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# Oxidized p-Phenylenediamine in Contact Allergy

## Clinical and Experimental Studies

EWA YOUNG | DEPARTMENT OF OCCUPATIONAL AND ENVIRONMENTAL DERMATOLOGY  
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## Oxidized p-Phenylenediamine in Contact Allergy



# Oxidized p-Phenylenediamine in Contact Allergy

Clinical and Experimental Studies

Ewa Young



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DOCTORAL DISSERTATION

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To be defended at Lilla Aulan, Medicinskt Forskningscentrum,

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on Friday 7th September at 13:15.

*Faculty opponent*

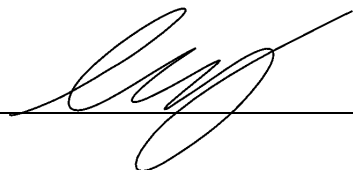
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<b>Abstract</b>  <p>The potent allergen p-phenylenediamine (PPD) is a common color substance in oxidizing permanent hair dyes. The complex hair dye cocktail has many ingredients. When the hair dye is used, oxidative agents are added and PPD can be transformed into new substances. These substances are partly unknown. Contact allergy to hair dyes is prevalent in hairdressers and their consumers. Contact allergy is diagnosed by patch test. The recommended routine patch test concentration for PPD has been 1.0% petrolatum since it was included in baseline set-up of patch test allergens. For over a decade, it has been discussed whether it is safe to routinely patch test PPD in this concentration due to the risk of patch test sensitization. Late-appearing patch test reactions may reflect patch test sensitization, but they may also be due to a low degree of pre-existing reactivity. Disposable gloves are used by hairdressers to protect the skin from occupational exposure to hair dye allergens. Not all glove materials protect against PPD and related chemicals.</p> <p>The general aim of this thesis was to study contact allergy to PPD, with focus on oxidized PPD. Studies I–III were performed in volunteers with previous reactions to PPD 1.0%. In study IV hairdressers' gloves exposed to a mixture of permanent hair dye were investigated regarding permeation of PPD and other hair dye allergens.</p> <p>The work presented in this thesis shows the presence of several PPD-associated allergens that may be formed in a permanent hair dye, or through other processes in which PPD is oxidized. Furthermore, the results show that 4-nitroaniline and 4,4'-azodianiline, formed during oxidation of PPD, are potent allergens that a substantial proportion of PPD-sensitized patients react to. When patch testing with PPD, we could see a clear risk of missing contact allergy when the dose was reduced. We found no proof of late-appearing reactions being caused by the allergen as such or the dose. Finally, results of this work show that nitrile gloves should be recommended for hairdressers when dyeing hair.</p>		
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# Oxidized p-Phenylenediamine in Contact Allergy

Clinical and Experimental Studies

Ewa Young



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*To my patients*

Je suis de ceux qui pensent que la science a une grande beauté. Un savant dans son laboratoire n'est pas seulement un technicien : c'est aussi un enfant placé en face de phénomènes naturels qui l'impressionnent comme un conte de fées. Tout progrès scientifique ne se réduit pas à des mécanismes, des machines, des engrenages, qui d'ailleurs ont leur beauté propre.

Marie Curie, 1933


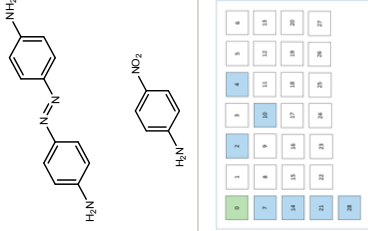

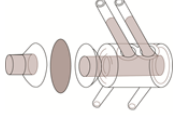


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# Thesis at a glance

Paper	Objective	Method	Illustration	Main findings/conclusions
I. Allergic contact dermatitis to p-phenylenediamine and some of its reaction products.	To investigate contact allergic responses to oxidized p-phenylenediamine (PPD) and some of its reaction products in PPD-positive volunteers.	PPD-positive volunteers were patch tested with dilution series of PPD and Bandrowski's base (BB), and also with thin-layer chromatography (TLC) strips of oxidized PPD.		Reactions to BB and to various parts of the TLC strips indicated the presence of several possible allergens formed during oxidation of PPD, and that PPD-sensitized individuals might react to these substances in various patterns.
II. Two sensitizing oxidation products of p-phenylenediamine patch tested in patients allergic to p-phenylenediamine.	To identify potential PPD allergen(s) possibly formed by oxidation as indicated in paper I and to patch test this/these substance(s) in PPD-positive volunteers.	A part of the TLC strip used in paper I was analyzed chemically, with two possible allergens being found: 4-nitroaniline and 4,4'-azodianiline. These two substances were patch tested in PPD-positive volunteers.		4-nitroaniline and 4,4'-azodianiline, formed during oxidation of PPD, are potent allergens. PPD-sensitized patients react to a high degree to concentrations of 4-nitroaniline and 4,4'-azodianiline that are equimolar to that of PPD.
III. 28-days follow-up of patch-test reactions to p-phenylenediamine and p-phenylenediamine dihydrochloride: a multicenter study on behalf of EECDRG.	To follow the positive patch test reactions to PPD and its salt PPD-dihydrochloride (PPD-DHC) in individuals already sensitized to PPD.	Patch test with PPD and PPD-DHC in equimolar dilution series. This was followed by observation of the reactions on 7 occasions over 28 days.		No late-appearing reactions were registered. We could clearly see a risk of missing contact allergy when the dose was decreased.
IV. Permeation of p-phenylenediamine and resorcinol in disposable hairdresser gloves exposed to a mixture of permanent hair dye and developer.	To investigate the presence of and glove permeation of PPD, BB, 4-nitroaniline, 4,4'-azodianiline and resorcinol during exposure to a mixture of permanent hair dye and developer.	Material from nitrile, natural rubber latex and polyvinylchloride (PVC) gloves was exposed to black oxidative hair dye containing PPD and resorcinol, and studied using the Franz permeation cell.		Nitrile gloves give good protection against PPD. PVC and natural rubber latex gloves are not suitable as protection against the hair dye allergens PPD and resorcinol. In experiments with PVC and natural rubber latex gloves, 4-nitroaniline was detected in the receptor fluid, but not BB or 4,4'-azodianiline.

## List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. **Allergic contact dermatitis to p-phenylenediamine and some of its reaction products.** Young E, Zimerson E, Svedman C, Bruze M. Jacobs Journal of Experimental Dermatology 2015 1(4):021
- II. **Two sensitizing oxidation products of p-phenylenediamine patch tested in patients allergic to p-phenylenediamine.** Young E, Zimerson E, Bruze M, Svedman C. Contact Dermatitis 2015 74:76-82
- III. **28-days follow-up of patch-test reactions to p-phenylenediamine and p-phenylenediamine dihydrochloride: a multicenter study on behalf of EECDRG.** Young E, Andersen KE, Bruze M, Gimenez-Arnau A, Ross-Hansen K, Johansen JD, Madsen JT, Zimerson E, Svedman C. In manuscript.
- IV. **Permeation of p-phenylenediamine and resorcinol in disposable hairdresser gloves exposed to a mixture of permanent hair dye and developer.** Young E, Dahlin J, Zimerson E, Bruze M, Svedman C. In manuscript.

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# Abbreviations

BB	Bandrowski's base
Da	Dalton
D	day
DHC	dihydrochloride
DKG	German Contact Dermatitis Research Group (Deutsche Kontaktallergie-Gruppe)
DO	disperse orange
EECDRG	European Environmental and Contact Dermatitis Research Group
FL	fluorescence spectrometric detection
HPLC	high-performance liquid chromatography
GCMS	gas chromatography mass spectrometry
GPMT	guinea pig maximization test
ICDRG	International Contact Dermatitis Research Group
IVDK	German Information Network of Departments of Dermatology (Informationsverbund Dermatologischer Kliniken)
4,4'-MDA	diphenylmethane-4,4'-diamine
ME-PPD	2-methoxymethyl-p-phenylenediamine
MHC	major histocompatibility complex
MW	molecular weight
p-	para-
pet.	petrolatum
PPD	p-phenylenediamine
PVC	polyvinylchloride
T cell	T lymphocyte, thymus cell
TDA	2,5-toluenediamine
TLC	thin-layer chromatography
UV	ultraviolet
v	volume
w	weight



# Introduction

This thesis is about contact allergy to p-phenylenediamine (PPD) and some of its oxidation products. The work is based on four clinical and experimental studies. PPD is a small molecule and the substance is colorless. It may change into colored substances if oxidized. PPD is therefore a common ingredient of permanent hair dyes. Contact allergy is a pathological reaction involving the skin. Contact allergy to PPD affects both hairdressers and users of hair dyes. The diagnosis and prevention of contact allergy and contact allergic dermatitis is covered in occupational and environmental dermatology. Thus, the Introduction starts with a brief description of the field of environmental and occupational dermatology. This is followed by descriptions of the skin barrier and of the immunological mechanisms in contact allergy. The concept of oxidization is also presented. The method of diagnosing contact allergy is described, as are the challenges in prevention of hand dermatitis. This is followed by a section dealing with PPD, both dermatologically and chemically. Finally, the studies are presented and discussed. The original articles are reproduced at the end of the book.

## Occupational and environmental dermatology

Occupational and environmental dermatology is a field of dermatology. It deals with diagnosis and prevention of pathological skin reactions from contact with chemical substances such as PPD or physical factors in our environment. The substances that cause skin disease may be present in our everyday or work environment. Sometimes exposure to the same substance occurs both in the workplace and at home (1). The work in the field of occupational and environmental dermatology requires the cooperation of several professions. Competence in chemistry, toxicology, social insurance, occupational psychology, vocational guidance, allergy, and dermatology is needed (2). In addition, experience in performing the diagnostic testing and evaluation of exposure is essential.

The most frequent occupational skin disease is dermatitis, synonymous to eczema (3). Hereon the term dermatitis will be used. Dermatitis is a group of diseases with inflammation of the skin. Characteristically, they present with erythema, pruritus,

and a rash. Dermatitis may be due to exogenous factors or endogenous factors, and there may be interplay between the two in one individual.

Contact dermatitis is exogenous. It is caused either by physical factors, such as mechanical rubbing, or by substances. Contact dermatitis caused by substances can be either irritant or allergic. Irritant contact dermatitis is non-specifically provoked by the exposure of skin to substances and/or physical factors that damage the skin. The irritants activate unspecific innate immune responses (1). Substances may be non-toxic in low doses, but a higher exposure may cause irritation. Even a substance one might consider harmless, such as water, will cause irritation of the skin upon extensive contact. This can happen due to excessive hand washing or prolonged work under wet conditions. Other common irritants are soaps and detergents.

Contact allergy may arise in an individual from exposure to an allergen, also called a sensitizer. An allergen is a substance that may cause a specific memory of the substance in our immune system. If the contact allergic individual is again exposed to the same substance, a contact allergic dermatitis may arise as a result of immune recognition of the substance. Both the innate and the adaptable immune mechanisms cause the inflammatory reaction in allergic contact dermatitis (1, 4). Based on the diagnosis of contact allergy to a particular substance and the evaluation of skin exposure, preventive measures can be taken. This thesis is about contact allergy to PPD and prevention of contact allergic dermatitis caused by PPD. When working with contact allergic patients, it is important to know that in contact dermatitis both etiologies, allergic and irritant, are often present in the one individual (1).

Environment- and work-associated diseases other than those of the skin are covered in occupational and environmental medicine. Bernardino Ramazzini (1633-1714) is recognized as being the father of both occupational medicine and occupational dermatology. His book *De Morbis Artificum Diatriba*, translated as *Treatise of the Diseases of Tradesmen*, appeared in 1700 in Modena (5).

## Human skin – an interface with our surrounding environment

In order to study contact allergic reactions, it is important to know about the structure and function of the skin. The human skin has many functions that are vital to our survival, and is a large organ with three major layers. The subcutis is the bottom layer, the dermis is the middle layer, and the epidermis is the outer layer. The skin is a mechanical barrier against the surrounding environment, and

protects humans from environmental hazards such as microbes and chemicals (constituting a barrier from the outside to the inside) (6, 7). It also inhibits undesirable water loss (constituting a barrier from the inside to the outside).

Although we may not be aware of it, there is a constant interaction between our skin and the chemical substances that are in contact with it. This may be oxygen and nitrogen molecules in the surrounding air, water molecules when enjoying a bath (accompanied by, for example, preservatives and fragrances in our bath foam), or the multiple substances in the juice while peeling an orange. The skin is a very efficient barrier, but it is by no means impermeable. Penetration of substances through the skin is dependent mostly on the surface layer of epidermis, which is called the stratum corneum (2). This layer is approximately 0.01 mm thick and is made up of around 10 layers of cells; it is in direct contact with the surrounding environment. The composition of this layer has its origin in the cell layers beneath. A description therefore follows, from the bottom of the epidermis and upwards (i.e. outwards).

The most prevalent type of cell in the epidermis is the keratinocyte. Epidermal keratinocytes have the ability to regenerate, which is the case in wound healing, for example (8). The epidermis is separated from the underlying dermis by a basal membrane. In the bottom layer of the epidermis, called stratum basale, there is constant regeneration of keratinocytes. Proliferating keratinocytes move upwards from the stratum basale, towards the surface of the skin. During this movement, the keratinocytes undergo differentiation (9). As the keratinocytes move into the stratum spinosum, intracellular lipids are synthesized and secreted into the space between the cells. The differentiation continues in the stratum granulosum, where important proteins (including filaggrin) are produced. Finally, the keratinocytes reach the stratum corneum – the surface layer of the epidermis (10).

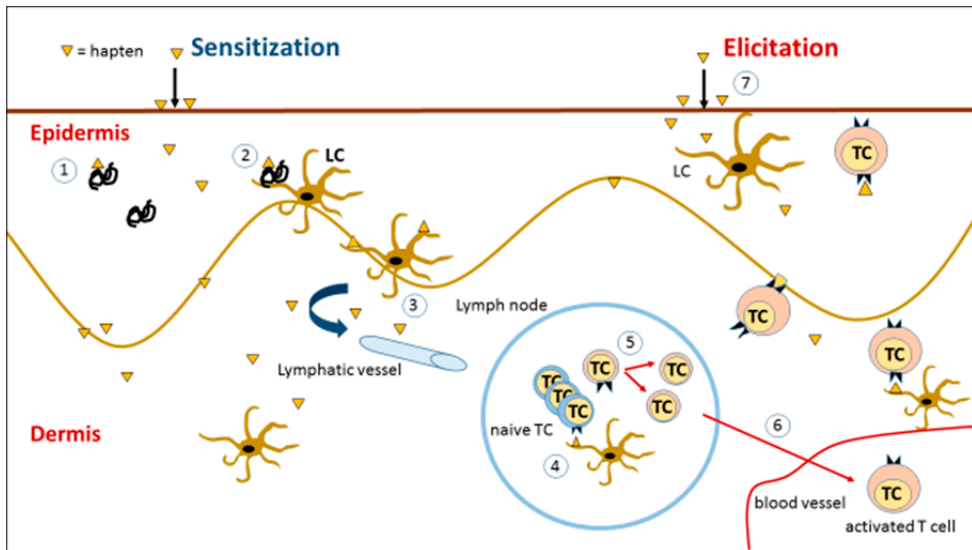
At the end of their maturation, the keratinocytes undergo apoptosis, which is programmed cell death. These dead cells, which have no nucleus, have an important function by forming a mechanical barrier and a barrier to water. The plasma membrane of the keratinocytes is replaced by an almost insoluble protein structure called the cornified envelope, which functions as a scaffold for lipid attachment. The intracellular space consists of fat (ceramides, fatty acids, and cholesterol) and the protein keratin. Moreover water-binding substances such as amino acids, lactic acid, urea, and salt are found in the stratum corneum. Lipids in the intercellular space become covalently bound to the cornified envelope. The final result is a strong, multilayered structure that simplistically resembles bricks (the dead keratinocytes and the cornified envelope) and mortar (the hydrophilic lipids). The stratum corneum is mechanically stable and it is a good defense against most chemicals, water, and water-soluble substances, but it is not very efficient against lipophilic substances. If the water content is less than 10%, the

stratum corneum becomes hard and cracks. Endogenous factors such as filaggrin polymorphism, and exogenous insults, for example, trauma, lead to a disturbance of the skin barrier. In contact dermatitis, irritants and allergens penetrate the epidermis and this may lead to inflammation (9, 11).

The keratinocytes are continuously being shed from the surface of our skin. This desquamation counterbalances the formation of new keratinocytes at the basal layer. The normal keratinocyte differentiation from stratum basale to desquamation takes around 28 days. Normally, one layer of corneocytes is shed every 24 hours. At this pace, the desquamation is hardly noticeable (7, 9, 10).

# The mechanism of contact allergy

Allergic reactions require an interaction between exogenous substances and the immunological system of an organism. Contact allergy in the skin is regarded as delayed cell-mediated hypersensitivity, which often presents clinically as dermatitis (12). Contact allergy is mainly driven by the interaction between an exogenous allergen such as PPD and the Langerhans cells and allergen-specific T cells of the adaptable immune system (13). In addition, innate immune mechanisms such as toll-like pattern recognition receptors are involved in the inflammatory reaction in allergic contact dermatitis (1, 4).



**Figure 1. The mechanism of contact allergy.** The main aspects of the adaptive immune reaction in sensitization (left) and elicitation (right). Innate immune mechanisms are not shown. (1) Binding of haptens to endogenous proteins. (2) Uptake of hapten-modified proteins by Langerhans cells. (3) Hapten-induced activation of Langerhans cells, which migrate and process hapten-protein complexes. (4) Presentation of antigens by Langerhans cells to naive T cells. (5) Proliferation of antigen-specific T cells; memory T cells are formed. (6) Hapten-specific memory T cells leave the lymph node and enter the circulation. (7) Re-exposure to and recognition of the hapten leads to release of cytokines and chemokines, attracting cells to the skin from the circulation. The inflammatory response occurs within 28-48 hours and produces symptoms of allergic contact dermatitis. LC, Langerhans cell; TC, T-cell. Illustration: Ewa Young. Modified from Ann-Therese Karlberg, Chem. Res. Toxicol, 2008, and from lecture slide by Stefan Martin, September 2015 Göteborg University, Göteborg, Sweden.

Our skin accommodates an immunological apparatus consisting of around  $2 \times 10^9$  Langerhans cells. This type of cell is a dendritic antigen-presenting immune cell. Langerhans cells have protruding dendrites that reach far between the abundant keratinocytes in the epidermis. The main function of Langerhans cells is to scan the epidermal environment to initiate immune responses. After stimulation by, for

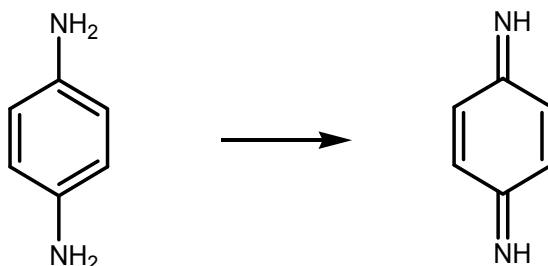
example, microbial threats or specific substances, Langerhans cells move down to the dermis – to lymphatic vessels (6). The T cell is a form of lymphocyte, a white blood cell, which matures in the thymus. The subset of T cells involved in the adaptive immune system has the ability to recognize specific molecules.

In order for an individual to become contact allergic to a substance, sensitization must occur. Sensitization is the phase of induction of the immunological memory. Elicitation is the phase of recognition of the allergen and activation of the inflammatory response. The contact allergens are usually small, reactive molecules, usually < 500–1000 Da. These small molecules must bind covalently to endogenous protein, forming a hapten-protein complex, to give a complete allergen that is recognizable by the immune system. They are therefore called haptens, from the ancient Greek word *háptein*, ἅπτειν, “to fasten”. The sensitization phase (Figure 1) begins when the allergens penetrate the skin barrier and covalently bind to endogenous epidermal and dermal molecules (Figure 1). This is followed by association with major histocompatibility complex (MHC) on epidermal Langerhans cells. The immune response is aimed at both the hapten and the protein. After processing of the haptens, MHC triggers the migration of epidermal Langerhans cells through lymph vessels to lymph nodes, where they encounter naive T cells. A subset of the T cells recognize the allergen-protein complex and the cells are triggered to become specific memory T cells. These cells proliferate clonically.

Upon new exposure, the hapten is recognized and elicitation may occur (Figure 1). This leads to the release of cytokines and chemokines, attracting various cells that contribute to the inflammatory reaction to the skin from the circulation including natural killer cells, mast cells and neutrophils (14). The hapten-specific memory T cells are recruited. The activation by the hapten leads to an immunological response. The reaction results in skin inflammation and clinically allergic contact dermatitis (12, 13, 15). Upon re-exposure to a hapten in a sensitized individual, the T cell activation of hapten-specific T cell clones will usually peak within 18–72 h. In some cases, the elicitation phase may be longer than the usual 1–4 days and sometimes 2–3 weeks may pass between exposure and the clinical presentation of allergic contact dermatitis (16–19). The strength of the reaction and thus severity of dermatitis will depend on several factors, such as the dose of allergen and repeated exposures. Some allergens have been shown to give varying elicitation reactivity in the same individual over time. This has been studied systematically regarding metals (20–25).

## Oxidation of allergens

Many known contact allergens require chemical transformation before they can act as haptens (13, 15). These substances may be transformed even before penetrating the skin, or be transformed in the skin after penetration. The reactions may occur spontaneously or be driven by enzymes in the skin. One type of chemical reaction by which haptens may be formed is oxidation. Such reactions involve the transfer of electrons from one molecule to another (26). As for other organic compounds, oxidation of PPD involves either gaining oxygen atoms or losing hydrogen atoms (27). On oxidation of PPD to quinonediimine (p-benzoquinonediimine) two hydrogen atoms are lost (Figure 2) (28).



**Figure 2. Transformation of p-phenylenediamine to quinonediimine by oxidation.** p-Phenylenediamine is shown on the left and the oxidation product quinonediimine (p-benzoquinonediimine) is shown on the right. N, nitrogen; H, hydrogen.

By repeated oxidation of a substance such as PPD, and also reactions with other substances that are present, new substances are formed. The substances formed in these oxidation reactions may be haptens.

