CHAPTER

General discussion and future perspectives

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The immunogenic potential of \( p \)-phenylenediamine (PPD) has been recognized since the 19th century \(^1,2\) and PPD is still an important allergen in scientific literature. Based on the local lymph node assay (LLNA) PPD is regarded as an extreme sensitizer \(^3\) with the potency to sensitize 100\% \((n = 24)\) of the humans exposed to 10\% PPD, as shown by the historical human maximization test published in 1966 by Kligman.\(^4\) Currently, this extreme sensitizing potency of PPD is clearly reflected when individuals - often children and adolescents - are exposed to PPD containing temporary ‘black henna’ tattoos. In contrast to natural red henna \( (Lawsonia inermis)\) which is considered relatively safe and rarely sensitizing,\(^5\) ‘black henna’ is an artificial mixture of red henna and PPD, although some mixtures do not even contain the red henna component.\(^6,7\) In my opinion these tattoos can be considered as a ‘modern’, although unintentional, human maximization test, because some of the ‘black henna’ mixtures have been shown to contain even higher percentages of PPD \(^8\) than used in the - nowadays considered unethical - human maximization test. It is therefore highly understandable that the application of PPD in temporary henna tattoos is prohibited in Europe and the United States.\(^9,10\) In fact, in Europe PPD is currently only allowed to be used in hair dyes, with an upper concentration limit of 4\% \((or 2\% as final on-head concentration after being mixed to the oxidizing agent)\).\(^11\) Although hair dyeing is still considered the most common cause of PPD sensitization,\(^12\) other sources including the temporary ‘black henna’ tattoo as well the diagnostic patch test are increasingly reported.\(^5,13\) Often, the source of exposure is unknown; a large study \((N = 3307)\) showed that in about 44\% of the subjects diagnosed to be sensitized to PPD, the probable source of exposure could not be identified.\(^14\) In addition, a positive patch test to PPD might also be due to cross-reactivity. Commonly reported cross-reacting allergens are structurally related chemicals such as the hair dye precursor \( p \)-toluenediamine (PTD), the sunscreen component para-aminobenzoic acid (PABA), other azo dyes frequently used in textiles \((Disperse Orange 3, Disperse Yellow 3, Disperse Blue)\), local anaesthetics such as benzocaine and black rubber \( N \)-isopropyl-N’-phenyl-\( p \)-phenylenediamine; IPPD).\(^15,19\) In fact, a recent study by Thomas \(et al\). (2014) suggested that the rate of cross-reactivity increases with increasing strength of the PPD patch test.\(^20\) This should be taken into account when studying subjects which are strongly sensitized to PPD, especially in the absence of a clear history of exposure.

The discussion on the extent of active sensitization to PPD by the diagnostic patch test is unresolved, with widely varying figures and opinions in the literature.\(^13,21-23\) Late reactions to a patch test, i.e. \textit{de novo} positive reactions after day 7-10 or later \(^24\) might indicate active sensitization, although late reactions may also be inherent to the chemical (e.g. for
corticosteroids or metals)\textsuperscript{25,26} or reflect a subject’s low responsiveness.\textsuperscript{27} A large prospective multcentre study performed by the German Contact Dermatitis Research Group (Deutsche Kontaktallergie-Gruppe; DKG) which found that PPD elicited late reactions in 1.5\% of routine patch tests, considered this indicative of an actual risk of active sensitization and therefore the DKG decided to withdraw PPD from the baseline series in Germany in 2005.\textsuperscript{13} The European Society of Contact Dermatitis (ESCD) did not endorse this opinion.\textsuperscript{28} In the context of active sensitization, Geier et al. (2013) assessed whether lowering the patch test concentration to 0.3\% PPD pet. could be a safer alternative with still sufficient sensitivity,\textsuperscript{29} since a study by the DKG already showed that lowering the patch test concentrations to 0.5\%, 0.4\% and 0.35\% PPD pet. decreased the risk of active sensitization. Although 26\% (32/123) of the subjects with a positive reaction to 1\% PPD pet. did not elicit a positive reaction to the 0.3\% PPD pet. patch test, the majority (69\%) of these ‘missed cases’ were not clinically relevant. Furthermore, with good quantitative parameters of diagnostic selectivity, i.e. the Reaction Index and Positivity Ratio,\textsuperscript{30,31} the authors recommended replacement of the 1\% PPD pet. by the 0.3\% PPD pet. In addition, Sested et al. (2006) showed that in a small population (N = 15) of subjects sensitized to PPD, 1/15 (6.7\%) reacted to a 48 hours patch test containing 0.0038\% PPD in petrolatum, whereas 0.05\% PPD in petrolatum increased this rate to 73\% and a further increase in PPD concentration to 0.5\%, resulted in 87\% of positive elicitation response.\textsuperscript{32} The high response rate to a PPD concentration of as low as 0.05\% seems surprising, but this might be explained by the fact that the strength of the previous diagnostic patch test was not reported (except for one subject graded 2+ previously), and that this group may have consisted of strongly sensitized subjects for whom the elicitation threshold is assumed to be lower than for the weakly sensitized subjects.\textsuperscript{33}

