dermatitis patient which included studies from 1965 to 2005 showed that the estimated median prevalence of PPD sensitization was 4% in Europe, 4.3% in Asia and 6.2% in North America. In Europe, initial high prevalence rates were followed by a decrease in the 70s, with a relatively stable prevalence of 2-6% since. Within Europe, sensitivity rates appear to be lower in Scandinavian centres, presumably reflecting the predominance of blond individuals who tend to colour their hair with lighter shades and hence, lower amounts of PPD in the used hair dye formulations. Most prevalence data on PPD sensitization in the general population are estimates, based on extrapolation of patch test data in dermatitis patients. Prevalence rates based on both population-based epidemiological studies and estimations, ranged from 0-1.5%, although studies performed after 1997 showed a considerable lower prevalence of 0.3%. A more recent epidemiological study on the prevalence of PPD sensitization in the general population revealed a PPD prevalence of 0.8%.

**Susceptibility to sensitization: genes and exposure**

Although some allergens - like PPD - are considered extreme sensitizers, only a minority of the exposed individuals become sensitized. This suggests that besides exposure, genetic factors are involved. Furthermore, other factors such as ethnicity, age, high induction dose and pre-existing inflammatory skin conditions also influence the susceptibility to develop contact allergy. The activity of several xenobiotic metabolizing enzymes has been studied for their role in PPD sensitization. Brands et al. found that both the Ala(16)Val peptide polymorphism of the primary antioxidant enzyme Manganese Superoxide Dismutase (MnSOD) enzyme does not or at least not importantly contribute to an increased susceptibility of contact sensitization to PPD. On the other hand, a logistic regression analysis showed that the combined Tumor Necrosis Factor (TNF)-308 (A/G + A/A) genotype was associated with sensitization to PPD. Furthermore, a small study of 90 PPD sensitized cases suggested that an insertion/deletion polymorphism of the angiotensin-converting enzyme (ACE) gene was associated with an increased risk of allergic contact dermatitis to PPD. In addition, Blömeke et al. (2009) found a reduced susceptibility to PPD sensitization for the rapid acetylator genotype of the N-acetyl transferase 1 (NAT1) enzyme whereas Westphal et al. (2000) found no association between
this genotype and contact sensitization to para-substituted aryl amines.\textsuperscript{44,46} As briefly described above, the formation of reactive oxygen species (ROS) following PPD skin application\textsuperscript{22} may exert an important role in the sensitization phase of allergic contact dermatitis to PPD.\textsuperscript{47} It is known that Glutathione S-transferases (GSTs) - a family of detoxification enzymes - play an important role in detoxification, by reducing the harmful effects of ROS. Disruption of the murine genes for cytosolic GSTs has demonstrated that their presence is protective against various agents and factors.\textsuperscript{48} Furthermore, their upregulation induced by oxidative stress suggests that they are part of an adaptive mechanism. Thus, absence of GSTs through gene deletions or a single nucleotide polymorphism (SNP) resulting in absence, enhanced or reduced protein, influences the detoxification of compounds and elimination of ROS-related secondary metabolites.

**Elicitation response; diagnostic versus daily-life exposure**
The elicitation response in sensitized subjects is only partly understood and depends on several factors. Commonly examined factors influencing the elicitation response are application time and (induction) dose, frequency of exposure, anatomical region and vehicle.\textsuperscript{49} For diagnosing sensitization to PPD, the gold standard is a patch test with either 1\% PPD in petrolatum or 90 μg PPD cm\textsuperscript{-2} in polyvidone (TRUE test\textsuperscript{®})\textsuperscript{50} applied for 48 hours. The elicitation response is assessed 72 h and 1 week after application. However, typically daily life exposure to PPD as in permanent hair dyeing differs from this diagnostic patch test situation in both the exposure time and the vehicle, as well as the anatomical region. To assess the elicitation response to PPD under real-life exposure conditions, Goebel\textit{et al.} (2010) performed a study which compared the elicitation response in PPD sensitized subjects under both diagnostic and typical hair dye use conditions, i.e. 48 hours versus 30 minutes.\textsuperscript{51} Furthermore, it was examined to which extent the actual dose becoming available for elicitation was influenced by these two exposure scenarios. By applying a typical hair dye product (with 2\% PPD in an oxidative hair dye formulation) during 30 minutes, it was shown that from 38 PPD allergic subjects, all 20 individuals diagnostically graded ++ or +++ according to the guidelines of the International Contact Dermatitis Research Group (ICDRG) classification, responded with a positive elicitation response. However, from the 18 subjects originally graded + in the diagnostic patch test only 8 subjects elicited a positive reaction, whereas 4 reacted with a doubtful response and 6 individuals did not react after the 30 minutes application of the typical hair dye formulation. Interestingly, a study using \textit{ex vivo} pig skin showed that the actual dose becoming available for elicitation under simulated hair dye exposure conditions was more than an order of magnitude lower than under patch diagnostic patch test conditions.\textsuperscript{51}
**Hair dye molecules**

Hair dyes are commonly classified according to their colour fastness. With increasing fastness, these categories are temporary, semi-permanent and permanent hair dyes. The latter, comprising 80% of the hair dye market share in the European Union[39] belong to the so-called oxidative hair dyes. A typical oxidative hair dye contains a precursor, such as PPD or PTD, and couplers (e.g., resorcinol or m-aminophenol) which are mixed with hydrogen peroxide under alkaline conditions. The alkalizing agent, usually ammonia or ammonia substitutes, serves to open up the hair cuticle thereby allowing penetration of hair dye precursors, lowers the internal pH for optimal dye chemistry and catalyses bleaching of melanin. Hydrogen peroxide on the other hand removes the resident hair pigment melanin and all other artificial colours. Simultaneously, hydrogen peroxide promotes the oxidation of precursors that have penetrated the hair shaft which then are coupled to the coupler molecules. This will result in formation of colour molecules that are too large to diffuse out of the hair, thereby providing a permanent hair dye which will last until the hair grows out or is shedded. Various combinations of precursors and couplers provide a spectrum of reaction products and as such, a spectrum of hair shades.[52]