Allergen oxidation has been thoroughly studied in several fragrance substances that are common in consumer products, such as limonene and linalool when in contact with oxygen in the air (12). It has been shown that oxidized forms of for example linalool, geraniol, and d-limonene are strong allergens (1).

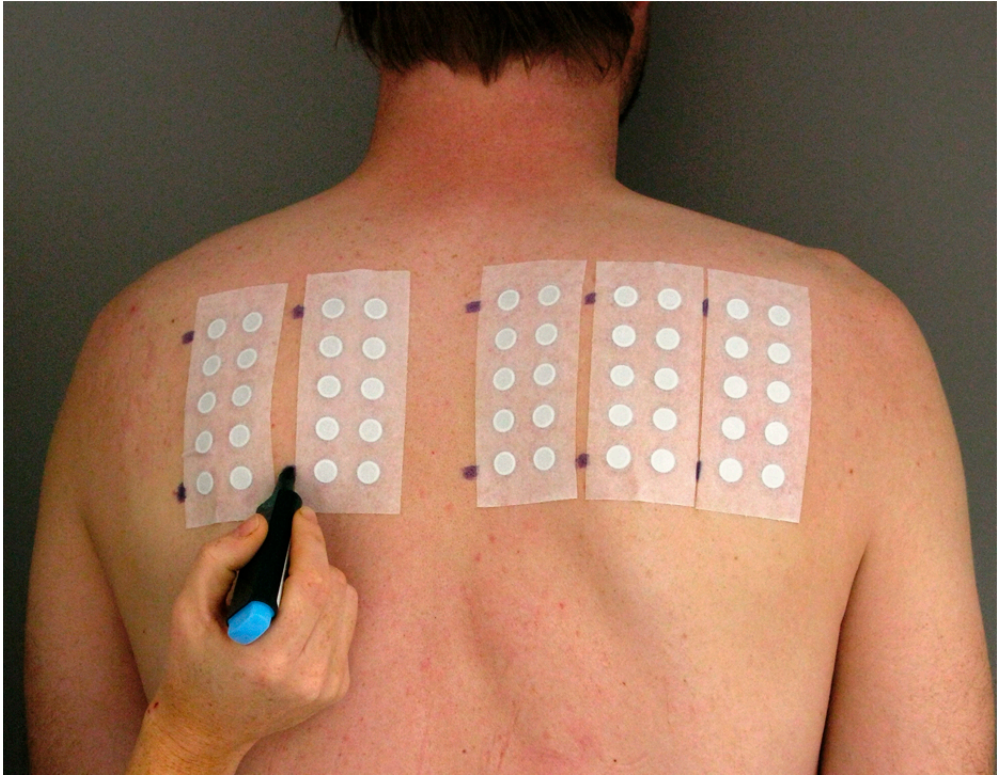
# Diagnosis of contact allergy

## Patch testing

In order to establish that there is contact allergy to PPD or other contact allergens in an individual, patch testing (epicutaneous testing) is performed. In the patch testing process, provocation with substances on the skin is followed by reading of the patch test, i.e. evaluation of the result (1, 12). If the patient is allergic to the substance applied, a small area with dermatitis occurs on that specific spot. The patch test substances are applied in a specified dose to small containers (chambers) with variable area of approximately 0.5-1 cm<sup>2</sup> and these chambers are attached to the skin using tape, normally on the back of the patient (Figure 3). The day of application of the patch tests is designated day 0 (D0). According to standard procedures, patch tests are physically removed on D2, i.e. after 48 h. The patch test can be removed by the patient at home or by healthcare personnel at a clinic.

The test chambers in different test systems vary in size, form (round or square), material (metal or plastic), and method of attachment. The chambers are provided either prefilled with substances or empty. In our department, the most common patch test chambers used are Finn chambers®, 8 mm in diameter and attached with Scanpore tape. The test substance may be delivered in a liquid solution where the vehicle varies, e.g. water or acetone may be used. The proper amount of test material applied to an 8-mm Finn chamber has been standardized to 15 µl if the substance being tested is a liquid or has been diluted in a solution (29) and 20 mg if the substance is applied in petrolatum (pet.), which is a common vehicle in patch testing (30).

Based on the patient history, known exposure, and the distribution and presentation of dermatitis (present, described by the patient, or from photographs) patch test substances are carefully chosen. In most cases, at least a set-up of more than 30 patch tests included in a “baseline series” is used in an investigation. Common allergens that are encountered in the environment such as metals, fragrance allergens, preservatives, and PPD are included in the baseline series. Besides the common allergenic substances included in a baseline series, further specialized series with substances of relevance to specific occupations or other exposures are also patch tested as part of the investigation, for example the hairdressers’ series, cosmetic series or glove series. In order to fully investigate the culprit allergen(s) responsible for a patient’s allergic contact dermatitis his or her own products may be patch tested. This may include patch testing of the material as it is, in dilution or as ultrasonic bath extracts (31).



**Figure 3. Patch testing.** Fifty patch test chambers with preparations of allergenic substances, fastened on tape, have been applied to the skin on the back of a man going through an occupational and environmental dermatological investigation. Marks are made to facilitate reading of the test once the patches have been removed. Photo: Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö.

## Patch test reading

Patch test reading is the procedure for examination and evaluation of the patch tested area of skin. If there is a reaction, the morphology is scored according to a standardized system. The tests are considered positive if there is at least a + reaction registered, according to the criteria of the International Contact Dermatitis Research group (ICDRG), corresponding to the location of the test chamber (32). According to the criteria, the patch test is either negative, irritant (IR), or positive. The criteria for positive reaction include at least erythema and infiltration. The positive reactions are scored + (weak positive), ++ (strong positive) or +++ (extreme positive). The reaction may be scored as being +? (doubtful) if it is not possible to distinguish between negative and positive.

The relevance of a positive patch test is assessed in relation to the exposure, the patient's present or past dermatitis, site, course and relapses (33). Use tests, chemical analysis and work-site visits can be of help. The evaluation of the relevance is difficult and requires "the dermatologist's skill, experience, and curiosity" (33). Advice to the patient is then given based on the combination of patch test reading (diagnosis of contact allergy) and the evaluation of the relevance.

The time for reading differs between centers and countries. In Malmö, patch tests are evaluated and scored according to the European recommendations: after 3 or 4 days (D3/4) and after one week (D7) (34). The department in Malmö has used this practice since the early 1990s (35).

### **Late-appearing reactions and patch test sensitization**

One possible adverse effect of patch testing is to make non-sensitized patients sensitized by exposure to a substance in a patch test. The term "patch test sensitization" is used synonymously with active sensitization. If a patient who was not previously allergic becomes sensitized, the elicitation might take place from the same delivered dose of allergen and may present clinically as a late-appearing positive reaction. Patch test sensitization is usually indicated if a positive patch test reaction develops after D7, and often after D10-14 and beyond. If a subsequent re-test with the same preparation gives a positive reaction within 7 days, the initial late-appearing positive reaction is due to suspected active sensitization (1, 36).

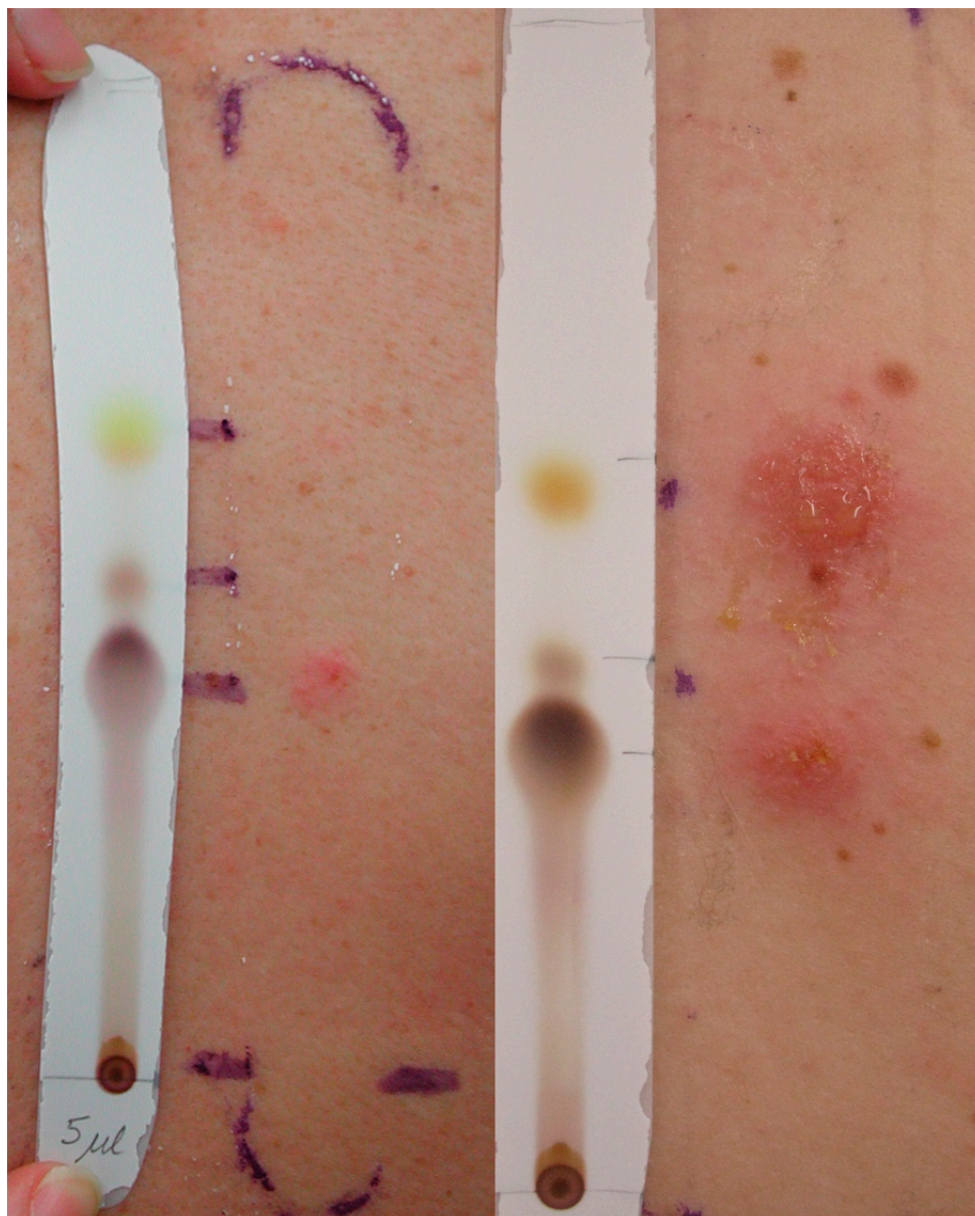
Most patch tested substances induce a positive reaction in the sensitized individuals within 7 days. A late-appearing patch test reaction is, however, not always due to patch test sensitization (20, 21). Causes of variation in reaction time to an allergen may be the chemical properties, skin permeation, inter-individual variation in the expression of the metabolic enzymes involved and in some cases anti-inflammatory influence from the allergen. Late patch test reactions have been observed for some contact allergens. This has been described with corticosteroids, gold, acrylates, and isocyanates (20, 37-39). Gold-allergic patients have been patch tested with dilution series of gold sodium thiosulfate, and in one of 10 patients the lower test doses elicited positive reactions on D10 and D21 while reactions to the highest concentrations developed within 7 days (20). Thus, there is a possibility when patch testing serial dilutions of PPD that reactions to the lower concentrations might appear later than reactions to the highest concentrations – and that a higher concentration could also give a later reaction, especially in a patient with lower reactivity who would therefore react only to the highest concentration in a dilution series. When a late-appearing reaction after a patch test is observed, the common routine is re-testing to investigate whether the subsequent positive reaction would appear earlier, which strengthens the suspicion

of patch test sensitization. In Malmö, re-testing is also performed with dilution series of the allergen, based on a paper published by Bruze in 1984 (40), although a retest with a dilution series will not always discriminate between patch test sensitization and a late appearing positive patch test.

## **Reactions to cross-reactive allergens**

The complex process of hapten recognition by T cells during elicitation depends chiefly on the type of chemical group involved and the spatial geometry of the allergenic molecule (41, 42). This recognition is specific, but not completely specific to the initial sensitizing hapten. Similar haptens may thus cause allergic contact dermatitis in an individual who has been first sensitized to one specific hapten. This phenomenon is called cross-sensitization, and can lead to cross-allergic reactions (41, 42). In addition to similarity of haptens, cross-sensitization as described above may also be explained by two substances being transformed to the same hapten by metabolism, oxidation, or some other mechanism.

In the laboratory environment, it is possible to investigate cross-sensitization in animals whose skin exposure to chemicals can be meticulously controlled. The guinea pig maximization test (GPMT) is a contact allergy animal test and the gold standard for establishing cross-reactivity patterns. The results from GPMT may in most cases be applied to human skin. GPMT enables study of the induction phase, the elicitation phase, and also cross-reactions (43). In the clinic, it is virtually impossible to establish whether allergic contact dermatitis in one individual from two or more allergens is caused by cross-reactivity between structurally related chemicals or by concomitant sensitization, which may occur if the individual is exposed to several similar substances in the environment and has become sensitized to these separately (1).



**Figure 4. Patch testing with thin-layer chromatography strips.** Examples of test reactions on D3 to thin-layer chromatograms of oxidized p-phenylenediamine in two test patients in study I. 5 µl of the test preparation was deposited on each strip. Left: patient 10; right patient 13; D, day. Photo: Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden. Published with the permission of the patients.

## **Patch testing with thin-layer chromatography strips**

Thin-layer chromatography (TLC) is a method that often enables complex mixtures of substances to be separated. The chemical's molecular size, polarity, affinity for the mobile phase, and affinity for the material of the TLC sheet determine how far that particular chemical will be carried on the plate, and therefore the quality of the separation. Different substances may thus be separated along the chromatogram, and some can be visualized as spots (Figure 4).

When an individual, in a patch test situation, reacts to a complex solution consisting of several different substances, it is not known to which substance(s) the individual has reacted. One way to investigate this is to separate the substances with TLC and then to patch test with the TLC strip (Figure 4), thus enabling identification of the allergens (44, 45). Bruze et al. developed a method for using plastic TLC strips epicutaneously in patch testing of individuals (44-47).

When patch testing with TLC strips is performed, the strips are prepared in duplicate and one copy of the TLC strip serves as the test protocol (Figure 4). Possible reactions can be correlated to the exact site of the TLC strip, which can then be used for identification of substances in the correct area. Infiltration and redness in the area corresponding to where the substances have been eluted would be regarded a + reaction. If the reaction also has papules, it would be regarded as a ++ reaction. If there is intense redness, papules, and even vesicles, it would be regarded as a +++ reaction.

Reading of thin-layer chromatograms is complicated, since the amount and distribution area of different possible allergens is unknown even when the original amount of the mixed substances is known. The whole area of skin that has been covered with the TLC sheet and where the substances have eluted will be evaluated. This means that an area, corresponding to an area on the duplicate TLC in which there is no visible spot, can still be positive. One reason being that the chemical is not detectable in the light used.

After patch testing, the spot that an individual has reacted to may be extracted from the thin-layer chromatogram. The extract may then be analyzed in order to identify possible allergens. The substance(s) found in a spot in a thin-layer chromatogram, i.e. the possible allergen(s), must be patch tested in the individual to prove that it is actually the culprit allergen. The substance must also be patch tested in control individuals if it is not a previously known allergen. This enables the distinction between an irritation reaction and a positive reaction.

## Prevention of hand dermatitis – a challenge

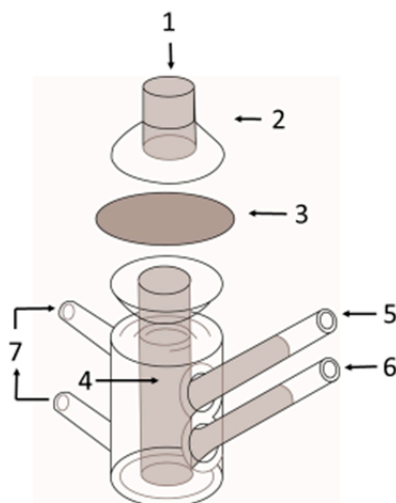
Contact dermatitis may develop in any location of the body, depending on the exposure. The hand is the location that is most exposed to substances and is therefore most affected by exogenous dermatitis (1, 12). This applies to irritants such as water and detergents, and also contact allergens such as PPD. A risk factor for occupational irritant hand dermatitis is wet work. There is no internationally accepted definition of wetwork. The following is one definition of wet work which is used in the German regulations:

“Wet work includes activities where the workers:

- have their hands in a wet environment regularly for more than 2 h/day.
- must wash their hands frequently (e.g. 20 or more times per day) or intensively.
- wear waterproof gloves; the time of wearing such gloves is added to the time in a wet environment if no effective measures are taken to regenerate the skin.” (48)

Gloves are used as a personal protective measure for the protection of hands from irritating and allergenic substances. The use of gloves is also a risk for irritation, since the occlusion makes the skin wet, as indicated in the definition of wet work. Thus the use of gloves may increase the irritant dermatitis. Moreover, if the inside of the gloves becomes contaminated, the substances will more easily penetrate the skin when covered by glove material. These risks can be reduced by shorter time spent doing wet work and more limited use of gloves.

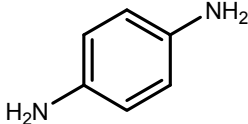
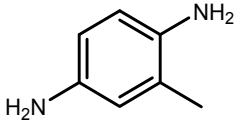
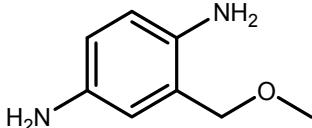
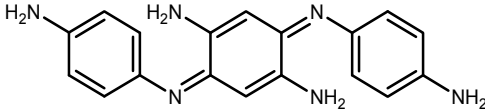
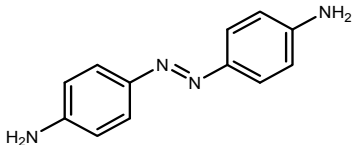
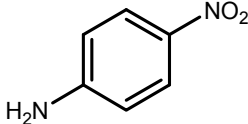
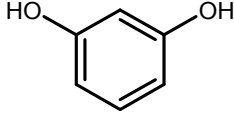
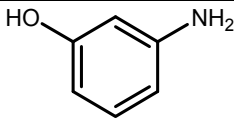
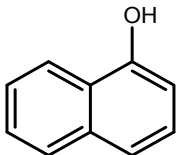
Another risk with glove use is that their use may give a false impression of being safe to chemicals if they protect against water, but this may not be the case, since different glove materials vary in how well they protect against permeation of other chemicals, such as hair dye allergens. An individual might consider that he/she is protected, but not all glove materials are suitable for protecting against certain substances. Gloves themselves may even contain allergens. Individuals working in occupations involving the use of irritating and allergenic substances, such as hairdressers, need to understand proper glove use. Wrong use of gloves may increase the risk of hand dermatitis. The manufacturers of gloves are constantly developing and changing their products. Therefore it is important as a clinician to have up to date information about the gloves on the market that are used by the patients in different occupations. For this purpose continuous investigation and research on the protective ability of gloves is crucial in work on prevention of hand dermatitis. This accounts both for studies in vitro and in vivo.



**Figure 5. The Franz permeation cell.** 1: donor compound; 2: donor compartment; 3: membrane; 4: receptor compartment; 5 and 6: sampling ports; 7: heater/circulator. The receptor compartment (4) is surrounded by a water jacket with circulating water which enables heating. Illustration: Tina Ljungberg, Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden.

## Franz cell for glove permeation studies in vitro

One way to investigate the protective capacity of gloves against substances is to study the permeation in vitro. Permeation refers to “the migration of chemicals through the protective glove material on a molecular level (sorption, diffusion, desorption)” whereas penetration refers to “the passage of chemicals through macroscopic holes or pores” (49). The study of permeation through gloves (and other membranes, e.g. the skin) may be performed using permeation cells. The Franz cell (Figure 5) is one type of permeation cell. Franz cells have two compartments (50). The material under study is clamped as a membrane between these two compartments. The donor compartment is open to the ambient air. The substance whose permeation is to be investigated is placed in the donor compartment. The receptor compartment is filled with a receptor solution that is stirred continuously with a small magnetic bar. Sampling is performed from the receptor compartment through sampling ports (Figure 5). A permeation cell may be surrounded by a temperature jacket with circulating liquid to maintain the desired temperature (50). If gloves are investigated the surface temperature of the hand may be used in order to assess chemical reactions and permeation as accurate as possible.

<b>a</b>	p-Phenylenediamine <sup>1</sup> Synonyms: 1,4-phenylenediamine, 1,4-diaminobenzene, PPD CAS: 106-50-3 MW: 108 log P <sub>ow</sub> : 0.43	
<b>b</b>	2,5-Toluenediamine <sup>1</sup> Synonyms: p-toluenediamine, 2,5-diaminotoluene, TDA CAS: 95-70-5 MW: 122 log P <sub>ow</sub> : 0.92	
<b>c</b>	Methoxymethyl p-phenylenediamine <sup>1</sup> Synonyms: Me-PPD, 2-(methoxymethyl)benzene-1,4-diamine, 2-methoxymethyl-p-phenylene-diamine CAS: 337906-36-2 MW: 155 log P <sub>ow</sub> : 0.22	
<b>d</b>	Bandrowski's base <sup>2</sup> Synonym: 4-diamine,3,6-bis((p-aminophenyl)imino)-4-cyclohexadiene-1 CAS: 20048-27-5 MW: 318 log P <sub>ow</sub> : -0.86	
<b>e</b>	4,4'-Azodianiline <sup>2</sup> Synonym: p-diaminoazobenzene CAS: 538-41-0 MW: 212 log P <sub>ow</sub> : 2.86	
<b>f</b>	p-Nitroaniline <sup>2</sup> Synonym: 4-nitroaniline CAS: 100-01-6 MW: 138 log P <sub>ow</sub> : 1.01	
<b>g</b>	Resorcinol <sup>3</sup> Synonyms: m-hydroquinone, 1,3-benzenediol, 1,3-hydroxybenzene, benzene-1,3-diol, 3-hydroxyphenol CAS: 108-46-3 MW: 110 log P <sub>ow</sub> : 0.8	
<b>h</b>	m-Aminophenol <sup>3</sup> Synonyms: 3-aminophenol, 3-hydroxyaniline, m-hydroxyaniline CAS: 591-27-5 MW: 109 log P <sub>ow</sub> : 0.2	
<b>i</b>	1-Naphthol <sup>3</sup> Synonym: Naphtalen-1-ol CAS: 90-15-3 MW: 144 log P <sub>ow</sub> : 2.64	

**Figure 6. Chemical structures of nine hair dye allergens.** The hair dye allergens are verified or potential. CAS, Chemical Abstracts Services number; MW, molecular weight.