Data from studies presented in this thesis contribute to this discussion of active sensitization by patch testing with 1\% PPD in petrolatum. In chapter 3 the human \textit{in vivo} penetration study learned us that after a short application of only 30 minutes of a 1\% PPD in petrolatum patch, already significant amounts of PPD (90 mg PPD/g keratin) can be found on the skin’s surface (0 micron depth). This actually corresponds to a level of 9 mass-\% PPD of dry skin. Applying a 1\% PPD petrolatum patch for 2 hours or even 3 hours (with refreshment of the patch every hour) resulted in PPD levels of 150 mg PPD/g keratin and 330 mg PPD/g keratin respectively, measured directly after removal of the patch. Furthermore it was shown that within the time frame of 27 h, approximately 330 mg PPD/g keratin had penetrated from the skin surface into the living epidermis and almost no PPD was left (17.2 mg PPD/g keratin compared to the detection limit of 13 mg PPD/g keratin). In addition, a 23 h patch demonstrated that directly after removal of the patch, the amount of PPD in the skin approached the amount found after the 30 min application (i.e. 90 mg PPD/g keratin), implying that within the time span of 23 h, the PPD patch, or at least its layer closest to the skin surface, is depleted of PPD.
This calls into question the duration of the application of the diagnostic patch test (i.e. 48 hours), in addition to the previously discussed concentration of PPD. However, we acknowledge the fact that other factors such as practicability and standardization of application time may be much more decisive in the patch test procedure than single chemical depletion time. The high level of PPD penetration after application of 1% PPD in a petrolatum formulation (such as used for patch testing) was also shown in ex vivo experiments, as previously published by our group.34 This study used pig skin in flow-through diffusion cells, to study the actual exposure levels of PPD, expressed as the Measured Exposure Level (MEL) which was calculated as the sum of PPD associated with the skin (dermis and epidermis including stratum corneum) and the receptor fluid. Interestingly, it was shown that under diagnostic patch test conditions (48 h application of a 1% PPD in petrolatum patch) the MEL is more than 10-fold higher than the MEL for hair dyeing conditions (2% PPD in hair dye formulation applied for 30 min with rinsing). When considering the applied doses for both conditions, i.e. 3000 µg PPD cm\(^{-2}\) for the hair dye condition versus 400 µg PPD cm\(^{-2}\) for the patch test condition,34 the fundamental difference between the dose applied and the dose actual becoming available for elicitation (the MEL) becomes even more evident. As demonstrated in chapter 6 of this thesis, in which available MEL data were examined against human elicitation responses in relation to applied dose and exposure time, we confirm that the MEL is a useful tool to better characterize thresholds of elicitation than the applied dose alone. In this context, it should be noted that the PPD TRUE test\(^{®}\) has an estimated MEL of almost a fifth of the MEL estimated for the 1% PPD in petrolatum patch test (45 µg PPD cm\(^{-2}\) compared to 205 µg PPD cm\(^{-2}\)).

As described for PPD in chapter 2, studying penetration and haptenation is intriguing since PPD is unstable, leaving several intermediates with its own (often only partly- or unknown) penetration and haptenation characteristics. Complex auto-oxidation processes have also been described for other compounds such as the fragrances limonene, citronellol, linalool and geraniol.35-38 Rüdback et al. recently described that not the parent compound citronellol, but its primary auto-oxidation products, the hydroperoxides, are the main sensitizers.36 Similar to PPD, citronellol is not directly protein-reactive and therefore it has a very low sensitizing potency itself. As for PPD, auto-oxidation of citronellol renders the more sensitizing derivatives and hence, both chemicals are classified as pre-haptens.36,39 However, when comparing the degradation processes of the parent compounds into the auto-oxidation derivatives, a striking difference is found in the rate of auto-oxidation. PPD has been shown to be degraded to 50% of its initial concentration after 8 hours of air oxidation,40 whereas as much as 10 weeks of air oxidation were needed to degrade 16% of the citronellol into its oxidation products.36 This example emphasizes the instability of PPD and therefore the complexity of studying its immunogenic characteristics. Moreover, the fact that in daily life PPD is used in a hair dye
formulation containing other chemicals such as couplers and an oxidizing agent makes it even more challenging. Nevertheless, by reviewing papers published on penetration of PPD under different conditions (i.e. varying formulations, species and applied doses) we have shown that despite these differences, PPD penetration values after a short exposure time of around 30 minutes are quite similar.\textsuperscript{41}