PPD and PTD are the most commonly used precursors in oxidative hair dyeing[53] and from all other chemicals in hair dyes, they are considered the most important allergens associated with hair dye-related allergic contact dermatitis.[54] Since the cross-reactivity between these two substances is high - meaning that being sensitized to one of these chemicals also gives a high chance of a positive reaction in the patch test to the other substance - usage of hair dye products containing PTD poses a risk for PPD-allergic individuals.

The industry had extensively searched for possible alternative oxidative hair dye molecules. Recently a new hair dye molecule, 2-methoxymethyl-p-phenylenediamine (ME-PPD), has been developed. This PPD derivative was shown not only to have good hair colouring properties, but also had considerably reduced skin sensitizing properties.[55] Whereas the EC3 value in the LLNA is established at 0.1% for PPD and 0.17% for PTD, ME-PPD had an EC3 value of 4.3% which indicates a moderate skin sensitizing potency. In addition, a quantitative risk assessment investigating the skin sensitizing potency under consumer hair dye usage conditions also indicated a much lower risk of induction of skin sensitization by ME-PPD compared to PPD or PTD. However, this sensitizing potency of a chemical is probably not the only determinant affecting cross-reactivity and elicitation responses, since it was shown that another PPD derivative, hydroxyethyl-p-phenylenediamine sulphate (HE-PPD), which is considered a strong sensitizer as well in the LLNA (EC3 value of 0.57%),[56] has less potential for cross-reactivity compared to PPD and PTD.[57]
Aim and outline of the thesis

PPD has been recognised as an extreme sensitizer since the late 19th century and is still a highly appreciated molecule in scientific literature. Despite all the acquired knowledge on PPD over the years, the exact pathways leading to sensitization and elicitation responses are not yet completely elucidated. Therefore, this thesis aims to get more insight into certain aspects required for sensitization; (in vivo human) skin penetration and elicitation (chapter 2 and 3) and a subject’s susceptibility to become sensitized to PPD (chapter 4 and 5). Furthermore, by examining elicitation responses to PPD under daily life conditions, i.e. hair dyeing, we aimed to gain better understanding of the factors dosage and exposure time, determining the elicitation response (chapter 6). In addition, to make a translation to daily practice in which patients express the desire to continue to dye their hair, a newly developed hair dye molecule was studied for its cross-reactivity in subjects sensitized to PPD (chapter 7).

It is commonly accepted that penetration and the ability to bind to proteins, i.e. to ‘haptenate’ proteins, are the first two hurdles that an allergen has to overcome to be able to sensitize. Since PPD is a molecule with a structure which is altered on skin contact or in solution, studying its penetration (pathway) both qualitatively and quantitatively is a challenging task. Subsequently, this challenge also applies to examining the protein binding of PPD and its derivatives. With the review in chapter 2 we aimed to present a clear overview of literature on PPD penetration through skin (analogues) and studies on the amino acids that are targeted by PPD. To complete this overview, inextricably linked auto-oxidation and N-acetylation steps involved in PPD metabolism are described. Despite the experimental knowledge on PPD and on formation of its oxidation and acetylation products, a clear in vivo picture on where and when these products are formed is lacking. To study the real-time penetration of a topically applied drug in vivo, non-invasive Confocal Raman Microspectroscopy (CRM) is the method of choice. In chapter 3 we used this CRM to investigate the in vivo penetration characteristics of topically applied PPD in humans as a function of depth in the skin and as a function of time. In addition, we examined if PPD-oxidation or -acetylation products could be detected in vivo, after topical application of PPD. The human body is equipped with xenobiotic metabolizing (XME) enzymes, such as the phase II N-acetyl transferase (NAT) and Glutathion S-tranferase (GST) enzymes which exert a detoxifying role by contributing to the elimination. Activity for GST has been found in the soluble cell fraction of intact human skin and human keratinocytes and therefore, the human skin is equipped with a strong protective system against oxidative stress. However, genes for human cytosolic GSTs display polymorphisms which are likely to contribute to interindividual differences in responses to xenobiotics. In chapter 4 we examined the role of GST polymorphisms in sensitization to PPD and other xenobiotics, by performing an
association study as well as a meta-analysis. In chapter 5 important differences in mode of action of the glutathione S-transferase enzymes are addressed. In chapter 6 we analysed the elicitation response in a highly selected, well described subset of individuals that had a weak response to a diagnostic PPD patch test, but who failed to respond to a previous conducted hair dye model test. By using varying exposure conditions, with increasing exposure times and hence, increasing dose, we aimed to get detailed insight into the dose-dependency of the elicitation responses of these subjects. In chapter 7 we evaluated whether the new hair dye molecule ME-PPD shows cross-elicitation responses in PPD-allergic individuals with a documented history of hair dye-related allergy and different diagnostic patch test response grades.
References


57. Frosch PJ, Kugler K, Geier J. Patch testing with hydroxyethyl-p-phenylenediamine sulfate - cross-reactivity with p-phenylenediamine. Contact Dermatitis 2011; 65: 96-100.