<sup>1</sup>Hair dye precursor; <sup>2</sup>Possible PPD allergen; <sup>3</sup>Hair dye coupler.

## The contact allergen p-phenylenediamine

p-Phenylenediamine (PPD), a potent contact allergen and the focus of this thesis, is a molecule consisting of an aromatic carbon ring and two amino ( $\text{NH}_2$ ) groups in para position (Figure 6a). In its pure form, the compound consists of colorless crystals and easily becomes oxidized in air to compounds that appear brown, and later black, in color. Although the sensitizing capacity of PPD has been known for over 100 years, it is still a standard color substance in oxidative (permanent) hair dyes (51-53). Other uses of this molecule (including substituted PPD), apart from being a component of permanent hair dyes, are – or have been – in textile and fur dyes, black rubber, developers for photography, and black henna tattoos. Black henna tattoos are a kind of temporary tattoo that can often be acquired in tourist destinations on streets and beaches. In addition to hair dyes, the use of these tattoos is an important risk factor for contact allergy to PPD (54). Central to this work are PPD allergens in oxidative hair dyes.

## Transformation of PPD

Hair dyeing has been performed for centuries, but the kinds of permanent oxidative hair dyes presently used were introduced in the nineteenth century. Their production became possible after the discovery that hydrogen peroxide can both bleach hair and react with chemicals, e.g. aromatic amines such as PPD, to give dark dyes that have permanent effects when hair is treated (55). The mechanisms of induction and elicitation of contact allergy by PPD are not fully understood. PPD can be transformed into new substances by processes such as oxidation, metabolism, and reaction with other chemicals (56, 57), and these substances may be important in connection with PPD and hair dye allergy. PPD in itself is not immunogenic. Oxidation of PPD results in the formation of p-benzoquinonediimine (Figure 2) and further other reactive substances that have the capacity to act as haptens (58-60). The extent to which patients who are PPD-allergic may react to different oxidation products that are formed is not fully known, and we also do not know the extent to which different reactivity patterns to the products formed might explain the different cross-reactivity patterns that are found in patients who are sensitized to PPD (61-70). The activation of PPD may be due to its oxidation occurring both on and in the skin.

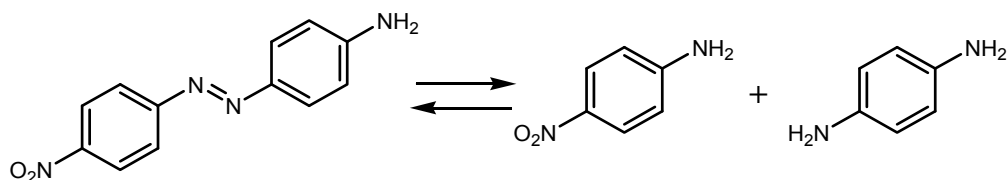
## PPD and cross-reactivity

For PPD, cross-reactions have been reported with other hair dye allergens and textile dyes, e.g. Disperse Orange (DO) 3 and DO1, as have concomitant reactions to sulfa drugs, benzocaine, and black rubber (41, 42). The majority of individuals who are allergic to permanent hair dyes have been exposed to PPD and have subsequently developed contact allergy to PPD – and possibly to other, similar substance(s) representing cross-sensitivity to PPD.

Regarding PPD and the textile dye DO3, there are contradictory suggestions about whether metabolism to PPD and 4-nitroaniline occurs in the skin (1) or in the anaerobic environment of the gastro intestinal tract (71) (Figure 7). Concerning another disperse textile dye, DO1, the degradation of DO1 to p-aminodiphenylamine and PPD has also been proposed (72).

Cross-reactions to diphenylmethane-4,4'-diamine (4,4'-MDA), in animals sensitized to PPD, in a GPMT have been shown by Hamada et al (73). However, no cross-reactions to PPD were found in animals sensitized to 4,4'-MDA.

In vitro studies have been used to investigate some allergens that are formed in hair dyeing with PPD (60). New allergens among the oxidation products of PPD may be established by patch testing these allergens in patients who are allergic to PPD. In order to establish that the individuals are actually exposed to these substances it is also necessary to prove the existence of these allergens in the hair dye and mixed hair dye.



**Figure 7. Suggested degradation of Disperse Orange 3 into 4-nitroaniline and PPD, and the reverse reaction.**

## Formation of oxidation products of PPD in permanent hair dyes

Humans are the primates with the least hair on their body, and our hair has only minor physical value, but on the other hand human hair is of major psychosocial

significance. The natural hair color is defined by combinations of different types of the pigment melanin. It is common worldwide to dye the hair on the head and face as an expression of fashion, and in order to fulfil expectations of today's society. Facial hair that can be dyed includes the beard, the moustache, the eyebrows, and the eyelashes. Not only external intentional hair dyeing, but also ingestion of some drugs may unintentionally influence hair color as a side effect; for example, hair may become lighter by oral intake of the antimalarial drug chloroquine (74).

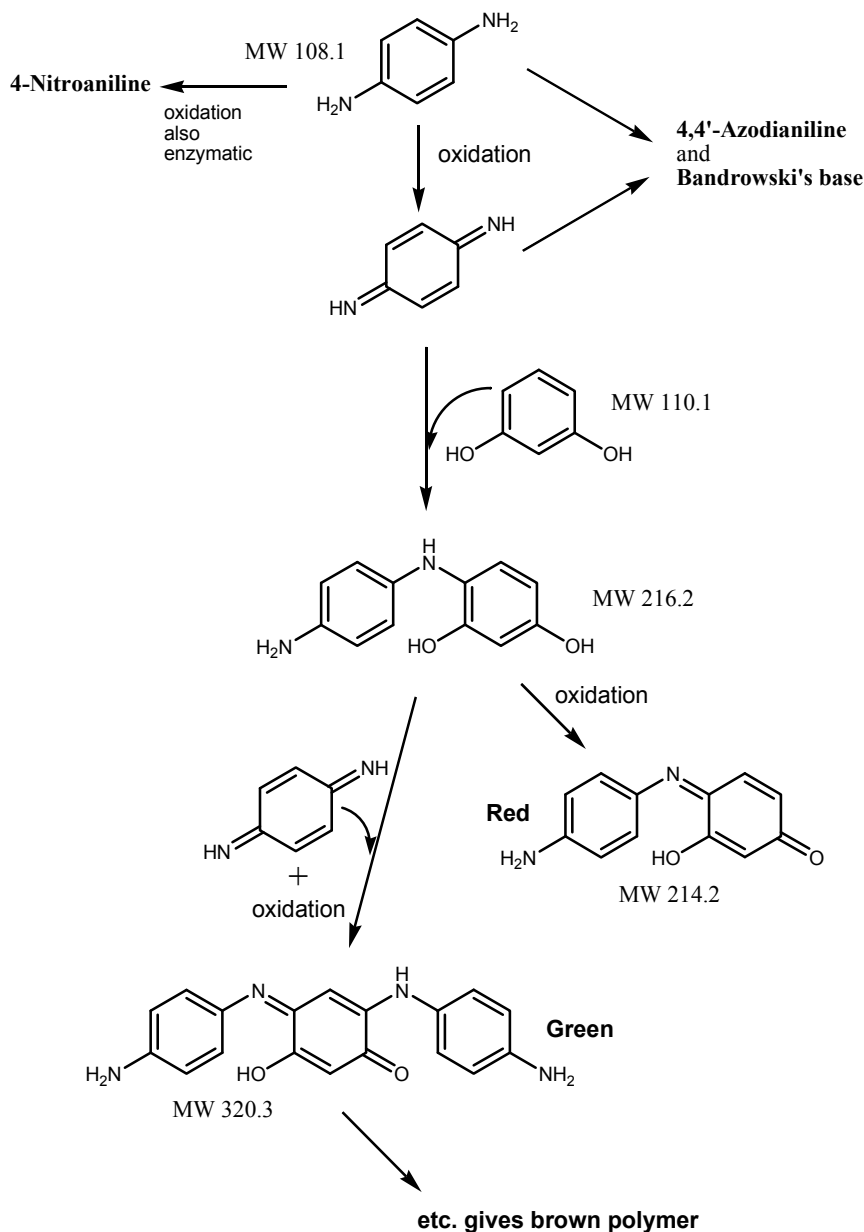
There are numerous kinds of hair dyes on the market that color the hair through various physicochemical mechanisms. The dyes have variable durability – from transient ones that only last one wash to permanent dyes that only disappear if the dyed hair shafts are shed from the scalp. The permanent dyes are also called oxidative hair dyes, since the formation of the color molecules from a colorless mixture is dependent on oxidation (75). Non-oxidative hair dyes are often referred to as semi-permanent, temporary or direct (76).

A typical oxidative hair dye has 3 main functional components: precursors, couplers, and an oxidizing agent. PPD and 2,5-toluenediamine (TDA) (Figure 6b) are examples of precursors and resorcinol, m-aminophenol, and n-naphthol are examples of couplers (Figure 6g-i). The oxidative hair dyes are typically provided by the cosmetics industry as two components, the color crème and the developer, to be mixed when used. The color crème, containing a precursor and one or more couplers, is mixed with the developer just before application to the hair. In the developer solution, hydrogen peroxide is often used as the oxidizing agent. After mixing of a PPD-based oxidative hair dye, PPD oxidation products are formed, which can then react with the coupler(s) to create the desired color molecules (28, 77). With numerous precursors and couplers in different concentrations, a multitude of colors and shades can be produced. Due to its strong sensitizing capacity, PPD is permitted in amounts of up to 2% “on-head concentration” in Europe, meaning after the mixing of the color crème and the developer (78). PPD has been prohibited in hair dyes in Sweden from 1943 to 1992 (79).

PPD may be found in all shades of hair dye, even blonde, but usually the darker the color the higher the PPD concentration (53). Due to the strong sensitizing capacity of PPD especially and of other, similar, color substances (51), efforts are being made to find substitutes with less allergenic potency (80, 81). PPD and TDA are still the most commonly used precursors in permanent hair dyes (82). Regarding both these substances, contact allergy is a problem – and since hair dyeing is becoming ever more common, there is a need for an understanding of how these substances act as allergens and to find less allergenic equivalents. Lately, hair dyes with 2-methoxymethyl-p-phenylenediamine (ME-PPD) have been launched on the consumer market. Methoxylated PPD is used in order to

sensitize less than PPD (80) but according to the data that are available a considerable number of PPD-sensitized patients still react to ME-PPD (81, 83).

The hairdressers and the consumers are also exposed to a cocktail of other irritating substances or contact allergens in hair dyes such as perfumes, in addition to the main functional components of the color crème, precursors, and couplers (84, 85). This may be of importance when investigating suspected contact allergic reactions to hair dyes.



**Figure 8. Possible reaction pathway for the formation of green and red pigment by oxidation of p-phenylenediamine (PPD) in the presence of the coupler resorcinol.** By these reactions, red and green pigments are formed (86). For the formation of red pigment, the ratio of PPD to resorcinol is 1:1. With the addition of a second PPD molecule, green pigment is formed, by coupling of two PPD molecules by resorcinol. The ratio of PPD to resorcinol is 2:1 for the green pigment. Further coupling/polymerization of PPD will result in a brown polymer. Oxidation in the absence of the coupler (resorcinol) may result in the formation of Bandrowski's base, 4,4'-azodianiline, or 4-nitroaniline.

## **Resorcinol – a coupler used together with PPD**

Resorcinol is a hair dye substance used as a coupler. Like PPD, resorcinol (Figure 6g) is a small molecule and has been used in several previous studies regarding permeation of hair dye allergens. It is an infrequent allergen in the clinical setting. When tested in hairdressers' series in Belgium, less than 0.1% of 1,187 patients were positive to resorcinol (87). At the Occupational and Environmental Department in Malmö, 0.3% (1 in 390) of the patients who were patch tested with resorcinol had positive reactions to resorcinol during the period 2004-2017. Resorcinol has been part of our hairdressers' patch test series since 1995. Other couplers that are known to be contact allergens are m-aminophenol and 1-naphthol (Figure 6h and i) (88, 89). The local lymph node assay suggested a sensitizing capacity approximately two orders of magnitude lower for resorcinol than for PPD (90).

There is a multitude of formulas for oxidative hair dye shades formed when choosing among different precursors and couplers in various concentrations. One possible combination is the pigment formation from oxidized PPD and resorcinol (86). In the reactions, red and green pigments are formed (Figure 8). For the formation of red pigment, the ratio of PPD to resorcinol is 1:1. Since the two molecules both have a molecular weight of around 110, the red pigment in particular would be predicted to form if the same w/w % of PPD and resorcinol was used. With the addition of a second PPD molecule, green pigment is formed, through coupling of two PPD molecules by resorcinol (Figure 8). The optimum ratio of PPD to resorcinol is thus 2:1 for the green pigment. The red and green colors are complementary. Thus, the green and red pigment together may absorb all light and the color will be perceived as being black. Further coupling of PPD will result in a brown polymer (86).

## **PPD oxidation in the absence of couplers**

Oxidation without the presence of the coupler (resorcinol) may result in the formation of Bandrowski's base (BB), 4,4'-azodianiline, or 4-nitroaniline (Figure 6d-f). BB has already been implicated as one substance that is possibly responsible for PPD-related contact allergy (60, 91-93). Individuals may be exposed to high concentrations of PPD without couplers in temporary tattoos, so-called black henna tattoos. Usually no couplers are added in such tattoos. The dark shade in these tattoos is due to a mixture of PPD oxidation products, possibly formed by auto-oxidation due to contact with air.

## Inactivation of PPD

Through enzymes, not only activation but also deactivation of haptens may occur. Acetylation is a chemical reaction by which an acetyl group ( $\text{CH}_3\text{CO}$ ) is attached to a molecule. N-acetylation, the binding of an acetyl group to nitrogen, is catalyzed by the enzymes N-acetyltransferase (NAT)-1 and NAT-2. Regarding the metabolism of PPD, N-acetylation appears to be a route of deactivation. It has been shown that a proportion of the PPD molecules that penetrate the skin upon exposure are metabolized to their mono- and diacetylated derivatives and are thus inactivated. The variation in human genes coding for NAT-1 and NAT-2 may result in both rapidly and slowly acetylating phenotypes. This polymorphism seems to result in reduced detoxifying capacity in slow acetylators, and thus increased susceptibility to allergic contact dermatitis (56, 93-96).

## Patch testing with PPD

A few years after the introduction of PPD as a color substance for materials in contact with the skin, such as hair dyes, fur, and textiles, it was realized that PPD was causing adverse skin reactions. As early as the 1930s, Bonnevie reported cases of PPD contact dermatitis associated with reactions to fur collars, and advocated the use of PPD as a test substance in patients with this history (97). A few years later one of the early baseline series in the history of occupational and environmental dermatology was introduced by Bonnevie, in 1939. This early series contained PPD 2% pet. as a patch test marker for hair dye allergy (97). Almost 80 years later, PPD is still the most relevant one clinically (69). In the baseline series of the ICDRG 1974, PPD was included as 1% pet. (98). PPD is commonly used in patch testing in its pure form, but in the period 1984–1988 it was included in the baseline series as its salt, PPD dihydrochloride (PPD-DHC) (98). On recommendation of the European Environmental and Contact Dermatitis Research Group (EECDRG) and ICDRG, this was abandoned in favor of going back to testing with PPD, since too many allergies were being missed (98). The recommended routine patch test concentration for PPD in order to establish that there is contact allergy in sensitized patients has been 1.0% pet. and PPD is currently still included in the European baseline series as 1.0% pet. (34). For over a decade, it has been discussed whether PPD is a safe contact allergen for routine patch testing due to the risk of making non-sensitized patients sensitized by the patch test itself, active sensitization, and whether or not the dose used in the patch test should be reduced for the same reason (42, 99-111).

When the patch test statistics of the German Information Network of Departments of Dermatology (IVDK) in the years 1995–2004 were analyzed 4% of 83,030

individuals tested were PPD-positive (102). In this material a high rate (44%) of PPD reactions without any known current relevance was found (102). Hillen et al. performed prospective investigations in order to study late occurring reactions in routine patch testing with PPD 1% pet. (108). Readings were performed on D3, D7, D14 and D21. The patch tests were placed on the arm and the patients were told to make daily evaluations and call if they suspected a positive reaction. Among 1428 individuals, 3.2% (n=46) had positive reactions on D3 and 1.9% (n=21) had positive reactions on D7 and beyond. Among the 21 patients with late reactions three had de novo reactions at D7 and the remaining 18 had positive reactions appearing at readings on D11–D37. Seven of the 21 patients were retested with PPD 1% and in 5 of 7 there was a positive reaction within 3 days on re-testing (108). Based on these result Hillen et al. estimate an active sensitization rate of around 1% in patients routinely patch tested with PPD 1.0% applied for 48h (108).

Based on this data removal of PPD at 1.0% from the baseline patch test series was recommended in Germany by DKG (German Contact Dermatitis Research Group), with the suggestion of only performing aimed testing (108). To avoid active sensitization, patch testing was only recommended if there was a clear suspicion of possible allergic contact dermatitis due to PPD from the patient's medical history and distribution of eczema. Lower patch test concentrations of 0.30–0.35% PPD have been proposed (99, 108). The delayed patch test reaction may also be due to other reasons than active sensitization. The drawback of only performing aimed testing is that the patient's history may not disclose exposure to PPD. Apart from PPD being a substance for diagnosing contact allergy to hair dyes in general, it is known that several other allergens can induce cross-reactions with PPD. Thus, excluding it from the baseline series might lead to missed contact allergy diagnoses in patients who have been sensitized to, for example, hair dyes, black rubber, and textile dyes.

In order to avoid extreme reactions measures can be taken when PPD is patch tested in patients with a history of strong reactions after exposure to known or suspected PPD. In our clinic lowering of the dose to 0.1% PPD or even 0.01% is performed in such cases. The patch test may be placed on the upper arm instead of on the back. This makes it possible for the patient to easily remove the test earlier than 48h if the patch test causes severe itching and do so without removing a whole panel with 9 other allergens under investigation.

## Epidemiology – hair dyeing and contact allergy to PPD

The frequency of hair dyeing, and thus the exposure to PPD, varies over time, from one geographical location to another, and between genders. In the beginning of the twentieth century, hair dyeing was mostly used to cover up gray hair. This trend has changed. It has been estimated that in 1995, over one-third of women over the age of 18 and more than 10% of men over the age of 40 in Europe and North America used some kind of hair dye (112). In a contact allergy survey performed in the general European population by Diepgen et al. 10,425 individuals were interviewed (54). Approximately 51% of these individuals reported having used hair colorants at least once in their lifetime (78% female, 20% male) (54). 35% had used hair colorants during the last 12 months.

Contact allergy to oxidative permanent hair dyes is common in hairdressers and their consumers worldwide (113-118). When considering the prevalence of contact allergy, one must differentiate between (i) the prevalence in particular risk groups, (ii) the prevalence among patch tested patients, and (iii) the prevalence in the general population.

Hairdressers are a risk group for contact allergy to PPD due to both occupational exposure and more frequent personal use of hair dyes (118-120). Lind et al. registered a prevalence of self-reported hand eczema of 18% in hairdressers and 12% in controls (118). Studies on contact allergy to PPD in hairdressers have shown a prevalence of 15–54% (Europe), 41% (Australia), and 45,5% (Thailand) (113-115, 121, 122). Bregnhøj et al. found hair dyeing frequencies among hairdresser apprentices at 6.6 times per year compared with 3.7 times per year in a control group (120).

The contact allergies of patch tested dermatitis patients are generally well documented. This group would be expected to have a higher prevalence of contact allergy than in the general population since these individuals have been selected on the basis of a suspected contact allergy or skin pathology where contact allergy needs to be ruled out. The prevalence of PPD contact allergy in dermatitis patients of 2% (Australia), 4% (Europe), 6.7% (Chile), 4.0–7.0% (North America) and 0.4–15.2% (Asia) has been reported (100, 123-127). Sparse data from Africa is available. From a small study in Ethiopia 1.7% PPD positive reactions were reported in dermatitis patients (128).

Diepgen et al. performed patch testing of 2,739 individuals from the general population in five European countries and 0.8% of the patch tested individuals were contact allergic to PPD 1.0% (54). Elseways patch testing of the general population is infrequently performed.



**Figure 9. Hand eczema.** Erythema, fissures, and squamation are seen on the ventral aspects of the fingers. Photo: Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden. Published with the permission of the patient.



**Figure 10. Hand eczema.** Erythema and interdigital squamation are seen on the dorsal aspects of the fingers. Morphology indicative of probable irritant contact eczema. Photo: Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden. Published with the permission of the patient.

## Clinical presentation of reactions to PPD

### **Allergic contact dermatitis from PPD**

The most common adverse reaction on exposure of the skin to PPD in an individual sensitized to PPD is allergic contact dermatitis at the area exposed. The severity ranges from mild dermatitis with erythema only, to increasingly severe reactions with papules, edema, vesicles, and oozing. Hairdressers who are in contact with permanent hair dyes on a daily basis are at risk of developing occupational contact allergic dermatitis to PPD and related hair dye allergens. Due to the manner of exposure the dermatitis is usually located on the hands (Figures 9 and 10). Since hairdressing work includes a lot of wet work and work with other irritants, such as shampoos, the dermatitis will often be a mixture of allergic contact dermatitis and irritant contact dermatitis (1, 129).

The contact allergic individual using hair dye with PPD often presents with a hairline dermatitis with more severe morphological features in areas such as the posterior neck and the periauricular area, whereas the part of the scalp with hair is affected less (Figure 11). In some cases, allergic contact dermatitis is caused “by proxy” or is connubial, which may happen with PPD from contact with a partner’s newly dyed hair (130).



**Figure 11. Allergic reaction to a black hair dye.** Erythema, papules, pustules, erosions, and edema. The periauricular area and posterior hairline are severely affected, whereas the area with hair is less affected. Photo: Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden. Published with the permission of the patient.