The review further shows that the major proportion (>80\%) of this low amount of PPD that penetrates the skin, will be acetylated to monoacetyl-PPD (MAPPD) and diacetyl-PPD (DAPPD) by the enzyme \textit{N}-acetyltransferase 1 (NAT1). This results in an even smaller proportion (i.e. <20\% of the already low amount of PPD that penetrates the skin) available for auto-oxidation and subsequent haptenation, yielding hapten-protein adducts that may induce sensitization. Whereas \textit{in vitro} assays are able to predict the haptenation potential of oxidized PPD derivatives, \textit{in vivo} correlation is still lacking since the exact fragments that are presented to the naïve T cells are unknown.\textsuperscript{42} Furthermore, there may be many unknown (oxidative) PPD derivatives, the impact of which is unpredictable. This warrants further investigation as currently attempted by combining data from \textit{in vitro}, \textit{in silico} as well as \textit{in chemo} assays.\textsuperscript{33}

In the \textit{in vivo} penetration study presented in \textbf{chapter 3} we demonstrated that in contrast to PPD, the acetylated PPD derivatives monoacetyl-PPD (MAPPD) and diacetyl-PPD (DAPPD) and the oxidation product Bandrowski’s base (BB) could not be identified within our experimental design. This is most probably caused by dilution, which occurs when these hydrophilic compounds enter the epidermis which has a relatively larger size and lower resistance to diffusion compared to the stratum corneum.\textsuperscript{44,45}

The experiments were performed within the existing safety margins, such as used in the diagnostic patch test procedure, but considering the high amount of PPD that penetrates the human skin after only a short application of the 1\% PPD in petrolatum patch, we would recommend using skin equivalents for future investigations in order to prevent possible sensitization.

One of our assumptions in this study was that - based on experimental data published in literature\textsuperscript{46} - \textit{N}-acetylation by NAT1 enzymes occurred in the viable epidermis, probably in particular in the basal cell layer.\textsuperscript{47} It was therefore, that additional experiments with prolonged measurements were performed at a fixed depth of 30 micron, which has been shown to represent the viable epidermis at the volar forearm.\textsuperscript{48} Interestingly, preliminary data from our group studying the localization of NAT1 in normal human skin suggests that this assumption should be revised, as NAT1 was shown to be abundantly present in the stratum corneum, in addition its perinuclear presence in the basal keratinocytes. (unpublished data)

By conducting an analysis of association as well as a meta-analysis we have shown in \textbf{chapter 4} that common polymorphisms in genes encoding the detoxifying phase II enzymes glutathione-
S-transferase (GST) do not play a major role in sensitization to PPD and other xenobiotics. In fact, even when considering the combined ‘high risk’ genotype GSTT1*0/GSTM1*0, to study a possible synergistic effect, no significant association with sensitization to PPD was found. This pointed to the conclusion that polymorphisms in GST genes have disease-modifying, rather than disease-causing effect and that they only have a weak biologic impact. As described above, the identification of susceptibility loci in complex, multifactorial diseases such as allergic contact dermatitis is demanding, in view of the relatively low frequency in the population, the presence of genes with minor effects and ethnic differences. This has also been addressed in chapter 5, in our response to a comment of Westphal et al. in which we explain the differences found in the meta-analysis by the fact that GST have different modes of action and that detoxification of the studied xenobiotics cannot be solely attributed to the examined GST genes. Consequently, as each individual has many single nucleotide polymorphisms (SNPs) that together create a unique DNA sequence, SNP-based genetic linkage analysis can be used to map disease loci, and hence determine disease susceptibility genes. The advances in SNP genotyping array technologies have made whole-genome association studies (WGAS) within reach. A single nucleotide polymorphism array is now a useful tool to study the whole genome for candidate genes. It is therefore, that we have established a consortium, comprising members of the European Society of Contact Dermatitis (ESCD), who will collect blood from subjects with a relevant sensitization to PPD or the closely related PTD together with a short questionnaire to obtain information regarding exposure. The participating countries are Germany (Heidelberg, Jena, Trier), the Netherlands (Amsterdam, Groningen, Nijmegen), Portugal (Coimbra), Singapore, Spain (Barcelona) and Sweden (Malmö). Blood samples of a specific control group, consisting of a large sample of the general population in which exposure and PPD patch test data have been established carefully, will be used as controls. By means of this network, we will be able to collect a large enough case series (at least N = 2000) to draw reliable conclusions, based on the genetic variations found. The participating group is experienced and has done preliminary analytical work. In addition, a European network will contribute to reduce the fragmentation in research investments.