In some rare cases, PPD-sensitized individuals may present with swelling of the face affecting periorbital and perioral areas (Figure 12). Cases occur with patients needing intensive care treatment after extensive swelling of the face, with the airways also being affected, when they have had their hair dyed. Contact urticarial and contact anaphylaxis due to PPD in hair dyes has been described (131, 132). A fatal case has been reported after hair dyeing, due to anaphylaxis (133).

Reactions to PPD from black henna tattoos are also reported in children and teenagers. The contact allergic reactions resulting from prolonged skin contact (several weeks) and often a high concentration of PPD may be severe, oozing, and edematous. After sensitization to PPD in a black henna tattoo there is an increased risk of developing severe allergic contact dermatitis, due to high reactivity, upon subsequent encounter with PPD (54).

Systemic contact allergic reactions due to PPD have been reported both after hair dye and black henna tattoo exposure (134, 135).

Apart from allergic contact dermatitis, contact allergy to PPD may also present as true erythema multiforme, erythema multiforme-like reactions, or lichenoid reactions (1). One unusual case at our department of systemic allergic contact dermatitis to topical PPD has been reported with a histological picture of neutrophilic cellulitis with marked neutrophilic infiltrate and variable spongiosis (136).



**Figure 12. Allergic reaction to hair dye in a 12-year-old girl.** Facial erythema and edema. Left periorbital edema prevents opening of the left eye. Photo: Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden. Published with the permission of the patient.

## **Skin contact with PPD may cause systemic exposure to PPD**

Like other substances, PPD may reach the blood circulation through skin exposure, via the blood vessels in the superficial or deep plexuses in the dermis. Since the most part of systemic PPD is excreted via the urinary system, the majority of the systemic uptake of PPD can be studied in urine. Several studies have been performed on systemic uptake from skin exposure to PPD. When studying skin penetration by PPD and its systemic uptake, one must take into account that PPD can transform into other molecules – and in order to estimate the uptake, these PPD products must be traced in addition to the PPD molecule itself. Nohynek et al. performed tests with radioactively labeled PPD molecules in hair dyes containing 1.0% and 2.0% PPD. Only acetylated PPD was excreted in the urine. The amounts of PPD detected in urine, plasma, and skin strips were judged to be unlikely to cause any health risk in consumers of hair dyes (137, 138).

## **Toxicity from orally ingested PPD in humans**

PPD is highly toxic after oral intake. In the literature, cases of attempted and successful suicide using PPD –especially in Africa and Asia – have been reported, as well as its use for the induction of abortion. The reported effects in adults of acute poisoning by PPD are rhabdomyolysis with acute respiratory failure, acute renal failure, and myocardial lysis. In a material from northern Africa, cardiac toxicity was reported in 10% of PPD intoxication cases with a high level of mortality, close to 100%. These patients may present with acute respiratory failure caused by major cervicofacial and oropharyngeal edema, which requires nasal intubation and mechanical ventilation. In one case, a young pregnant woman ingested PPD and the expelled fetus was found to have myocardial lysis caused by the PPD (139).

## **The use of gloves for prevention of hairdressers' hand dermatitis**

Preventive work is a main component of occupational and environmental dermatology. Prevention of contact allergy to – and allergic contact dermatitis from – a substance like PPD in an individual may be carried out in several ways, such as decreased or banned exposure through legislation or avoidance of the allergen. In some cases the products used by the individual may be exchanged to equivalents without the allergens. Change of work may become necessary.

The great amount of wet work and contact with irritant and contact allergenic substances in hairdressing requires proper protection of the hands with gloves, both to prevent irritation from wet work and to prevent contact allergic dermatitis.

Adequate gloves must be used in a correct way to (i) prevent sensitization from occupational exposure to hair dye allergens (i.e. primary prevention), and (ii) hamper contact allergic dermatitis in those already sensitized (i.e. secondary prevention). Insufficient use of gloves has been reported in several studies on preventive behavior in hairdressers. This includes lack of glove use, re-use of disposable gloves and washing of gloves intended for single use. Re-use of contaminated gloves inside-out has been reported, leading to the combination of allergen exposure and occlusion. Some reasons for the incorrect use are based on economic or practical considerations and others stem from lack of information on correct glove use (118, 140, 141).

Hairdressing is an apprentice-based profession. The young hairdressers are initially educated by teachers in vocational schools, which is followed by learning from their seniors during internship. It is therefore important that education about correct glove use should be addressed early in vocational schools and that information on correct prevention is made available to as many hairdressers as possible who are active in the trade, to benefit both them and their apprentices. Studies on education programs have shown good results concerning increased use of protective gloves and less dermatitis of the hand (141-144) .

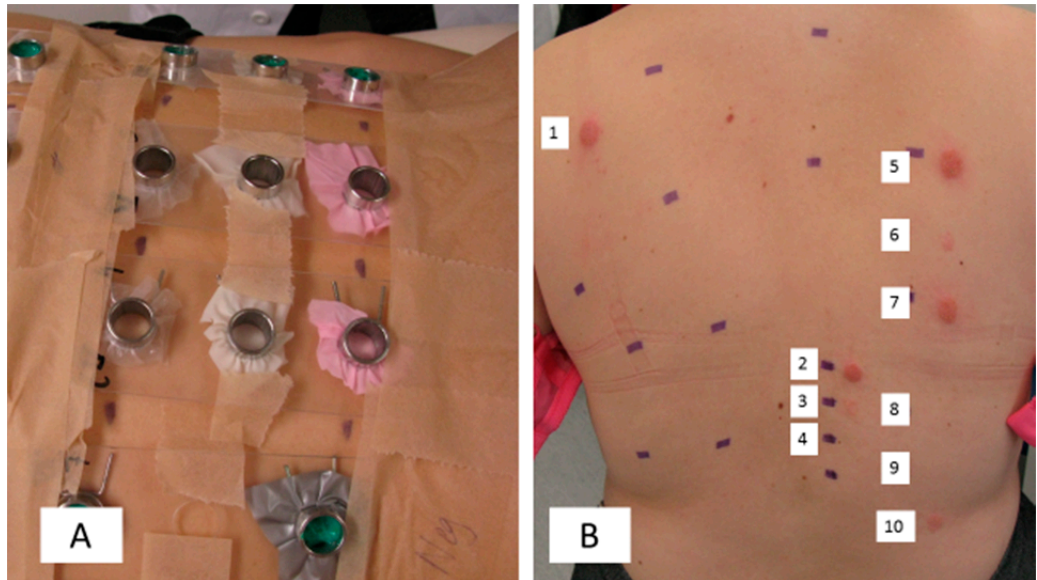
## Glove protection against a PPD-containing hair dye – a study in vivo

A method to study glove permeation of substances, such as allergens, in vitro has been described above (the Franz permeation cell). Studies in human volunteers, i.e. in vivo studies, may add important information about the biological response (dermatitis) from any permeated allergens. Such study in vivo was performed at our department in order to investigate how well disposable protective gloves protect against exposure to hair dye allergens (145). The study was performed on PPD-sensitized volunteers.

A hair dye for professional use containing PPD was used and mixed with a developer according to the instructions. The volunteers were exposed to the mixed hair dye directly on the skin, for up to 60 min. Furthermore the mixed hair dye was placed on pieces of the gloves to be investigated, which were in contact with the volunteers' skin and removed after 15, 30, and 60 min (145). For this investigation, a research patch test system developed by Andersson and Bruze

(146) was used. It consisted of chambers formed as tubes and made of stainless steel with an inner diameter 12 mm (Figure 13a). The glove material under investigation was mounted at the bottom of the tube. These chambers were then placed in a plastic support tray. The tubes and trays were mounted on the back of the volunteer using tape, while he/she was lying down (Figure 13a). The glove material was in touch with the skin and the test substance was placed inside the tubes.

The black hair dye used (Infinity permanent hair color 1.0 black; Affinage, Salon Exclusive, Milano, Italy) was mixed with a developer. The PPD concentration in the hair dye was 1.8%, corresponding to 0.9% in the mixed dye. The gloves investigated were of natural rubber latex (latex), polyethylene, polyvinylchloride (PVC), or nitrile. After removal of the chambers, no hair dye was visible on the skin. The patients returned for reading of the patch test D3 or D4 and on D7. Gloves of PVC, polyethylene, and latex did not prevent permeation of allergen(s). A small area with eczema did develop on the spot where the glove and hair dye had been placed (Figure 13b). Gloves made of nitrile prevented dermatitis (145). Results from this kind of in vivo study give information about the presence of permeated allergens, but not which allergens or the permeated amount. In order to establish which allergens have permeated the glove material other studies need to be performed such as permeation studies with the Franz cell.



**Figure 13. Patch test system for evaluation of glove protection in vivo (A) and patch test reading in the hair dye and glove study on day 4 (B).** A. Three test chambers were placed on each tray. Glove material was mounted at the bottom of the chambers. Several gloves and durations could be tested on the same occasion. B. One of the volunteers in the hair dye study performed by Antelmi et al. (145), at reading of the test on day 4. Numbers 5–10 indicate areas exposed to glove material and hair dye for 60 min. The reactions and scoring of selected patch tests are (from the left) 1: +++ for positive control (mixed dye directly on the skin for 60 min); 2: +++ for 0.1% PPD; 3: + for 0.01% PPD; 4: - for 0.001% PPD; 5 and 10: +++ and ++, locations for test with glove of polyvinylchloride; 6: + location for test with glove of polyethylene; 7: +++ location for test with glove of natural rubber latex; 8 and 9: - and -, locations for test with glove of nitrile. This volunteer had negative reactions in all areas that were exposed to glove material and hair dye for 15 and 30 min. Photos: Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden. Published with the permission of the patient.



# Aims

The general aim of the work presented in this thesis was to gain knowledge on contact allergy to the hair dye allergen PPD, regarding PPD allergens formed by oxidization and patch test reaction patterns to oxidized PPD, and also to compare how PPD, oxidation products of PPD, and the hair dye coupler resorcinol permeate hairdressers' disposable gloves in vitro – based on our previous study on glove permeation in vivo (145).

More specifically, studies I and II mainly concerned contact allergic responses to PPD-associated haptens. PPD is oxidized in hair dyes and the formed haptens are not fully known, including their possible clinical relevance. The objectives of studies I and II were:

- To study possible oxidation products formed from PPD based on a simplified hair dye model and using TLC (Study I and II).
- To investigate patch test reactions in individuals who were already sensitized to PPD, to substances that are formed when PPD is oxidized, including the PPD trimer Bandrowski's base (Study I).
- To extract and identify possible PPD allergen(s), based on the findings from study I, and to patch test with this/these substance(s) in PPD-positive volunteers (Study II).

Study III concerned patch testing with PPD. The routine patch testing of the allergen PPD is discussed regarding dose and patch test duration. The purposes of this study was:

- To follow the positive patch test reactions to PPD and its salt PPD dihydrochloride in serial dilutions over 28 days in order to characterize reaction patterns concerning time and dose in a cohort of individuals who were already sensitized to PPD (Study III).

Study IV concerned the use of gloves to protect against PPD-containing hair dyes and was based on the results from our previous study on hairdressers' glove permeation *in vivo*. The aims were

- To expose disposable, hairdressers' gloves to a mixture of a developer and black, permanent hair dye with PPD, and to study the presence and glove permeation of PPD, BB and resorcinol, as well as the two substances identified in study II, 4-nitroaniline and 4,4'-azodianiline, through the gloves. (Study IV)
- To compare the outcome of study IV with results from the study of hairdressers' gloves and mixed hair dye that was performed previously in PPD-sensitized volunteers (*in vivo*) at our department (145).

# Materials and methods

The materials and methods section has been simplified. The reader is kindly referred to the articles attached for technical data, suppliers, and other specifications regarding the equipment and chemicals used in studies I–IV.

The dilution series for patch testing (studies I–III) were prepared as follows. Stock solutions of substances to be tested were prepared and these were diluted further according to the study protocols (see below). The concentrations of substances for patch testing in dilution series were equimolar to the concentrations of PPD used. When a concentration of a substance was insoluble in the vehicle chosen, this concentration was tested in an alternative vehicle as indicated in the tables. Patch testing with the dilution series was performed on the backs of the volunteers using Finn chambers with a diameter of 8 mm. A micro-pipette was used to apply 15  $\mu$ l (29) of each test solution to the test chamber. For petrolatum preparations 20 mg (30) per chamber was applied. All test materials were removed after 2 days.

Patch test reading was performed according to the criteria of the ICDRG (32, 147). Treatment with topical corticosteroids was performed if necessary when the reaction was severe (e.g. oozing or extensive itching) at the reading of the test. If treated, the area was not read further.

## Study I

### **Study volunteers**

The inclusion criterion was a previously positive PPD patch test. Volunteers were individuals who had been previously tested with the baseline series at the Occupational and Environmental Dermatology department in Malmö, because of dermatitis and suspected contact allergy, and had tested positive to PPD in the previous 10 years. Fourteen female volunteers, mean age 53 years (range 20–77), were included. PPD allergy was due to professional exposure (hairdressers) or exposure as a consumer of hair dye and/or black henna tattoo.

## **Preparations for patch testing with PPD and oxidized PPD**

In order to study reaction patterns to PPD and its oxidation products, volunteers in study I were patch tested with dilution series of PPD, the PPD trimer Bandrowski's base (BB), and oxidized PPD prepared as described below.

### *Dilution series of PPD and Bandrowski's base*

The dilution series of PPD and BB tested are presented in Table 3. In study I, dilutions of PPD and BB were prepared with the highest concentration of PPD (1.0%) corresponding to 2.9% BB. Five additional concentrations per substance were prepared in 10-fold serial dilutions.

### *Thin-layer chromatograms of oxidized PPD*

As a measure to test the volunteers with oxidation products of PPD, a test preparation was prepared with PPD and hydrogen peroxide. This test solution contained unknown oxidation products. The substances in this solution were separated with thin-layer chromatography (TLC). These TLC strips with oxidized PPD were then used for patch testing (Figure 4).

The test preparation of oxidized PPD was prepared approximately 24 h before patch testing, as follows. A mixture according to these ratios was prepared: 40  $\mu$ l 30% w/w hydrogen peroxide was added to 1.0 ml 1.0% PPD in acetone. Without delay, 20  $\mu$ l distilled water was added to avoid formation of reaction products between acetone and PPD. The solution was stored at room temperature. The substances in this oxidized PPD solution were separated with TLC. In this case, acetone was chosen as the mobile phase for separation of the oxidized PPD solution because it showed the best separation of spots, so the samples were eluted on the TLC sheets using a mobile phase of 100% acetone.

We performed patch testing of TLC strips with oxidized PPD. The amounts of oxidized PPD solution applied to the TLC strips for testing were 5  $\mu$ l, 25  $\mu$ l or 50  $\mu$ l. The detectable spots (in daylight and/or under UV radiation) were numbered from 1 to 4, where number 1 indicated the site of application. Spots 1–3 were brown whereas spot number 4 was yellow (Figure 4). The TLC sheets were cut into strips of about  $2 \times 16$  cm, with a band of spots on each (Figure 4).

## **Patch testing**

### *Patch testing with dilution series of PPD and BB*

Patch testing was performed as described above, with preparations of PPD and BB as specified in Table 3. The individuals who had the highest reactivity at first PPD patch test had their highest test concentration of PPD reduced to 0.01%. If a

patient presented with a negative reaction on D3/4, the higher concentrations up to 1.0% PPD were tested. Thus, these reactions were only read once, on D3/4 after patch testing.

### *Patch testing with thin-layer chromatography strips*

The site of contact with the spots on the TLC strip were marked out on the patient's back and on the edges of the TLC strip. The chromatograms were applied with Scanpor tape and removed on D2. Initially, the patients were tested with TLC strips with 5 µl and 25 µl of an oxidized PPD solution. A patient who presented with a negative reaction to these TLC strips on D3/4 was tested using TLC strip with 50 µl of oxidized PPD, which was read on D3/4 after patch test application only.

### **Patch test reading**

Patch test reading of the dilution series and testing with TLC strips was evaluated and scored on D3/4 and D7, as described above.

### **Controls**

In study I, 15 consecutively patch tested dermatitis patients who were negative against PPD and other hair dye allergens were patch tested with 0.29% w/v BB in acetone, and served as controls. For comparison of test patients and controls, two-sided Fisher's exact test was used. Any p-value < 0.05 was considered significant. Data analysis was performed with the statistical software SPSS version 22.

## **Study II**

### **Extraction and chemical analysis of “the yellow spot”**

An analytical chemistry procedure used for identifying, detecting, and quantifying substances at the Occupational and Environmental Dermatology department in Malmö is gas chromatography (GC) separation followed by mass spectrometric (MS) detection (GCMS). In study I the yellow spot, spot number 4, was the one that most patients reacted to, and it was therefore chosen for further analysis in study II. The spot was extracted from a TLC of oxidized PPD (Figure 4) and samples were analyzed with gas chromatography mass spectrometry (GCMS).

For extraction, 5 TLC sheets with oxidized PPD were prepared for analysis in the same way as the ones that had been used for patch testing of patients (study I). Spot number 4 on the TLC sheets (Figure 4) was then scraped into a beaker and extracted with acetone, and then with methanol. These extracts were combined, filtered, and treated at 30°C using a rotary evaporator. The residue was dissolved in 1.0 ml ethyl acetate and this solution was used for chemical analysis.

Separation of components in the sample was performed with an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-MSI capillary column (Agilent Technologies). The carrier gas was helium. The injection volume was 1 µl. The gas chromatograph was connected to a Jeol GCmate II mass spectrometer (Jeol Datum Ltd., Tokyo, Japan). The National Institute of Standards and Technology (NIST; Gaithersburg, Maryland, USA) library of mass spectra was used for identification.

GCMS analysis identified 4,4'-azodianiline and 4-nitroaniline (Figure 6e and f) in spot number 4 from TLC strips of oxidized PPD. These substances were used to patch test PPD-sensitized volunteers.

### **Patch testing with 4-nitroaniline and 4,4'-azodianiline**

The 14 PPD-positive volunteers who participated in study I, and who were previously tested with the TLC strips of oxidized PPD, participated in the investigation. They were tested with dilutions of 4-nitroaniline and 4,4'-azodianiline as specified in Table 3. Patch testing and patch test reading of dilution series was performed as described above for dilution series.

### **Patch test reading**

Patch tests of the dilution series in study II were evaluated on D3/4 and D7.

### **Controls**

Fifteen consecutively patch tested dermatitis patients who were negative against PPD were patch tested with 4-nitroaniline (1.0% w/v in ethanol:acetone, 40:60) and 4,4'-azodianiline (0.020% w/v in acetone) and served as controls. For comparison of test patients and controls, two-sided Fisher's exact test was used. Any p-value < 0.05 was considered significant. Data analysis was performed with SPSS version 22.

## Study III

### Study volunteers

The inclusion criteria were an age of at least 18 years and a previous positive test reaction to PPD. In four study centers, dermatitis patients with previous positive reactions to PPD were identified from patch test data records, contacted, and invited to participate either by phone call or by letter. Altogether, 34 study subjects were included (6 in Barcelona, 10 in Gentofte, 6 in Odense, and 12 in Malmö).

### Preparation of PPD and PPD-DHC dilution series

In order to study reaction patterns to PPD over time, volunteers in study III were patch tested with dilution series of PPD and its salt, PPD-DHC. The concentrations of the two substances were equimolar and dissolved in the same vehicle (ethanol/water, 70:30 v/v). The substances tested are presented in Table 4. All solutions were prepared at the Department of Occupational and Environmental Dermatology in Malmö. The solutions were transported cooled, either by air or by road, to the three participating test centers outside of Sweden. The staff at the participating clinics were instructed to store the solutions in a refrigerator, and to keep track of the preparation date. The test preparation was supposed to be tested within one week.

### Patch testing and patch test reading

Patch testing of the dilution series of PPD and PPD-DHC was performed as described above for dilution series.

Patch tests were evaluated and scored after 2 days (a minimum of 30 min after patch test removal), followed by readings on D3/4, 7, 10, 14, 21, and 28. For each volunteer seven protocols were used, one for each patch test reading. The protocol consisted of two separate tables for PPD and PPD-DHC. A list for scoring was included, as well as a list of specified morphological evaluation features such as erythema, infiltration, papules, squamation, and pustules. Each of the 22 test spots was evaluated according to the morphological features, which were marked if present. This morphological evaluation served as a help for the patch test readers to make the overall scoring. Based on the morphological scoring, each reader of a test made an overall evaluation with score +/++/+++/-doubtful/irritant/no reaction. This scoring by the reader was then used in further analysis.

# Study IV

## Glove permeation experiments

### Gloves

For inclusion of gloves in the experiment, we (i) investigated the selection of gloves in four hairdresser retailer shops in Malmö and Lund, (ii) investigated the types of gloves used in previous studies, and (iii) ran pilot permeation tests, up to 4 hours, without hair dye to exclude gloves that release substances that might interfere with PPD and resorcinol in the HPLC analysis. The four gloves specimens included were of nitrile, polyvinylchloride (PVC), and of natural rubber latex (hereon referred to as latex) (2 sources). Substances from the excluded nitrile gloves that were retailed from the hairdresser shops interfered with the analysis, and were replaced with a nitrile examination glove that was used at the hospital.

**Table 1. Gloves used.** The 4 samples of disposable gloves used in the study were all white. The thickness was measured with a digital micrometer

Glove description	Glove name Distributor/ Manufacturer	Material Powdered/powder-free	Thickness, mm	Supplier
Nitrile	Examination gloves Evercare; OneMed Group Oy, Helsinki, Finland. Made in Malaysia	<b>Nitrile</b> Powder free	0.08 <sup>#</sup>	Glove used at the hospital in Malmö; same brand and material used by hairdressers.
Latex 1*	Guanti in lattice Il Monouso a Cincque Stelle Ro.ial. S.r.l. Agliaia, Italy. Country of manufacture not specified	<b>Natural rubber latex</b> Powdered	0.09 <sup>#</sup>	Hairdresser retailer, Bari, Italy.
Latex 2	Senso Skin Medika Medizintechnik GmbH, Hof, Germany. Country of manufacture not specified	<b>Natural rubber latex</b> Powder-free	0.13 <sup>#</sup>	Hairdresser retailer (Headbrands), Lund, Sweden.
PVC	Semper Guard disposable vinyl gloves Semperit Investments Asia Pte Ltd., JTC Summit, Singapore.	<b>Polyvinylchloride</b> Powder-free	0.07 <sup>#</sup>	Hairdresser retailer (HÅR för frisör), Malmö, Sweden.

\*glove that was used in our previous *in vivo* study with hair dye (145).

<sup>#</sup>Thickness measured 3 times; median value.

PPD, *p*-phenylenediamine; latex, natural rubber latex; PVC, polyvinylchloride.

The thickness of the glove specimen was measured with a digital micrometer (Mitutoyo Corp., Kawasaki, Japan). The latex gloves differed in thickness, the thin latex glove being designated latex 1 and the thick latex glove latex 2. Information on the materials and the thickness of the material measured is given in Table 1.

### *Hair dye and developer*

The hair dye Infinity permanent hair color 1.0 black (Affinage, Salon Exclusive, Milan, Italy) and Creme Developer with 6% hydrogen peroxide (Affinage, Salon Professional, Milan, Italy) were used for exposure in study IV. Labeled ingredients of the two products are presented in Table 2. This particular dye was used since the same dye had been used previously in a study in vivo on hair dye and glove protection, which was performed at our department and has been described above (145).

**Table 2. Oxidative hair dye and developer.** The product information and ingredients of the oxidative hair dye and developer used in the study IV, as specified by the manufacturer on the package.

<b>Product: Infinity permanent haircolour 1.0 black (Affinage, Salon Exclusive, Milano, Italy; made in Italy)</b>
Specified ingredients: Aqua, Glycol cetearate, Cocamide MEA, Cetearyl alcohol, Ceteareth-25, Myristyl alcohol, Cocamidopropyl betaine, Ammonia, Butyrospermum parkii (shea butter), Argania spinosa kernel oil, Datem, Oleth-5-phosphate, Dioleil phosphate, Sodium sulfite, Sodium hydrosulfite, Parfum (fragrance), Disodium EDTA, Laureth-3, <b>p-Phenylenediamine, Resorcinol</b> , p-Methylaminophenol sulphate, 4-Amino-2-hydroxytoluene, 2-Amino-3-hydroxypyridine, N,N'-bis(2-Hydroxyethyl)-p-phenylenediamine sulphate, 4-chlororesorcinol, 2-methylresorcinol, 4-amino-m-cresol, 6-amino-m-cresol, 2-amino-4-hydroxyethylaminoanisole sulfate, HC Red no. 3, HC Yellow No. 2, HC Blue No.2, Ethylhexyl methoxycinnamate
<b>Product: Creme Developer 6% 20 vol (Affinage Salon Professional, Milano, Italy; made in Italy)</b>
Specified ingredients: Aqua, Hydrogen peroxide, Cetearyl alcohol, Cetrimonium chloride, PEG 40-hydrogenated castor oil, Oxyquinoline sulfate, Disodium EDTA, Phosphoric acid, Dimethicone.

Datem, diacetyl tartaric acid ester of monoglycerides and diglycerides, E472, emulsifier.

### *Franz permeation cell*

A Franz permeation cell with two sampling ports (diameter 9 mm, receptor volume approximately 5 ml; Perme Gear, Hellertown, PA, USA) was used to study the chosen gloves made of nitrile, latex or PVC (Figure 5). The black oxidative hair dye was deposited on the glove material. The concentration of PPD and resorcinol in the receptor fluid was investigated over time, as was the presence of BB, 4-nitroaniline, and 4,4'-azodianiline.

In study IV, the receptor compartment was filled with distilled water. It was chosen as receptor fluid since non-polar solvents might interfere with the glove materials under study.

Pieces measuring  $2 \times 2$  cm were cut from the palm area of the four glove samples. The piece of glove was mounted between the compartments of the diffusion cell with the side intended to face the skin facing the lower compartment. In this way, the receptor fluid corresponded to the skin of the hairdresser. The temperature of

the cell was set to 32°C in accordance with normal palmar skin surface temperature (148).

The hair dye and developer were mixed 1:1 w/w according to the instructions for use. Using a 1-ml syringe, 0.3 ml of the mixed hair dye was drawn immediately after mixing, as is done when applied to the hair, and deposited carefully in the donor compartment of the Franz cell. The initially pearly whitish color crème started to become light brown immediately after mixing. Samples were drawn with a 100- $\mu$ l syringe from the receptor compartment of the Franz cell approximately every 30 min, for 4 hours. These samples were analyzed without delay.

### **Detection of PPD, resorcinol, and PPD oxidation products**

HPLC separation followed by UV spectrometric detection is an analytical chemistry method that is widely available and useful for detecting and quantifying substances. This method was used in study IV, with the addition of fluorescence spectrometric detection (FL), which was necessary in order to detect very low concentrations of PPD and resorcinol.

The concentration of PPD and resorcinol in the receptor fluid was investigated over time, as was the presence of BB, 4-nitroaniline, and 4,4'-azodianiline. For detection of the 5 substances in the receptor fluid, HPLC separation was used followed by FL for PPD and resorcinol and UV spectrometric detection for BB, 4-nitroaniline, and 4,4'-azodianiline. The HPLC system consisted of an L-2130 pump (La Chrome Elite; Hitachi High-Technologies Corp., Tokyo, Japan), a manual injector valve (Rheodyne 7125) equipped with a 20- $\mu$ l loop and a spectrofluorometric detector (RF-10A, Shimadzu, Kyoto, Japan) connected in series with an L-2455 diode array UV detector (La Chrome Elite; Hitachi High-Technologies Corp., Tokyo, Japan). The excitation wavelength for PPD and resorcinol was set to 285 nm and the emission wavelength was set to 350 nm (149). For monitoring of HPLC analysis, Azur 4.0 (Datalys, Martin D'Herès, France) and subsequently Chromeleon 7.2 (Thermo Scientific) was used. The UV-spectrometer was software-monitored by Ezchrome Elite software (Agilent Technologies, Inc., Santa Clara, CA, USA).

We used a silica-based Zorbax SB-C3 liquid chromatography column (4.6 internal diameter  $\times$  250 mm, and particle size 5  $\mu$ m; Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of water (A) and acetonitrile (B), both with 0.1% v/v trifluoroacetic acid (TFA). The elution was performed with a gradient starting at 15% B and was isocratic for 4 min; thereafter, the concentration of B was increased linearly to 95% over 3 min. The elution was then isocratic for 8 min, and thereafter the concentrations returned to the initial

conditions. Standards of 0.1–0.3 ppm PPD and 0.05–0.15 ppm resorcinol were freshly prepared for each permeation test. Analysis were done by manual injection.

The concentrations of PPD and resorcinol in the color crème were established with HPLC-FL. Analyses of PPD and resorcinol were also done over time in the complete hair dye, i.e. in the mixture of color crème and developer. For these analyses the hair dye was prepared in the same way as for the donor compartment in Franz cell. This was performed in the same system as described above after dilution 1,000 times.

## Main chemicals and patch test material (Studies I–IV)

The patch tested allergens in this work were 4,4'-azodianiline (95%; Acros Organics, Geel, Belgium), BB (ICN Biomedicals Inc, Aurora, Ohio, USA), 4-nitroaniline ( $\geq 99\%$ ; Sigma-Aldrich, Steinheim, Germany), PPD ( $> 99\%$ ; Sigma-Aldrich, St Louis, MO, USA), PPD-DHC ( $> 99\%$ ; Fisher Scientific, Bridgewater, NJ, USA), and resorcinol (Aldrich Chemie, Steinheim, Germany). For patch testing, Finn chambers 8 mm in diameter (Epitest OY, Tuusula, Finland) were used, and for patch testing with TLC strips, silica gel plastic roll (TLC plastic roll  $500 \times 20$  cm with silica gel 60F254; Merck KGaA; Merck, Germany) were used. For further details of chemicals and test materials, the reader is referred to the manuscripts.



# Ethics

In studies I–III the volunteers were informed about the procedures, the expected eczematous reactions, and possible adverse reactions. Informed written consent was obtained from all the subjects. These studies were approved by the Regional Ethical Review Board, Lund, Sweden (Study I and II: 2007, No. 327/2007; Study III: No.473/2011). In addition study III was also approved by ethical boards in Denmark (Videnskabsetisk komité, Projekt id S-20140002) and Spain (Comité de Ética de Investigación con medicamentos (CEIC), Barcelona, No. 2013/5260/I). These studies were conducted in accordance with the ethical standards specified in the Declaration of Helsinki.

All photographs in this thesis have been used with the permission of the individual who features.



# Results

## Study I

### **Patch test results with dilution series of PPD and BB**

The results of patch testing with dilution series of PPD and BB are shown in Table 3.

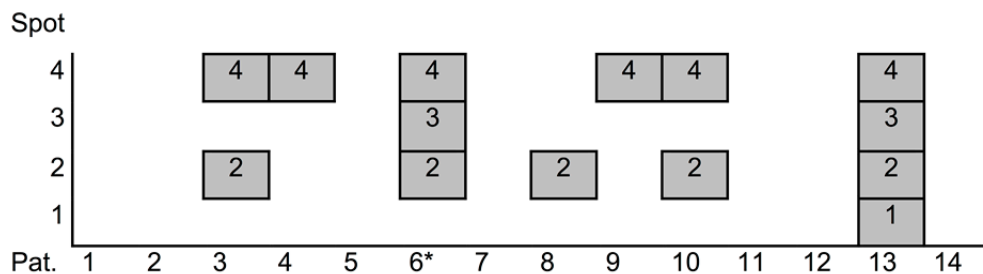
Of the previously PPD-positive individuals, 13/14 reacted to PPD. 7/13 (54%) reacted to BB. Of those volunteers who reacted to both PPD and BB, 6 volunteers had reactions to 0.10% PPD or less and one volunteer reacted to PPD 1.0%. Those who did not react to BB reacted to PPD at 1.0% only, and one of these volunteers did not react to PPD at all (6 of 6 vs. 1 of 8;  $p = 0.0047$ , Fisher's exact test, two-sided). In the test volunteers, 5/14 reacted to BB at 0.29% or less and none of the 15 controls reacted to 0.29% BB (5/14 vs. 0/15;  $p = 0.017$ ).

### **Patch test results with the TLC strips of oxidized PPD**

On the TLC strips, oxidized PPD was divided into 4 visible spots. Figure 4 shows the TLC strips and two examples of the patients' reactions to the TLC strips with oxidized PPD. The results from patch testing with the TLC strips of oxidized PPD are summarized in Table 3 and Figure 14. Seven patients reacted to one or more of the TLC spots in various patterns. Figure 14 is an overview of the different reactivity patterns, with boxes representing reactions to the spots on the TLC strips with any volume of oxidized PPD used (5, 25, or 50  $\mu$ l), whereas the reactions are shown separately in Table 3.

Altogether, of the 13 patients who were PPD-positive on patch testing with dilution series, 7/13 (54%) had a positive reaction to BB and the same 7/13 (54%) had a positive reaction to one or more of the TLC spots. Eight patients, the ones who did not have a reaction to the thin-layer chromatograms with 5  $\mu$ l or 25  $\mu$ l oxidized PPD on D3/4 were also tested with thin-layer chromatograms with 50  $\mu$ l. Patient number 9 was only tested with a thin-layer chromatogram with 5  $\mu$ l because of her high reactivity.

\*Patient 6 was tested with thin-layer chromatograms with 10  $\mu$ l and 20  $\mu$ l oxidized PPD; Pat., patient.



**Figure 14. Overview of the reactions to thin-layer chromatograms with oxidized PPD (study I).**

This figure indicates the TLC spots to which the test volunteers had a positive reaction, irrespective of the severity of the reaction and the amount of oxidized PPD on the thin-layer chromatogram (5  $\mu$ l, 25  $\mu$ l, or 50  $\mu$ l) to which the patient reacted

**Table 3. Results from patch testing with p-phenylenediamine, Bandrowski's base (BB), thin-layer chromatography (TLC) strips of oxidized PPD, 4-nitroaniline, and 4,4'-azodianiline (studies I and II)**

Test substance	Patient no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Concentration (%)*														
PPD	1.0	+	-	nt	nt	++	+++	++	+++	nt	nt	+	+++	nt	+
	0.10	-		++#	+	-	-	-	+++	++	nt	+	-	nt	-
	0.010			-	-	-			-	-	+	-	-	++	
	0.0010			-					-	+	-			-	
	0.00010									-					
	0.000010														
BB	2.9**	-	-	++	+	-	nt	-	nt	nt	nt	-	-	nt	-
	0.29		-	+#	-	-	+++	-	+++	nt	+	-	-	+++	-
	0.029			-			++		++	+	-			+#	
	0.0029						-		-	-				-	
	0.00029									-					
	0.000029									+					
4-nitroaniline	1.0***	-	-	-	-	-	nt	-	-	nt	+	-	-	nt	+
	0.13	-	-	-	-	-	++	-	-	nt	-	-	-	nt	-
	0.013	-	-	-	-	-	-	-	-	++	-	-	-	+	-
	0.0013	-	-	-	-	-	-	-	-	+	-	-	-	-	-
	0.00013	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.000013	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4,4'-azodianiline	1.0 <sup>§</sup>	-	-	nt	+	-	nt	-	nt	nt	nt	-	nt	nt	nt
	0.20	-	-	++#	+	-	+++	-	++	nt	++	-	+	nt	+
	0.020	-	-	+	-	-	+++	-	+	nt	-	-	-	+++	-
	0.0020	-	-	+#	-	-	+	-	-	+	-	-	-	+++	-
	0.00020	-	-	-	-	-	-	-	-	+	-	-	-	+	-
	0.000020	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TLC testing	Spot no.	Applied amount													
		1	50 µl	-	-	nt	-	-	nt	-	nt	nt	nt	-	-
			25 µl	-	-	-	-	-	-	-	nt	-	-	-	+++
			5 µl	-	-	-	-	-	-	-	-	-	-	-	-
	2	50 µl	-	-	nt	-	-	nt	-	nt	nt	nt	-	-	nt
		25 µl	-	-	+	-	-	++	-	+	nt	+	-	-	+++
		5 µl	-	-	-	-	-	+++	-	-	-	+	-	-	+++
	3	50 µl	-	-	nt	-	-	nt	-	nt	nt	nt	-	-	nt
		25 µl	-	-	-	-	-	+++	-	-	nt	-	-	-	+++
		5 µl	-	-	-	-	-	+++	-	-	-	-	-	-	+
	4	50 µl	-	-	nt	+	-	nt	-	nt	nt	nt	-	-	nt
		25 µl	-	-	+++	-	-	+#	-	-	nt	++	-	-	+++
		5 µl	-	-	-	-	-	-	-	-	++	-	-	-	+++

\*The vehicle was acetone and concentration % w/v except for:

\*\*2.9% w/w of BB prepared in petrolatum;

\*\*\*1.0% w/v of 4-nitroaniline. Vehicle was ethanol/acetone 40:60;

§1.0% w/w of 4,4'-azodianiline prepared in petrolatum.

PPD, p-Phenylenediamine; BB, Bandrowski's base

#Test reaction stronger on day 7 than on day 3/4.

Blank means negative. The strongest reaction is noted in the table.

## Study II

### **Analysis and detection of a yellow thin-layer chromatography spot**

Based on the reaction pattern from patch test results in study I, spot number 4 – the spot to which most patients reacted – was chosen for chemical analysis. The identification was performed by GCMS analysis, which revealed the presence of 4-nitroaniline and 4,4'-azodianiline in the extract of the yellow spot used previously for patch testing in study I. In study II, these identified substances were used to patch test the patients who were previously found positive to PPD and in controls.

### **Patch test results with 4-nitroaniline and 4,4'-azodianiline**

The test reactions to 4-nitroaniline and 4,4'-azodianiline are shown in Table 3. Five patients reacted to 4-nitroaniline and 9 patients reacted to 4,4'-azodianiline. Of the 6 patients who reacted to TLC spot no. 4, four reacted to 4-nitroaniline and all 6 reacted to 4,4'-azodianiline. Of the 8 patients who did not react to spot no. 4, one reacted to 4-nitroaniline and 3 reacted to 4,4'-azodianiline. The lowest 4-nitroaniline concentration to elicit any reaction was 0.0013% ( $n = 1$ ). One patient reacted to 4-nitroaniline at 0.013%, one reacted to 4-nitroaniline at 0.13%, and 3 reacted to 4-nitroaniline at 1.0% as the lowest concentration. The lowest 4,4'-azodianiline concentration to elicit any reaction was 0.00020% ( $n = 2$ ). Of the 15 controls, none reacted to 4-nitroaniline at 1.0% (0/15 vs. 5/14;  $p = 0.042$ ) and none reacted to 4,4'-azodianiline at 0.020% (0/15 vs. 5/14;  $p = 0.042$ ).

## Study III

### **Patch test results with dilution series of PPD and PPD-DHC**

The results of patch testing with dilution series of PPD and PPD-DHC are shown in Table 4. Of the 34 study subjects included in study III, 26 completed the study, i.e. they were patch tested with dilution series of PPD and PPD-DHC and the test area was read 7 times according to the protocol as presented in Table 4. 23/26 (88%) reacted to PPD at 1.0%, whereas 69% reacted to 0.32%; 42% and 27% reacted to the corresponding equimolar concentrations of PPD-DHC. No new reactions after day 7 were observed at any concentration tested, either with PPD or its salt (PPD-DHC), in study III. 8 (31%) of the previously PPD-positive test subjects and 5 (22%) of those who were currently positive to PPD at 1.0% were

negative on testing with 0.32% PPD on days 2–7, whereas the same number (n = 17) were positive to PPD at 1.0% and 0.32% on day 2. Reactions that did not appear until day 7 occurred in 4 of 26 study subjects (15%; volunteers 17, 19, 22, and 23). This pattern, reactions not appearing until D7, was also seen for 0.32% PPD in study subjects 20 and 23 (2/26) and for 0.1% PPD in study subject 13. In no other volunteer did any new reactions to PPD occur that had not appeared until D7. After D7, there were no new positive patch test reactions to any of the PPD concentrations tested. In Figure 15, the total number of positive reactions over time is shown for the 3 highest concentrations of PPD tested. Generally, positive reactions appeared between days 2 and 7 and then decreased in intensity. All these had disappeared by day 28.

15 study subjects in study III had positive reactions to PPD-DHC at 1.7%. Reactions that did not appear until day 7 occurred in 2/26 study subjects (8%; volunteers 9 and 11). In no other volunteers did reactions occur to PPD-DHC that did not appear until D7. There were no new positive patch test reactions after D7 to any of the PPD-DHC concentrations tested. In Figure 16, the total number of positive reactions over time is shown for the 3 highest concentrations of PPD-DHC tested. Generally speaking, positive reactions appeared on days 2 to 7, then decreased in intensity. All these had disappeared by day 28.

The test areas that were treated with corticosteroid cream due to extreme reactions, which occurred in 6 study subjects in study III on D2 or D4, were not read further i.e. from test reading D4 or D7. When we analyzed the number of reactions over time, these treated areas were counted as positive up to D14 and negative from D21, in accordance with the trend observed for untreated +++ reactions.

**able 4 Part 1/3. Test results for 26 volunteers tested with p-phenylenediamine (PPD) and p-phenylenediamine dihydrochloride (PPD-DHC) (study III): PPD 1.0% and 0.32%; PPD-DHC 1.7% and 0.53%. D4-readings were performed on either D3 or D4. Volunteer 19 was read on D9 instead of D10 and on D29 instead of D28.**

Pat.	PPD 1.0 %							PPD-DHC 1.7 %							PPD 0.32 %							PPD-DHC 0.53 %						
	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28
1	+++	+++	+	+	-	-	-	+++	+++	+	+	-	-	-	+++	+++	+	+	-	-	-	+++	+++	+	+	-	-	-
2	++	++	+++	+	+	-	-	++	++	++	+	-	-	-	++	++	+++	+	+	+	-	++	+	++	+	-	-	-
3	+++	C	C	C	C	C	C	+++	+++	+++	-	-	-	-	+++	C	C	C	C	C	C	++	++	-	-	-	-	-
4	+++	+++	+	-	+	+	-	++	+	++	+	-	-	-	+++	+++	+	-	+	+	-	+	+	-	-	-	-	-
5	+++	C	C	C	C	C	C	+	++	++	+	-	-	-	+++	C	C	C	C	C	C	-	+	+	-	-	-	-
6	++	+++	C	C	C	C	C	-	++	+++	+	-	-	-	+	+++	C	C	C	C	C	-	++	+++	-	-	-	-
7	+	+++	+++	-	-	-	-	++	+++	+++	+	-	-	-	+	+++	+++	-	-	-	-	+	+++	+++	+	-	-	-
8	+++	+++	C	C	C	C	C	+++	+++	C	C	C	C	C	+++	+++	C	C	C	C	C	+++	+++	C	C	C	C	C
9	++	+++	++	+	-	-	-	++	++	++	-	-	-	-	++	+++	++	+	+	+	-	-	-	++	-	-	-	-
10	+++	C	C	C	C	C	C	+	++	C	C	C	C	C	+++	C	C	C	C	C	C	++	+++	C	C	C	C	C
11	++	++	++	+	+	-	-	++	++	++	+	-	+	-	++	++	++	+	+	+	-	-	-	+	-	-	-	-
12	+++	C	C	C	C	C	C	+++	+++	+	-	-	-	-	+++	C	C	C	C	C	C	++	+++	+	-	-	-	-
13	+	+++	++	+	+	IR	-	-	++	+	-	-	-	-	+	++	+	-	-	IR	-	-	-	-	-	-	-	-
14	++	+++	+++	+	IR	-	-	-	++	+	-	IR	-	-	++	++	++	-	-	IR	-	-	-	-	-	-	-	-
15	+	++	-	-	-	-	-	-	-	-	-	-	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	-
16	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	++	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	+	++	++	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
21	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	++	++	-	-	-	-	-	-	-	-	-	-	+	++	++	++	+	+	+	-	-	-	-	-	-	-
23	-	-	++	+	-	-	-	-	-	-	-	-	-	-	-	-	++	+	+	+	-	-	-	-	-	-	-	-
24-26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

PPD, p-phenylenediamine; PPD-DHC, p-phenylenediamine dihydrochloride; NT, not tested; +, weak positive; ++, strong positive; +++, extreme reaction; IR, irritant reaction; C, cortisone cream treatment.