The elicitation response to PPD has previously been shown to be dose and exposure-time related. This is not unique to PPD, as dose-response relationships in the elicitation phase also have been demonstrated for other compounds including nickel, methylisothiazolinone, isogeugenol and many others. However, the distinction made between the dose applied and the dose actually becoming available for elicitation, and the connection made to the elicitation response is unique. In chapter 6 it was shown that the elicitation dose needed to elicit allergic reactions in truly, but weakly PPD sensitized individuals, is between the dose becoming available by diagnostic patch testing and the dose becoming available by our model hair dye test exposure and that this dose...
was different for each individual. Importantly, these differences in MEL only seemed to be of importance in patients who belong to the lower end of the patch test reactivity spectrum, given the fact that all previously tested ++ and +++ responders (in diagnostic patch testing), reacted to the 2% PPD hair dye test model. Thus, for this particular group of subjects, the + diagnostic PPD patch test seemed not to have a direct relevance for reactivity to a PPD concentration of 2% under simulated hair dye conditions. In these cases, the PPD diagnostic patch test only indicates sensitization and does not predict whether a contact allergic reaction will occur during daily life PPD exposure, i.e. hair dyeing. However, since only a limited number of subjects were available for this study, a larger number of subjects is needed to substantiate the results found thus far.

The importance of the available dose (MEL) in evaluating contact allergic responses, regarding both the elicitation and the sensitization phase, has been reaffirmed in a report on quantitative risk assessment (QRA).\textsuperscript{61} It was shown that for PPD the MEL under in use conditions is in close proximity to the no-expected-sensitization-induction-level (NESIL), indicating a high risk of induction of sensitization when used up to its maximal use concentration of 2%. This is in concordance with animal data (LLNA), which categorizes PPD in the range of extreme sensitizers. For resorcinol, another common ingredient in oxidative hair dyes, the MEL was shown to be much lower that the NESIL. This points to a negligible risk of induction of contact sensitization and confirms LLNA data categorizing resorcinol as only a moderate skin sensitizer.

In line with the study described in chapter 6, chapter 7 describes the investigation of elicitation responses to a different hair dye molecule under use conditions in PPD allergic individuals. The hair dye used is based on a new, recently developed molecule, in which a methoxymethyl side chain was added to PPD, thereby forming methoxymethyl-PPD (ME-PPD).

ME-PPD was developed since the most commonly used hair dye precursors PPD and PTD are the main allergens associated with hair dye related allergic contact dermatitis\textsuperscript{62} and the demand for safe, permanent hair dye alternatives is high (author’s experience from daily practice). It is a challenge to develop a new hair dye precursor, because good hair colouring performances often come together with a strong sensitizing potency, owing to the characteristic low molecular weight, the ability to penetrate the hair shaft and follicle and the strong protein reactivity. In the past, hydroxyethyl-\textit{p}-phenylenediamine sulphate (HE-PPD), which has good colouring characteristics, had been shown to have less potential for cross-reactivity compared to for example PTD in PPD sensitized subjects, but turned out to be a strong sensitizer according to the LLNA (EC3 value 0.57%; SCCS opinion on HE-PPD).\textsuperscript{63} The recently developed ME-PPD holds the combination of a good hair colouring performance together with a moderate skin sensitizing potency (EC3 value LLNA 4.3%, compared to 0.1% and 0.17% for PPD and PTD) and could be promising for consumers who have not been
sensitized or exposed to the common dye precursors PPD and PTD. However, because a ME-PPD containing hair dye can still elicit reactions in at least 30% of the PPD-sensitized subjects, these individuals must be discouraged from using this hair dye.

**Concluding remarks**
People want to dye their hair. This carries some risk of becoming allergic. By gaining insight into the dose-response relationship of the ‘classic’ hair dye precursor PPD we hope to have contributed to the process of risk management of hair dyes.
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