**Table 4 continued. Part 2/3. Test results for 26 volunteers tested with p-phenylenediamine (PPD) and p-phenylenediamine dihydrochloride (PPD-DHC) (study III): PPD 0.1% and 0.032%; PPD-DHC 0.17% and 0.053%. D4-readings were performed on either D3 or D4. Volunteer 19 was read on D9 instead of D10 and on D29 instead of D28.**

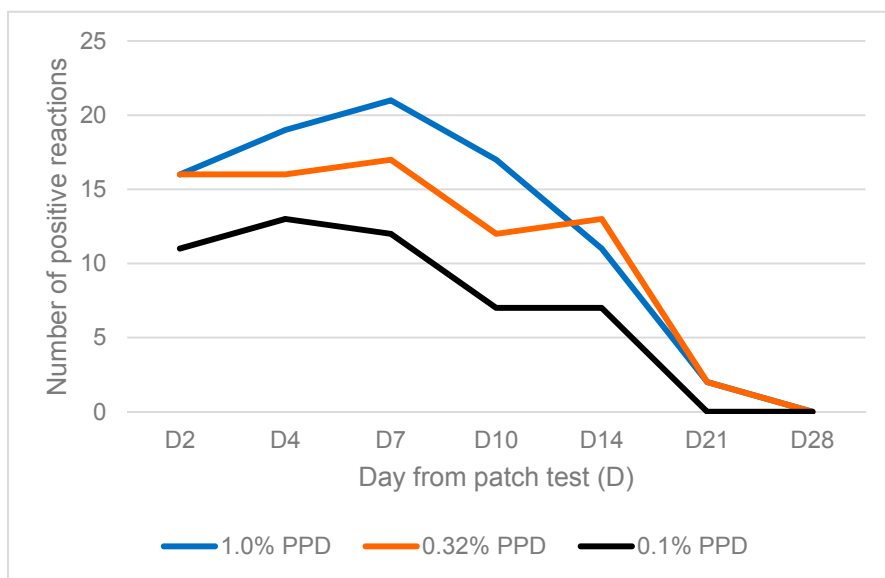
Pat.	PPD 0.1 %						PPD-DHC 0.17 %						PPD 0.032 %						PPD-DHC 0.053 %									
	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28
1	+++	+++	+	+	-	-	-	+++	+++	+	+	-	-	-	+++	++	-	-	-	-	-	++	++	-	-	-	-	-
2	++	++	++	+	+	-	-	+	+	++	+	-	-	-	++	++	++	+	+	-	-	+	+	++	+	-	-	-
3	+++	+++	C	C	C	C	C	-	++	-	-	-	-	-	+++	+++	C	C	C	C	C	+	++	+	-	-	-	-
4	+	++	-	-	-	-	-	-	-	-	-	-	-	-	+	++	-	-	-	-	-	+	++	-	-	-	-	-
5	++	C	C	C	C	C	C	-	-	-	-	-	-	-	-	++	++	+	-	-	-	-	-	-	-	-	-	-
6	-	+++	C	C	C	C	C	-	++	++	-	-	-	-	-	++	++	+	-	-	-	-	+	++	-	-	-	-
7	+	+++	+	-	-	-	-	-	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
8	++	++	C	C	C	C	C	++	+++	C	C	C	C	C	+	++	C	C	C	C	C	++	++	C	C	C	C	C
9	+	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	++	++	+	-	-	-	-	++	+++	C	C	C	C	C	+	++	-	-	-	-	-	+	++	+	-	-	-	-
11	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
12	++	+++	C	C	C	C	C	+++	+++	-	-	-	-	-	++	++	-	-	-	-	-	+	+++	-	-	-	-	-
13	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16-26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

PPD, p-phenylenediamine; PPD-DHC, p-phenylenediamine dihydrochloride; NT, not tested; +, weak positive; ++, strong positive; +++, extreme reaction; IR, irritant reaction; C, cortisone cream treatment.

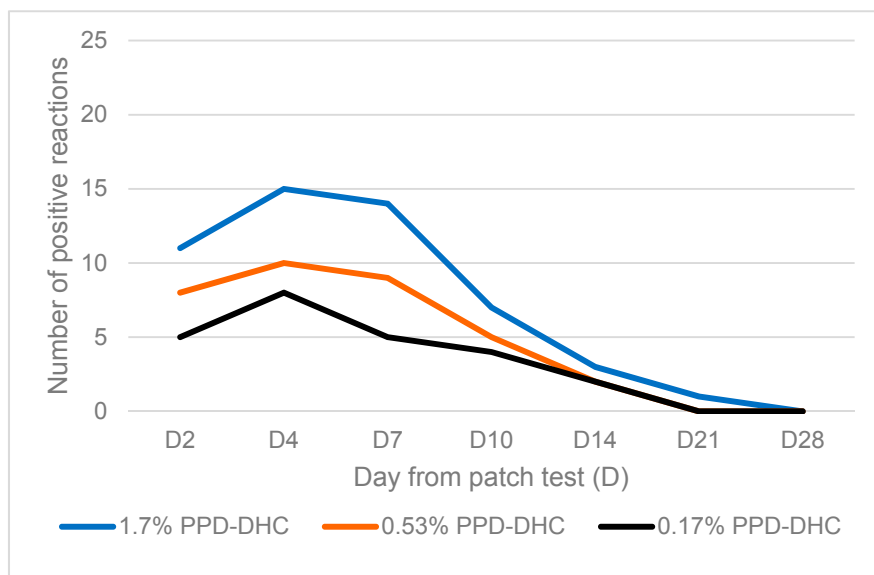
**Table 4 continued. Part 3/3. Test results for 26 volunteers tested with p-phenylenediamine (PPD) and p-phenylenediamine dihydrochloride (PPD-DHC) (study III): PPD 0.001% and 0.00032%; PPD-DHC 0.0017% and 0.00053%. D4-readings were performed on either D3 or D4. Volunteer 19 was read on D9 instead of D10 and on D29 instead of D28. Among the 3 lowest concentration tested for PPD and PPD-DHC not presented in this table volunteer 1 had + to 0.0001% and 0.000032% PPD.**

Pat.	PPD 0.001 %						PPD-DHC 0.0017 %						PPD 0.00032 %						PPD-DHC 0.00053 %									
	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28	D2	D4	D7	D10	D14	D21	D28
1	++	-	-	-	-	-	-	+	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

PPD, p-phenylenediamine; PPD-DHC, p-phenylenediamine dihydrochloride; NT, not tested; +, weak positive; ++, strong positive; +++, extreme reaction; IR, irritant reaction; C, cortisone cream treatment.



**Figure 15. Number of positive reactions to p-phenylenediamine (PPD) over time (study III).**

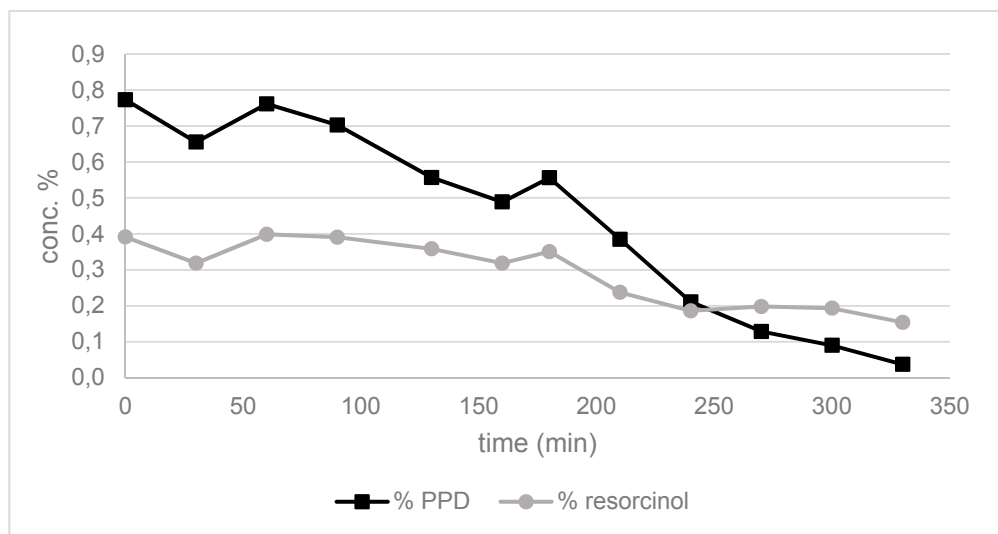


**Figure 16. Number of positive reactions to p-phenylenediamine dihydrochloride (PPD-DHC) over time (study III).**

## Study IV

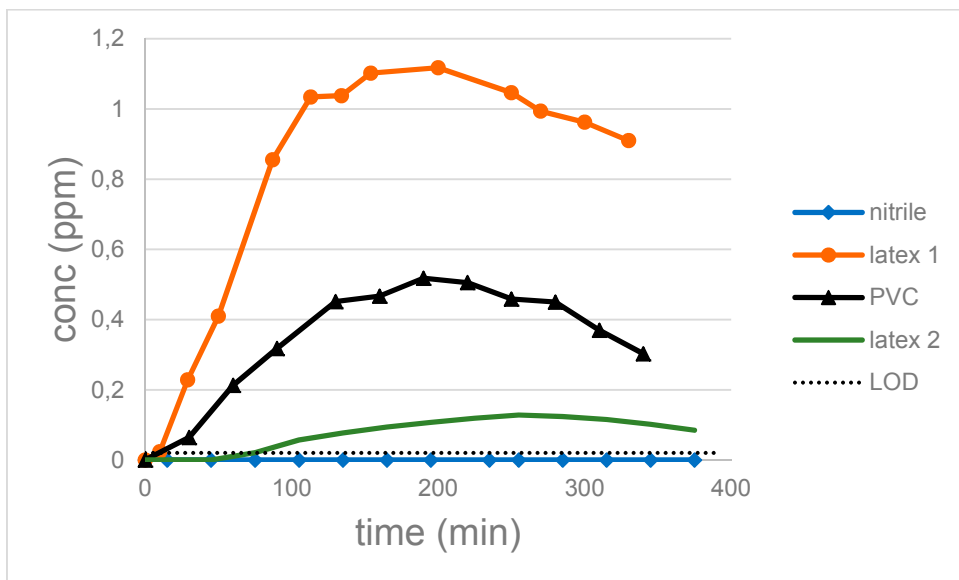
### PPD and resorcinol concentrations in the hair dye

The concentration of PPD in the color crème used was determined to be 1.8% and that of resorcinol to be 1.0%. After mixing the hair dye with the developer (50:50 w/w), the concentrations of PPD and resorcinol were analyzed over time (Figure 17). There was a delay of approximately 3 min due to mixing and diluting before the first samples were analyzed. The initially measured concentrations were 0.8% PPD and 0.4% resorcinol. Both PPD and resorcinol are consumed in the hair dye mixture. The concentration of PPD decreased faster than that of resorcinol (Figure 17). The mixed dye was kept at room temperature during the test period.



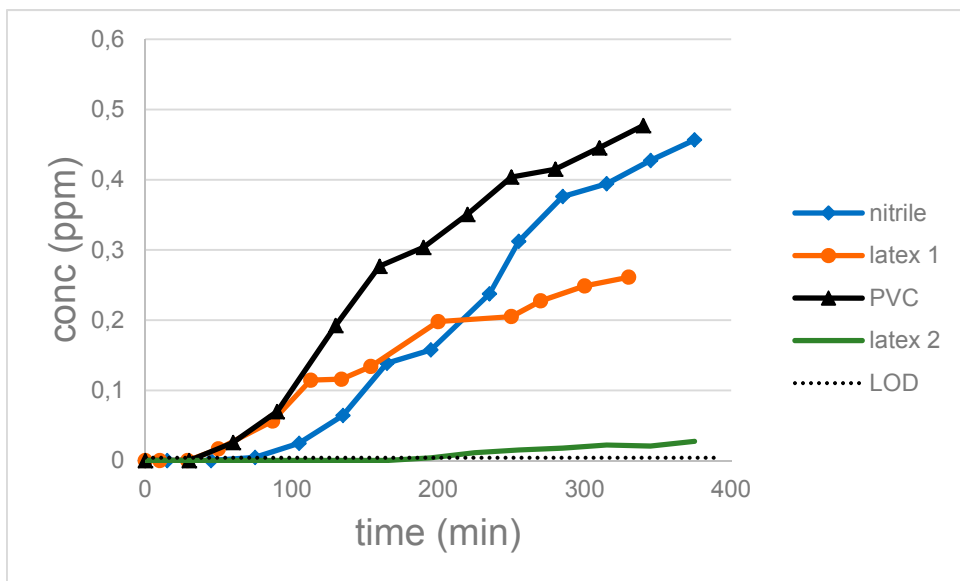
conc, concentration; PPD, p-phenylenediamine.

**Figure 17.** The concentrations of p-phenylenediamine and resorcinol in the mixed hair dye over time (study IV).



PPD, p-phenylenediamine; t, time from application of complete hair dye to the membrane; latex, natural rubber latex; latex 1, latex glove material with a thickness of 0.09 mm; latex 2, latex glove material with a thickness of 0.13 mm; PVC, polyvinylchloride; conc, concentration; ppm, parts per million; LOD, limit of detection (approx. 0.01 ppm).

**Figure 18.** The concentrations of p-phenylenediamine in the receptor fluid over time when using different glove materials as a membrane between mixed hair dye and receptor fluid in a Franz cell (study IV).



t, time from application of complete hair dye to the membrane; latex, natural rubber latex; latex 1, latex glove material with a thickness of 0.09 mm; latex 2, latex glove material with a thickness of 0.13 mm; PVC, polyvinylchloride; LOD, limit of detection (approx. 0.005 ppm).

**Figure 19. The concentrations of resorcinol in the receptor fluid over time when using different glove materials as membrane between mixed hair dye and receptor fluid in a Franz cell (study IV).**

## **Glove permeation of PPD and resorcinol**

The concentrations of PPD and resorcinol in the receptor fluid varied for the four gloves examined, as shown in Figures 18 and 19. The observed breakthrough time for PPD, i.e. the time when the first sample with measurable PPD concentration was drawn from the receptor fluid, was 10 min for the latex 1 glove, 30 min for the PVC glove, and 75 min for the latex 2 glove. No PPD was detected when the nitrile glove was analyzed for up to 330 min (Figure 18). Resorcinol breakthrough time was 50 min for the latex 1 glove, 60 min for the PVC glove, 75 min for the nitrile glove, and at 195 min with the latex 2 glove (Figure 19).

## **Glove permeation of BB, 4-nitroaniline, and 4,4'-azodianiline**

Neither BB, 4-nitroaniline, nor 4,4'-azodianiline was detected in the mixed hair dye. In the *in vivo* study, no BB was found in the hair dye (145). In the permeation experiments with PVC and latex gloves, 4-nitroaniline was detected in the receptor fluid with a detectable concentration after 230 min for the latex 1 glove, 375 min for the latex 2 glove, and 180 min for the PVC glove, and there was no detection for the nitrile glove. No BB or 4,4'-azodianiline was detected. The color of the receptor fluid remained colorless during the whole experiment for latex 2, nitrile and PVC glove. For latex 1 the color switched to yellow towards the end of the 4 hours. The color of the receptor fluid remained colorless during the whole experiment for the latex 2 and nitrile glove material. For PVC and latex 1, the color switched to yellow towards the end of the 4 hours, which may have been due to formation of 4-nitroaniline. The mixed dye in the donor compartment successively turned from light brown to dark brown to black over time.

# Discussion

After more than 100 years, PPD is still on the hair dye market and keeps causing contact allergic dermatitis worldwide, mainly from hair dyes and black henna tattoos (113-118). This, together with the clinical challenges of contact allergy to PPD described above, is the main reason for this thesis. When PPD is oxidized either by oxygen in the air or oxidizers in products, more than one possible PPD hapten may be formed. The haptens causing the allergic reactions are not entirely known.

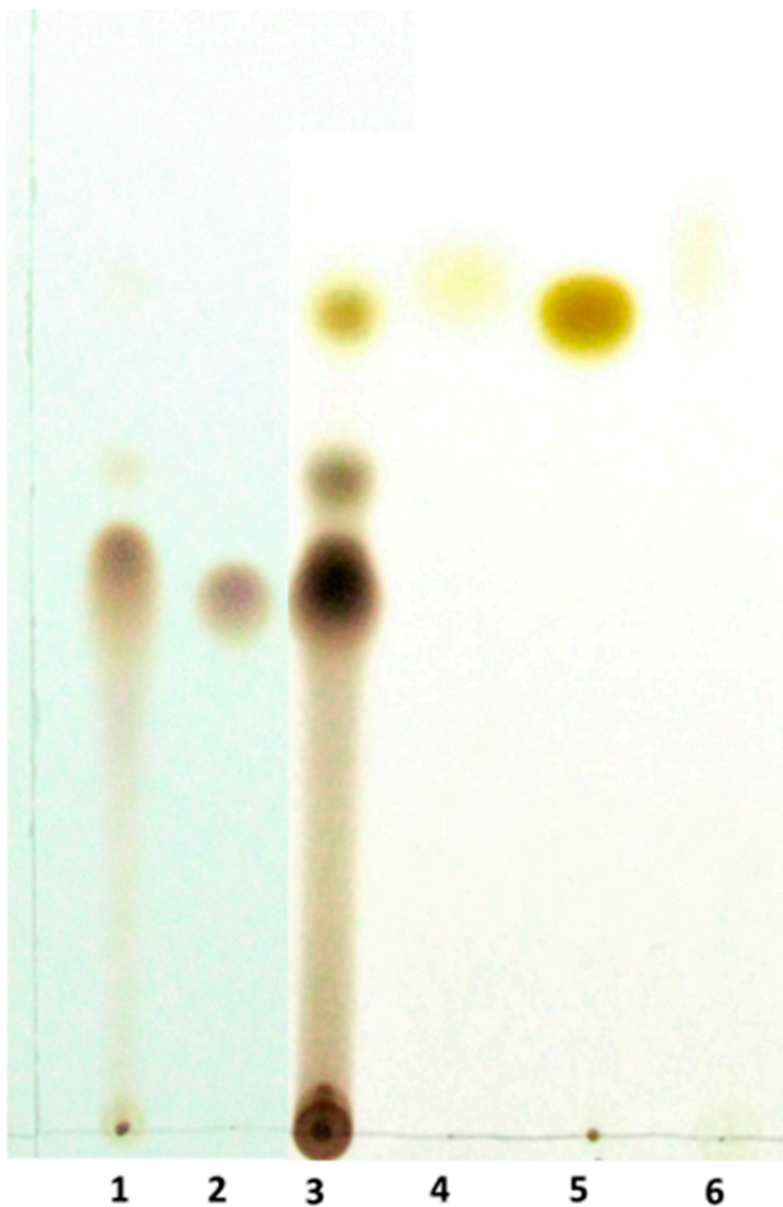
## Studies I and II

In patch testing, the aim is to expose the skin to the substances that the individual has been exposed to previously and to elicit a contact allergic reaction if the individual is sensitized. In study I, we aimed to patch test the PPD-sensitized individuals with substances – PPD oxidation products that are possible haptens – which correspond more to the exposure in hair dyes than the conventional PPD patch test. We wanted to find a way of looking at the possible haptens formed when PPD is used in hair dyes. The oxidized PPD model used, enables the study of possible contact allergic responses to the substances formed in the hair dye process, from the reaction between PPD and hydrogen peroxide. Patch testing with the oxidized PPD solution separated on thin-layer chromatograms allows differentiation between separate reactions to several substances, as opposed to testing the whole solution. Moreover, the oxidized PPD used in the present study was more analogous to exposure from hair dyes, than patch testing with unoxidized PPD.

The PPD-sensitized volunteers in study I reacted with different patterns to the spots, suggesting the presence of several allergens. The 4 TLC spots may each have consisted of one or several substances. Thus, the patients may theoretically have tested positive for a spot that contained one or more possible sensitizing compounds, i.e. oxidation products and other derivatives of PPD. Also, the presence of sensitizing contaminants must always be considered. Different

sensitization patterns to the TLC strips found in the test volunteers may be explained by factors such as differences in the exposure levels of PPD, varying enzymatic properties of the skin (94), or the existence of several allergens involved in contact allergy to PPD. If different potent allergens are involved in the sensitization of volunteers, they might show varying patterns of reactivity and cross-reactivity. Our results showed two allergens that are formed during oxidation of PPD: 4-nitroaniline and 4,4'-azodianiline (study II). These substances have been found by our group in one hair dye product in powder form (53).

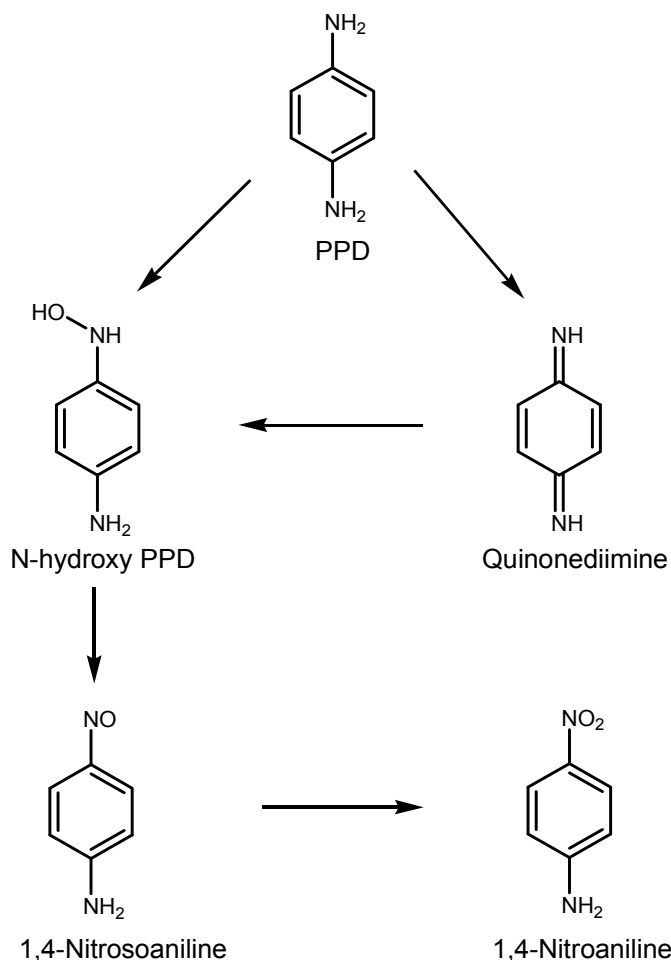
In Figure 20, comparative thin-layer chromatograms with BB, PPD, oxidized PPD, 4-nitroaniline, 4,4'-azodianiline, and an extract of TLC spot number 4 are shown. These chromatograms were eluted in the same system and for the same time as oxidized PPD. In the comparative TLC, the main BB spot is brown and located halfway up the TLC plate, slightly above the main PPD spot and spot number 2 of the oxidized PPD (Figure 20). The proximity might indicate that PPD may contain BB and vice versa. The association between the volunteers' reactions to BB and their reactions to the thin-layer chromatograms also suggests the possibility of impurities of BB in PPD and vice versa. However, the analytical HPLC analysis showed only  $< 0.2\%$  BB in the freshly prepared PPD solution and  $0.3\%$  PPD in the BB used. It is unlikely that the contamination demonstrated has any significance for the patch test results regarding PPD and BB. The concentration of BB in PPD may increase depending on time, the nature of the solvent, and temperature.



**Figure 20. Thin-layer chromatograms with oxidized PPD and reference substances (studies I and II).** From the left, the six solutions applied were (1) Bandrowski's base (BB), (2) p-phenylenediamine (PPD), (3) oxidized PPD, (4) 4-nitroaniline, (5) 4,4'-azodianiline, and (6) an extract of spot no. 4 from the thin-layer chromatogram of oxidized PPD in study II. Acetone was used as eluent.

The reactions to PPD and BB could also indicate cross-reactivity between the two substances. In a previous study where 43 PPD-positive patients were patch tested with BB, 7 (16%) were positive to either 0.1% or 1.0% BB (92). These results show a lower prevalence than in study I, where 6 patients of 14 (43%) reacted to BB at 0.29% or less. In total, 7 individuals who were hypersensitive to PPD tested positive for BB in study I (50%). Furthermore, 3/3 volunteers who tested positive with 0.01% PPD or less were positive for BB, 3/4 volunteers who tested positive down to 0.1% PPD were positive for BB but only 1/7 volunteers who tested negative for PPD or positive with no less than 1.0% PPD was positive for BB. These figures, with an association between the degree of reactivity to PPD and a simultaneous positive reaction to BB, suggest the possibility of cross-reactivity between the two substances.

4-Nitroaniline is mainly used industrially as a precursor to PPD, and is commonly used in the synthesis of dyes (150). It is a starting material for the synthesis of Para Red, one of the first azo dyes, which has been used since 1880 (151, 152). 4-Nitroaniline has been evaluated as a possible degradation product of Disperse Orange I (Figure 7) (92), which is a common allergen in the textile dye mix (63). A possible oxidation pathway of PPD to 4-nitroaniline is shown in Figure 21.

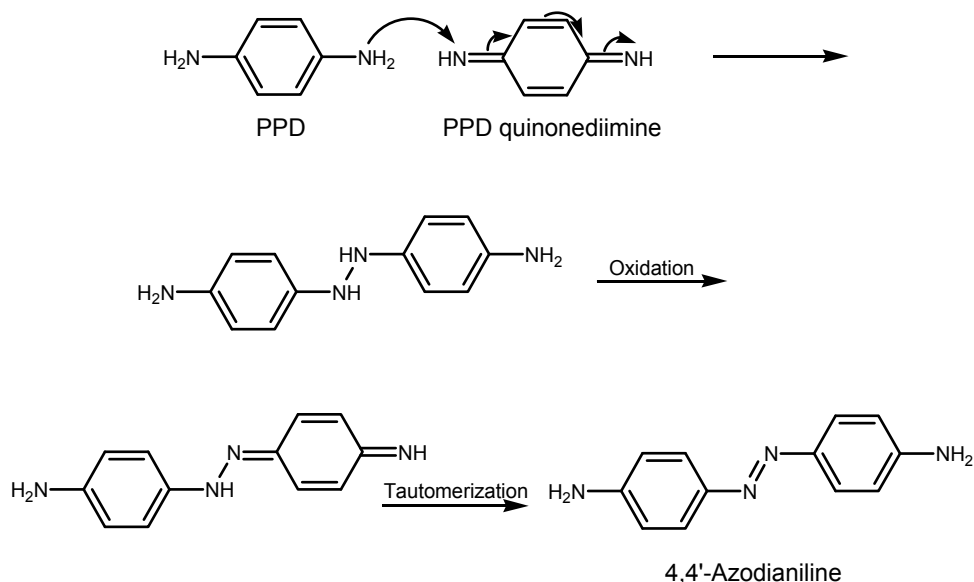


**Figure 21. Proposed reaction pathways in the formation of 4-nitroaniline from p-phenylenediamine (PPD) by auto-oxidation and/or oxidative metabolism (study II).**

4,4'-Azodianiline is an azo compound with the characteristic nitrogen-nitrogen double bond, called an azo group. When investigated contact allergy to BB, White et al. found 4,4'-azodianiline as an impurity in BB when purity analysis was performed on the test substance. In our material,  $n = 7/9$  of the volunteers who were positive for 4,4'-azodianiline showed reactions to BB (studies I and II) (92). The proposed oxidation pathway of PPD to 4,4'-azodianiline is shown in Figure 22. Of the 6 volunteers who reacted to spot number 4, all 6 reacted to 4,4'-azodianiline and 4 reacted to 4-nitroaniline. Interestingly, of the 8 volunteers who did not react to spot number 4, three reacted to 4,4'-azodianiline and 1 reacted to

4-nitroaniline. One explanation of this might be that the concentration of these substances was too low in the TLC spot to elicit a reaction.

The formation of several possible haptens might be part of the explanation of the association between PPD and other groups of allergens such as black rubber mix and textile dyes. The identification of haptens involved in contact allergy is of importance to enable evaluations of occupational and non-occupational exposure as well as prevention of contact allergy and contact allergic reactions to PPD. Investigating the reactions to substances formed in permanent oxidative hair dyes may be of importance for development of preventive measures, such as recommendations for protective gloves for handling of hair dyes or the development of new, safer hair dye products. Cross-reactivity to PPD might be an explanation for why PPD serves well as a marker for hair dye allergy, but little is known about this cross-reaction pattern. With regard to the identified allergens 4-nitroaniline and 4,4'-azodianiline, it is impossible to determine whether the simultaneous reactions found are due to cross-reactions or concomitant primary sensitization to these chemicals. To investigate cross reactivity animal studies such as guinea pig maximization test must be performed (68, 72).



**Figure 22. Possible reaction pathway from p-phenylenediamine (PPD) to 4,4'-azodianiline (study II).**

## Study III

Study III was designed to investigate reactions to PPD dilution series over time, and to determine whether we could detect any late reaction – first appearing at D10 or later. When patch testing with PPD, the test leaves a dark spot on the skin. In spite of the fact that in study III we did not treat PPD with an oxidizing agent for patch testing, as was done in study I, we are aware of the possible oxidation of PPD by for example oxygen in the air. Due to this oxidation process, patch tests with PPD may also contain the PPD oxidation products described.

In study III equimolar dilution series of PPD and its salt PPD-DHC were tested, and the patch test reactions were observed over time for 4 weeks in 26 already PPD-sensitized study subjects. None of the 26 study subjects had late-appearing reactions to PPD after D7 (Table 4). We did not see any late-appearing reactions to PPD-DHC either, although due to its high polarity this substance can be expected to have slower skin penetration and thus later elicitation. In study III, we were unable to show that slower penetration or lower concentrations would lead to new reactions after D7. However, lower concentrations produced later reactions in some cases. This concerned 3 volunteers with new reactions at reading on D7 (for PPD in subject no. 20 and for PPD-DHC in subjects no. 9 and 11). When comparing D2 and D4, we saw new reactions developing at D4, to lower concentrations in 8 cases (for PPD in subjects no. 2, 5, 6, 7, and 15, and for PPD-DHC in subjects no. 3, 5, and 7).

Hillen et al reported a rate of 1.5% (n=21/1,428) delayed PPD reactions, but patients who were positive on D7 were included. If we apply our definition of a late patch test reaction to the Hillen material, 3 patients can be excluded and the rate becomes 1.3%, which is still a high figure for possible active sensitization. The data presented by Hillen et al. together with other PPD patch test data constituted the basis for the recommendation in Germany in 2006 to stop routine patch testing with PPD at 1.0%, with subsequent recommendation to lowering the patch test concentration to 0.3%, to reduce the risk of active sensitization (108). Others have questioned these findings meaning that the risk for active sensitization has been overestimated and have promoted the keeping of PPD 1% for routine patch testing to avoid missing contact allergy to PPD (100, 107, 110, 153).

The time for patch test reading varies in different patch test centers. Some late reactions, regardless of the cause, will be missed if patch test reading is performed before the positive patch test reaction has appeared. Reading only on D2 in study III would miss 10/26 (38%) of the previously PPD-positive individuals, or 7/23 (30%) of the currently positive test subjects. In study III, 4/23 study subjects did not show positive reactions to 1.0% PPD until D7. This means that if the last reading had been on D4, we would have missed 17% of the sensitized volunteers.

Geier et al. recommended replacing PPD 1.0% pet. in the baseline patch test series with PPD 0.3% pet. (99). Also, removal of PPD from the baseline series has been suggested with the recommendation to only test with it when there is a direct suspicion of contact allergy to PPD, in order to avoid patch test sensitization (111). In study III, 23/26 study subjects reacted to PPD at 1.0%, whereas 18/26 reacted to 0.32% PPD. Lowering the routine patch test concentration to 0.3% might have missed diagnosis of contact allergy to PPD in 5 study subjects (22%) among those who showed reactions, and in 8 (31%) among subjects who were previously found to be PPD-positive. In a recent study good agreement was shown to PPD patch tests of equivalent doses of 1.0% and 0.3% pet. preparations (154).

The scoring criteria are adapted to patch test reactions that become positive within 7 days. In study III, we followed the reactions until D28. During the healing process, the appearance of the positive patch test reaction site could change to show a morphology that looks more like an irritant reaction. In the summing-up of the test results, the overall scoring of the test readers was used.

Regarding PPD and PPD-DHC, the molecules should give rise to common haptens due to their molecular likelihood and this would explain why the volunteers who reacted to PPD also reacted to a high degree to PPD-DHC. The reason for PPD giving generally stronger reactions than PPD-DHC might be due to the salt being more polar and thus being less liable to penetrate stratum corneum. Regarding PPD-DHC, one would expect few positive reactions due to its high polarity. Surprisingly we have seen multiple reactions. Antelmi et al. patch tested 718 consecutive patients with PPD at 1.0%, PPD at 0.1%, and PPD-DHC at 1.7% in petrolatum. Of these, 26 were positive to PPD at 1.0%, 8 were positive to PPD at 0.1%, and 6 were positive to PPD-DHC at 1.7%. Of the 6 patients who were positive to PPD-DHC, 5 had simultaneous reactions to equimolar PPD (155).

In conclusion, none of the 26 test subjects in study III reacted with late-appearing reactions to any concentration tested, either with the base or the salt. Even though late reactions to PPD that are due to causes other than patch test sensitization might occur, they appear to be infrequent. Chaudry et al. conducted a test reading once sometime between day 7 and day 14 and found no new positive reactions to PPD (156). Aalto-Korte et al. reported late reactions in a material of 826 patients tested with PPD, on day 10–14, in 6 cases (16), who then reacted early on re-test. The risk of patch test sensitization must be considered when routinely patch testing with 1.0% PPD. On the other hand, the risk of missed contact allergy must be considered, if reducing the test concentration of PPD or excluding PPD altogether from routine patch test. In study III, testing with 0.32% PPD at the most would have resulted in false negative reactions in 8 (31%) of the test subjects who were previously positive to PPD.

## Study IV

In study IV we focused on protective gloves for prevention of occupational hand dermatitis caused by hair dyes in hairdressers. Protection from hair dye allergens was investigated, but wet work, irritant substances, and other allergens are also risk factors for the development of hand dermatitis when performing hairdressing.

In study IV, four different gloves, made of materials commonly used by hairdressers when performing hair dyeing, were assessed regarding efficacy in protecting against PPD and resorcinol in a mixed oxidative hair dye. We also investigated the presence of the three PPD oxidation products (and possible hair dye allergens), BB, 4-nitroaniline, and 4,4'-azodianiline. We also wanted to compare the outcome with results from an *in vivo* study that had been performed earlier with the same hair dye at our department (145).

The four gloves (Table 1) were of nitrile, latex, and PVC. These are commonly used by hairdressers and were similar to those previously assessed regarding their efficacy in protecting against PPD and resorcinol. The exposure time in the present study was intended to exceed the duration of routine hair dyeing, up to 60 minutes, and lasted up to 4 hours to make comparison with other similar studies possible (145). Our results showed a superior protection towards PPD by the nitrile glove (Figure 18). The short permeation time for the PVC glove makes this type of glove unsuitable as protection against PPD. The two latex glove materials differed in their ability to protect, with the thin latex 1 glove having a similar, short breakthrough time and permeation of PPD to those of the PVC glove, whereas PPD permeated the thicker latex 2 glove to a lesser degree. Regarding the permeation of resorcinol, the lowest concentrations were detected for the latex 2 glove and this glove had the longest breakthrough time of more than 3 hours (Figure 19). The highest permeability of resorcinol was detected with the PVC glove.

Both protective gloves (latex and PVC) and medical examination gloves (nitrile) were examined in study IV. Protective gloves have higher requirements regarding permeation of chemicals, and are recommended for protection against hair dye chemicals. Even so, no PPD was detected using the nitrile glove material intended for medical examination in the present study. We conclude, based on the results from the present study that disposable nitrile gloves are well suited to protect against PPD in hair dyes.

The results were compared with previous permeation tests of gloves of latex, PVC, nitrile, and polyethylene (PE), performed by Lind et al., which had lasted up to 4 hours (157). Permeation of PPD, toluene-2,5-diaminesulphate (TDS), and resorcinol using the American Society for Testing and Materials (1-inch) test cell (ASTM cell) was examined (157). The exposed area in the ASTM cell used by

Lind et al. was  $4.15 \text{ cm}^2$ , as compared to  $0.64 \text{ cm}^2$  in the Franz cell used in study IV and an exposure area of  $1.13 \text{ cm}^2$  in the device used in vivo by Antelmi et al. (145). The study by Lind et al. (157) differed in many ways from study IV in addition to the different permeation cells, which makes comparison of the results difficult. Higher concentrations were used by Lind et al. in the exposure solutions: 5% PPD (w/v), 0.75% TDS, and 10% resorcinol. The higher concentrations used in the study by Lind et al. would theoretically result in shorter permeation times for PPD and resorcinol, for equivalent glove types, compared to study IV. Lind et al. found that permeated amounts of the substances tested were all below the level of detection for the latex glove (157), which contrasts with the results of study IV, where the latex 1 glove performed worst with PPD being detectable already after 10 min, and then after 75 min with the thicker latex 2 glove. This can partly be explained by the difference in thickness of the latex glove material used, which was thicker (0.2 mm) in the Lind study, but thinner in study IV (0.09 mm and 0.13 mm). The PVC glove gave the lowest protection against PPD and resorcinol in the study by Lind et al. (157). TDS did not permeate any of the gloves in the Lind study (157).

The glove permeation will be influenced by the material of the glove, but also other glove related properties such as thickness and age. Furthermore the temperature and the vehicle and mix of substances in contact with the glove and skin/receptor will influence the permeation. When investigating permeation of hair dye substances, one must take into account whether the exposure solutions under study are of single or mixed test substances. Lind et al. used single substance preparations. Contrary, in study IV a complete hair dye was investigated, which makes the study a better simulation of the real-life exposure in hairdressers.

A permeation study using hairdresser gloves of PVC, latex, and neoprene, which were challenged with single and mixed hair dye allergen solutions (of PPD and three other known hair dye allergens, p-aminophenol, m-aminophenol, and o-aminophenol with and without the oxidizer hydrogen peroxide) was performed by Lee et al. (158). Resorcinol was not tested. Nitrile gloves were not included. Ethanol solutions and 12% hydrogen peroxide solutions were used for exposure. Lee et al. found a PPD breakthrough time of  $> 240 \text{ min}$  in both single (4% PPD) and mixed (1% PPD) solutions with hydrogen peroxide for the PVC and latex gloves included. None of the substances investigated permeated the neoprene gloves exposed for 4 hours. The mixture and oxidizing agent makes the Lee et al. study closer to real-life exposure compared to the study by Lind et al., but Lee et al. used solutions with a limited number of substances and not a complete hair dye cream as in study IV. Furthermore, the permeation experiments by Lee et al. were performed at room temperature, which means that the real-life situation with higher temperature would result in higher quantities permeating than measured in the study. In this type of study, some of the oxidation reactions that happen in a

permanent hair dye with PPD can be expected to occur, with formation of some of the PPD-related contact allergens responsible for hair dye contact allergy.

Antelmi et al. performed an *in vivo* study with challenge in 9 PPD-sensitized test subjects who were exposed to mixed hair dye, with or without glove material between the skin and dye, for up to 60 min. The same hair dye with 1.8% PPD as in study IV was used. Latex gloves latex 1 were used. No visible hair dye contamination was seen on the skin after the removal of the tests. On test readings after 4 and 7 days, the exposed skin was evaluated for contact allergic dermatitis. Positive reactions were seen where latex and PVC gloves had been used. Nitrile gloves gave good protection in all 9 test subjects (145). The results in study IV are in consistency with the results of the *in vivo* study.

In study IV, the existence of 3 possible PPD allergens was investigated besides PPD, and we detected 4-nitroaniline in the receptor fluid in permeation experiments with the PVC and latex gloves. Neither BB nor 4,4'-azodianiline was detected. 4-Nitroaniline was shown to induce contact allergic dermatitis upon patch testing of PPD-positive individuals in study II. The presence of 4-nitroaniline further advocates the avoidance of gloves made of latex and PVC while handling hair dyes, even though the substance was detected late in the tests, at a time exceeding the 60 min of supposed hair dyeing activity. 4-Nitroaniline and other allergens may have been present in lower doses than we could detect, but still in doses that were high enough to induce an immunological contact allergic reaction.

In the glove experiment in study IV, 4-nitroaniline was not detected in the mixed hair dye studied for 4 hours, but it was detected in the receptor fluid coinciding with the lowering of the PPD concentration (Figure 18). We can therefore assume that some of the PPD which permeated the gloves was oxidized to 4-nitroaniline, as shown in Figure 21. In the hair dye other oxidation products may be favoured due to the presence of couplers and/or other dye molecules, and also hydrogen peroxide.

The gloves recommended when performing hair dyeing must give good protection against the current hair dye allergens and be harmless to the skin. In conclusion, our results in study IV indicated that disposable nitrile gloves give good protection against PPD in oxidative hair dyes. Resorcinol permeated the nitrile gloves in study IV, although this occurred after a long time that exceeds the duration of normal hair dyeing. Neither PVC nor latex gloves are suitable for protection against hair dye allergens according to our data. Nitrile gloves are recommended for dyeing hair and handling hair dyes.



# Summary of conclusions and concluding remarks

The work presented in this thesis concerns investigation of PPD haptens that individuals are exposed to in commercial products, the study of patch testing with PPD, and prevention of PPD contact allergy and contact allergic dermatitis. We demonstrated the presence of several PPD-associated allergens that might be formed in a permanent hair dye, or through other processes in which PPD is oxidized (study I). Furthermore, the results show that 4-nitroaniline and 4,4'-azodianiline, which are formed during oxidation of PPD, are potent allergens that a substantial proportion of PPD-sensitized patients react to (study II). Moreover, we studied the patch testing of PPD and its salt PPD-DHC. When patch testing with PPD, we could clearly see a risk of missing contact allergy when the dose was decreased (study III). We found no proof of late-appearing reactions being explained by the allergen as such, or the dose. However, the number of individuals tested was low (study III). Last but not least, according to the permeation study results (study IV), disposable gloves made of nitrile should be recommended for hairdressers while performing hair dyeing.

We believe that the research presented here will be of advantage to individuals affected by contact allergic dermatitis caused by PPD and related allergens. The recommendation about the use of disposable nitrile glove for protection is of benefit for the patients who are hairdressers, both for primary and secondary prevention. There is no question that the urge to dye our hair will continue, so new hair dye substances and technologies that are harmless to the human skin are needed. Therefore continuing the investigation of haptens involved in contact allergy to hair dye allergens and their effect on the human skin is important. This is important for understanding and predicting sensitization by existing and new substances aimed at the consumer market. This work must continue, both by researchers in the health care system and by those in the cosmetic industry.

For me personally, as a clinician and a researcher, I have gained a lot of experience from working on the PPD project. I am especially thankful for the experience of planning, performing, and analysing results from the experimental studies with regard to substances and chemical analysis. I learned about the difficulties and needed skills for setting up of systems for chemical separation and

analysis. Additionally, I learned about the various challenges working with an unstable chemical such as PPD.

Getting to know PPD has been a rewarding journey into occupational and environmental dermatology. This substance has become like a friend to me, but as it turned out, a complex one – needing much more time to get to know it in depth.

# Popular scientific summary in Swedish (Populärvetenskaplig sammanfattning)

Den medicinska specialitet som har hand om hudsjukdomar och sexuellt överförbara infektioner kallas dermatovenereologi, av grekiskans "derma" hud, "logos" kunskap och latinets "veneris", genitivform av Venus (den sinnliga kärlekens gudinna) som fått benämna könssjukdom. Yrkes- och miljödermatologi är den del av dermatovenereologin som omfattar diagnosticering, behandling och förebyggande av hudsjukdom orsakad av yttre faktorer (bland annat kemiska substanser) i vår omgivning, både inom arbete och miljön i övrigt. Varje dag omges vi människor av mängder med kemiska ämnen. Huden utgör en stor kontaktyta mot omgivningen, hos en vuxen individ ca 2m<sup>2</sup>. En del ämnen som kommer i kontakt med huden kan orsaka skada på huden. En kategori av ämnen som kan skada huden benämns kontaktallergen. Det kan vara parfymämnen eller konserveringsmedel i schampo, ämnen i konstgjorda naglar eller metaller, t.ex. nickel eller guld.

Kontaktallergen är ämnen som kan skapa ett immunologiskt minne vid tillräcklig hudkontakt. Processen där minnet skapas hos en individ kallas sensibilisering. Hos en sensibiliserad individ sätter ny hudkontakt med ämnet igång den icke önskade reaktionen i huden. I typiska fall uppstår rodnade, kliande och fjällande fläckar. Sjukdomen vid kontaktallergi visar sig oftast som eksem på den exponerade lokalen, kontaktallergiskt eksem. Kontaktallergi är en långsam typ av allergi. Det tar vanligen dagar innan den märks på huden.

Det viktigaste arbetsredskapet för att undersöka om en individ är kontaktallergisk är att lapptesta, dvs. att sätta små prover med substansen på huden, oftast ryggen. Dessa lappar sitter kvar 48 timmar och därefter kommer individen till hudläkare för undersökning (avläsning) efter 3 eller 4 dagar och ytterligare en gång efter 7 dagar. Lapptestning är en väsentligen standardiserad diagnostisk metod, men förfarandet skiljer sig mellan olika länder, avseende t.ex. vilka ämnen som testas, testdoser och tidpunkt för avläsning.

Denna avhandling med fyra arbeten (I-IV) fokuserar på kontaktallergi för ett färgämne som heter para-fenylen-diamin (PPD). PPD är ett starkt allergen som förekommer i bland annat permanenta hårfärger och ger mörka färger. Den kemiska strukturen för PPD är en bensenring med två motsatt placerade aminogru-pper. PPD har använts som beståndsdel i hårfärger över 100 år, trots att man känt till risken för kontaktallergi. Individer som färgar håret kan drabbas, man kan bli sensibiliserad även om man färgat håret i många år utan besvär. Frisörer löper en hög risk för kontaktallergi inom sitt yrke bl.a. för PPD. De som drabbas kan utveckla ett handeksem som leder till lidande, hindrar yrkesutövningen och i en del fall tvingas den drabbade individen att byta yrke.

PPD är till en början färglöst. I kontakt med syre sker en oxidation och ämnet mörknar. I en PPD-innehållande hårfärg finns olika molekyler som reagerar med varandra och tillsammans bildas i denna blandning färgmolekyler som ger den önskade nyansen, under inverkan av ett oxiderande ämne, vanligen väteperoxid. De som är allergiska för PPD kan därmed teoretiskt reagera på själva PPD-molekylen men även på andra ämnen som bildas när PPD reagerar med ex. syre på och i huden.

I arbete I har vi undersökt hur PPD-allergiska individer reagerar på oxiderad PPD. En lösning med blandning av PPD och väteperoxid, dvs. en förenkling av de kommersiella oxidativa hårfärgerna, har använts. I denna lösning kan många okända ämnen finnas, som bildas när PPD på olika vis reagerar. För att möjliggöra upptäckten av vilka ämnen som de PPD-allergiska individerna reagerar på gjorde vi en uppdelning av lösningen innan den lapptestades. Till det användes tunn-ski-cktskromatografi, en kemisk metod för separation av ämnen, på en mjuk plast-platta klädd med kisel. Efter uppdelning fick vi en remsa med 4 separata, synliga fläckar på rad, tre bruna och en gul. Dessa remsor testades på försöks-individernas ryggar enligt modifierad lapptestningsmetod som har utarbetats på vår enhet i Malmö. Vi fann att olika individer som är sensibiliserade för PPD reagerar på olika fläckar och i olika mönster. Detta talade för att det bildas flera olika potentiella allergen när PPD oxideras.

I arbete II fortsatte vi att undersöka remsorna med oxiderad PPD och fokuserade på den gula fläcken, eftersom flest individer hade reagerat på den. För att komma närmare vilka ämnen som fanns i området med gul färg gjordes analys av substanser från det gula området. Analysen visade förekomst av två potentiella allergen, oxidationsprodukter av PPD. Dessa var 4,4'-azodianilin och 4-nitroanilin. För att bekräfta vår teori lapptestades även dessa ämnen på individerna i arbete I. Testresultaten bekräftade att en stor andel som reagerat på den gula fläcken även hade reaktioner för 4,4'-azodianilin och 4-nitroanilin.

För höga doser vid lapptestning kan i sällsynta fall göra individer allergiska mot det ämne som testas. Detta är givetvis en icke önskvärd biverkan. PPD har länge

lapptestats som en beredning med 1.0% av ämnet för diagnostik av kontaktallergi. Bland en del experter på området har denna koncentration ifrågasatts med motivationen att det är en för hög dos som kan leda till sensibilisering genom lapptest. När en individ är kontaktallergisk brukar oftast ett eksem uppstå efter lapptestning inom en vecka. Om individen reagerar med eksem senare än efter 7-10 dagar kan detta tala för sensibilisering genom lapptest. I andra fall är det ett vanligt reaktionssätt hos redan sensibiliserad individ, vilket är känt för ämnen som kortisonämnen, guld och akrylater. I arbete III önskade vi lapptesta redan PPD-sensibiliserade individer för PPD i 1.0%, och ytterligare 10 lägre koncentrationer, samt följa reaktionerna över 4 veckor för att se om några reagerar sent, trots att det i denna grupp inte kan finnas sensibilisering genom lapptest. Vi hittade endast reaktioner inom 7 dagar.

Om en kontaktallergi har diagnosticerats kan sjukdom, i form av kontaktallergiskt eksem, undvikas om individens exponering för ämnet upphör. Eftersom detta inte är möjligt alla gånger, så behövs olika former av skyddsutrustning, t.ex. handskar för att undvika eller minska hudkontakt. Val av material i handskarna och hur dessa används är av yttersta vikt för att handskanvändningen ska skydda. Tidigare forskning på området har visat att bland frisörer, som färgar hår, förekommer felaktig användning. En del individer tvättar och återanvänder handskar för engångsbruk, vilket leder till kontamination. På vår avdelning har man tidigare studerat olika engångshandskars skydd mot en PPD-innehållande hårfärg på PPD-kontaktallergiska individer, studie in vivo. De undersökta handskarna av latex och av vinyl skyddade dåligt och även om huden skyddats med handske vid exponering för hårfärgen, så uppstod senare ett eksem. Detta talar för att PPD, PPD produkter och/eller andra allergen passerar genom handskens material. Ingen synlig hårfärg hade passerat. I in vivo-studien skyddade handske av nitril bra, inget eksem uppstod vid senare kontroller.

I arbete IV undersökte vi engångshandskars skydd mot PPD, 4-nitroanilin, 4,4'-azodianilin och resorcinol, ytterligare ett hårfärgsallergen, in vitro. Vi önskade också jämföra resultaten med den tidigare handskstudien. Vi använde ett redskap som kallas permeationscell, vilket möjliggör studier av hur ämnen passerar genom ett membran, t.ex. hud eller handske. I studien användes en typ, Franz cell, uppkallat efter dess uppfinnare. Franz cell består av två kammare som separeras av det studerade membranet. Som membran användes handskmaterial av nitril, vinyl och två sorter i latex med olika tjocklek. Handsken var i direkt kontakt med vätska i den nedre kammaren (receptorvätska). I den övre kammaren placerades nyss blandad hårfärg direkt på handskbiten. Därefter togs flera prover från receptorvätskan under loppet av 4 timmar. Proverna analyserades avseende koncentration av PPD och studerade substanser. För att underlätta jämförelse med in vivo-studien användes samma sort tunn latexhandske och samma slags hårfärg som användes i den tidigare studien. Resultaten visade att nitrilhandsken ger ett

bra skydd vid användning av hårfärg. För vinylhandske och latexhandske kunde PPD detekteras inom tidsintervall som motsvarar exponering vid hårfärgning (30-60 min). Resultaten i denna studie in vitro stämde överens med in vivo-studien. Nitrilhandske av engångstyp rekommenderas vid hårfärgning.

Sammanfattningsvis fann vi i denna avhandling att det finns flera PPD-associerade allergen som PPD-allergiska individer kan reagera på, varav två potentiella är 4,4'-azodianilin och 4-nitroanilin. Vi kunde inte se några senreaktioner för PPD hos redan sensibiliserade individer, men med tanke på liten studiepopulation kan man inte uttala sig om förekomst i hela gruppen PPD-sensibiliserade. Slutligen bekräftade vi att frisörer som färgar hår bör använda engångshandskar av nitril för ett bra skydd mot allergen.

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# Paper I





## Jacobs Journal of Experimental Dermatology

### Research Article

# Allergic Contact Dermatitis to p-phenylenediamine and Some of its Reaction Products

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### Abstract

#### Background

Contact allergy to hair dyes is prevalent among hairdressers and their consumers. The potent sensitizer p-phenylenediamine (PPD) is a common colour substance in oxidizing permanent hair dyes. The complex hair dye cocktail consists of many ingredients. When the hair dye is used oxidative agents are added and PPD can be transformed into new substances. The substances, being formed and applied to the hair, when PPD is used in hair dyes, are partly unknown.

#### Objectives

The aim of this study was to investigate contact allergic responses to oxidized substances of PPD and some of its reaction products in PPD-positive patients.

#### Methods

The methods used were high-performance liquid chromatography (HPLC), patch testing, thin layer chromatography (TLC) and patch testing with TLC strips. Purity analysis were made of PPD and its trimer Bandrowski's base (BB) using HPLC. 14 patients, previously tested PPD-positive, participated in the investigation. They were patch tested with dilution series of PPD and BB, as well as with TLC strips of oxidized PPD. Reaction patterns were compared.

#### Results

Of the 14 previously PPD-positive patients, 13 were repeatedly PPD-positive. 7/13 (54 %) reacted to BB. On the TLC strips, oxidized PPD was divided into 4 visible spots. The 7 BB-positive patients reacted to one or more of the TLC spots in various patterns.

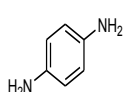
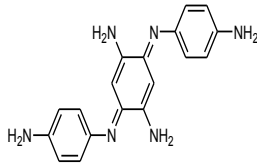
#### Conclusion

The results indicate the presence of several possible sensitizers formed during oxidation of PPD and that PPD-sensitized patients might react to these substances in various patterns.

**Keywords:** Bandrowski's Base; Contact Allergy; Hair Dye; Oxidation Products; P-Phenylenediamine; Ppd; Thin Layer Chromatography

## Introduction

Contact allergy to oxidative permanent hair dyes is common among hairdressers and their consumers [1,2]. Exposure to hair dye products means exposure to a complex mixture of substances that apart from the actual dye substances also includes perfumes, surface active agents and many other functional additives. The potent sensitizer p-phenylenediamine (PPD, Figure 1) is still a standard colour substance in oxidative hair dyes [3-5]. The hairdressers and the consumers are also exposed to a cocktail of other irritating substances or contact allergens in hair dyes [6,7]. Due to the strong sensitizing capacity especially of PPD and other similar colour substances [3], efforts are made to find substitutes with a less allergenic potency [8,9].

	<p><b>p-Phenylenediamine</b></p> <p>CAS no.: 106-50-3</p> <p>MW: 108</p>
	<p><b>Bandrowski's base</b></p> <p>CAS no.: 200-48-27-5</p> <p>MW: 318</p>

**Figure 1.** Chemical structures, Chemical Abstracts Services (CAS) numbers and molecular weights of p-phenylenediamine (PPD), and Bandrowski's base (BB).

PPD was introduced as a marker for hair dye allergy in 1939, and it still remains the clinically most relevant one [10]. Most hair dye allergic consumers and hairdressers have been exposed to, and developed contact allergy to PPD. Some of these might have been sensitized to another similar substance causing cross reactivity to PPD. The mechanisms of induction and elicitation of contact allergy by PPD are not well understood. PPD can be transformed into new substances by processes such as oxidation, metabolism and reaction with other chemicals [11,12] and these substances might be important in connection to hair dye and PPD allergy.

An oxidative hair dye contains 3 main functional components, a precursor, a coupler and an oxidizing agent. The colour crème, containing a precursor and one or more couplers, is mixed with the oxidizing agent just before application to the hair. The solution of the oxidizing agent, is often called the developer,

and contains hydrogen peroxide. After mixing of a PPD based oxidative hair dye PPD oxidation products are formed, which then can react with the coupler(s) creating the desired color. Certain couplers and oxidation products of PPD have been suggested to be involved in hair dye and PPD-related contact allergies [13]. One substance that can be formed by oxidizing PPD (in the absence of a coupler) is the trimer Bandrowski's base (BB; N,N'-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine, Figure 1). BB has earlier been pointed out as one substance possibly responsible for PPD-related contact allergy [14-16].

In vitro studies have been used to investigate some allergens formed in hair dyeing with PPD [17]. Among animal study models, the guinea pig maximisation test is used for investigations of cross reactivity [18]. However, patch testing in men is the best method for detecting allergies to new substances that have sensitized exposed individuals. New allergens among the oxidation products of PPD may be established by patch testing in patients allergic to PPD. It is then necessary to separate the substances found in the mixed hair dye. By using thin layer chromatography (TLC), complex mixtures of substances can often be separated. The more substances, the more complicated the separation.

When a mixture of substances is separated on a thin layer chromatogram, the substances move according to their physical and chemical properties. The different substances can be visualised, as distinct spots on the chromatogram. We wanted to find a way to look at the possible allergens formed when PPD is used in hair dyes. Since, PPD in hair dyes is used with couplers and hydrogen peroxide, the most clinically relevant way would be to use the thin layer chromatogram formed when all the three substances are mixed. However, in this study we chose to look at the oxidation products formed in the mixture of PPD and hydrogen peroxide. This makes it easy to study the oxidation products formed as they are formed in higher concentrations and are easier to identify in this less complex mixture. It is relevant to study the mixture of PPD and hydrogen peroxide, because the development of the same oxidation products also could occur in a complete hair dye.

Furthermore, individuals may be exposed to high concentrations of PPD in temporary tattoos, so called black henna tattoos. No couplers are added in black henna tattoos. The dark shade in these tattoos is due to a mixture of PPD oxidation products, possibly formed by auto-oxidation due to air contact. Some non-European hair dye products in powder form contain henna, PPD and an oxidizing agent also in powder form (i.e barium peroxide), but no coupler [19]. It is highly likely that products for black henna tattoos and the henna hair dyes with an oxidizer will expose the user to relatively high doses of PPD oxidation products.

When patch testing with PPD, the test leaves a dark spot on the

skin due to oxidation products formed from contact with air oxygen. Due to this oxidation process patch tests with PPD may also contain PPD oxidation products.

The aim of the present study was to investigate the importance of oxidation products of PPD and the role of BB in PPD-related contact allergy. The oxidation products have been separated using TLC and TLC strips of oxidized PPD have been patch tested in PPD sensitized patients.

## Materials and Methods

### Chemicals and test material

The following chemicals were used: Acetone ( $\geq 99.5\%$ , Scharlau Chemie SA, Sentmenat, Spain and  $99.9\%$ , VWR, Fontenay-sous-Bois, France), BB (ICN Biomedicals Inc, Aurora, Ohio, USA), n-butylamine (Acros, Fisher Scientific, Gothenburg, Sweden), distilled water (Millipore, Q-Guard 1, Molsheim, France), ethanol (Kemetyl, Haninge, Sweden), n-heptane (VWR, Fontenay-sous-Bois, France), hydrogen peroxide ( $H_2O_2$ , 30 % w/w, Merck KGaA, Darmstadt, Germany),

petrolatum (pet, Vaselinum album, Snow White Quality E from Apoteket Produktion & Laboratorier, Göteborg, Sweden) and PPD ( $>99\%$ , Sigma-Aldrich, St Louis, MO, USA). Silica gel plastic roll (TLC plastic roll 500 x 20cm with silica gel 60F254, Merck KGaA), Finn chambers, diameter: 8 mm (Epitest OY, Tuusula, Finland) on Scanpor tape (Norgeplaster A/s, Vennesla, Norway).

### Patch test preparation

### Dilution series

Dilutions of PPD and BB were prepared. The BB dilutions were equimolar to the PPD concentrations. A stock solution of  $1.0\%$  w/v PPD in acetone was prepared and further diluted into the test concentrations (Table 1). A stock solution of  $0.29\%$  w/v BB in acetone was prepared and further diluted into the lower test concentrations. Being non-soluble in acetone  $2.9\%$  w/w BB was mixed with petrolatum (Table 1). The dilution series were stored in a refrigerated environment for a maximum of one week. New stocks were prepared several times during the test period.

**Table 1.** Results from patch testing with p-phenylenediamine and Bandrowski's base, as well as testing with thin-layer chromatogram strips from oxidized p-phenylenediamine.

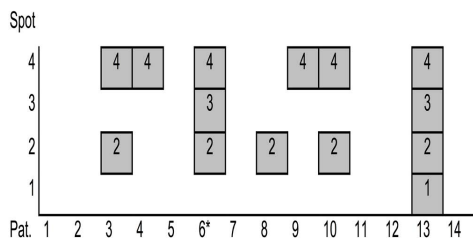
Test substance	Patient no.		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Concentration (%) <sup>*</sup>															
p-Phenylenediamine	1.0		+	-	nt	nt	++	+++	++	+++	nt	nt	+	+++	nt	+
	0.10		-		++ <sup>#</sup>	+	-	-	-	+++	++	nt	+	-	nt	-
	0.010				-	-	-			-	+	-		-	++	
	0.0010				-					-	+	-			-	
	0.00010															
	0.000010										-					
Bandrowski's base	2.9		-	-	++	+	-	nt	-	nt	nt	nt	-	-	nt	-
	0.29		-	-	++ <sup>#</sup>	-	-	+++	-	+++	nt	+	-	-	+++	-
	0.029				-			++		++	+	-			++ <sup>#</sup>	
	0.0029							-		-	-				-	
	0.00029									-	-					
	0.000029										+					
TLC testing	Spot no.	Applied amount														
	1	50 $\mu$ l	-	-	nt	-	-	nt	-	nt	nt	nt	-	-	nt	-
		25 $\mu$ l	-	-	-	-	-	-	-	-	nt	-	-	-	+++	-
		5 $\mu$ l	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	50 $\mu$ l	-	-	nt	-	-	nt	-	nt	nt	nt	-	-	nt	-
		25 $\mu$ l	-	-	+	-	-	++	-	+	nt	+	-	-	+++	-
		5 $\mu$ l	-	-	-	-	-	++ <sup>#</sup>	-	-	-	+	-	-	+++	-
	3	50 $\mu$ l	-	-	nt	-	-	nt	-	nt	nt	nt	-	-	nt	-
		25 $\mu$ l	-	-	-	-	-	+++	-	-	nt	-	-	-	+++	-
		5 $\mu$ l	-	-	-	-	-	++ <sup>#</sup>	-	-	-	-	-	-	+	-
	4	50 $\mu$ l	-	-	nt	+	-	nt	-	nt	nt	nt	-	-	nt	-
		25 $\mu$ l	-	-	++ <sup>#</sup>	-	-	+	-	-	nt	+	-	-	+++	-
		5 $\mu$ l	-	-	-	-	-	-	-	-	++	-	-	-	+++	-

\* = vehicle is acetone and concentration % w/v except for  $2.9\%$  w/w of Bandrowski's base prepared in petrolatum; # = test reaction stronger day 7 than day 3/4. The strongest reaction is noted in the table

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**Figure 2.** Overview of the reactions to thin-layer chromatograms of oxidized PPD.

This figure indicates to which TLC spots the test patients had a positive reaction disregarding the severity of the reaction and the amount of oxidized PPD on the thin layer chromatogram (5µl, 25µl or 50µl) to which the patient reacted.



Pat.=patient, \*Pat. 6 was tested with thin layer chromatogram 10µl and 20µl

### Test preparation of oxidized p-phenylenediamine for thin layer chromatography

Approximately 24 hours before separation with TLC a mixture according to these ratios was prepared: 40 µl 30 % w/w hydrogen peroxide was added to 1.0 ml 1.0 % PPD in acetone. Immediately 20 µl distilled water was added, to avoid formation of reaction products between acetone and PPD. The solution was stored at room temperature.

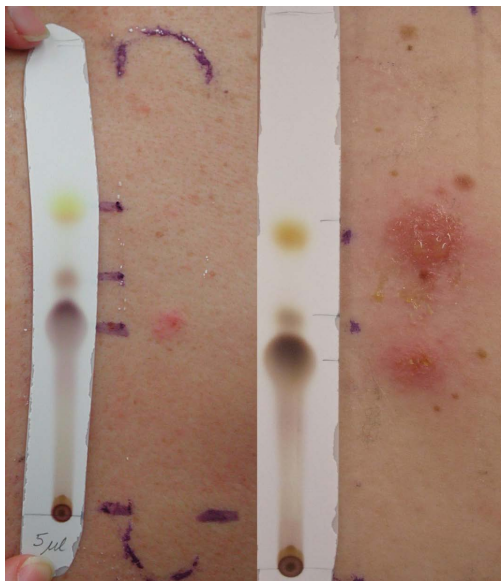
### Preparation of thin layer chromatograms

The use of thin layer chromatography (TLC) strips for epicutaneous testing is a method developed at the Occupational and Environmental Dermatology Department in Malmö [20-23]. The TLC strips were prepared 60-120 minutes before testing. The silica gel plastic roll was cut into 17.5 x 18 cm sheets. The oxidized 1.0% PPD solution was deposited using 5 µl capillaries on a line, marked with a pencil, 2 cm from the bottom of the TLC sheet. Six application spots were marked on the application line per sheet. For the thin layer chromatograms the amounts of oxidized PPD solution applied were 5µl, 25µl or 50µl.

Various combinations and concentrations of solvents were tested in order to find the optimal eluent for separation of the oxidized PPD solution. The chemicals' size, polarity, affinity to the mobile phase and to the material of the TLC sheet, determine how far the chemicals will be carried on the plate and thus the quality of the separation. In this case acetone was chosen as mobile phase for separation of the oxidized PPD solution because it showed the best separation of spots, thus the samples were eluted on the TLC sheets using a mobile phase of acetone 100%.

The TLC sheets were inspected in daylight and under UV radiation at 254 and 365 nm. All detectable spots were marked with a pencil. The spots were numbered from 1 to 4, where number 1 indicates the site of application. The TLC sheets were cut into strips about 2 x 16 cm, with a band of spots on each (Figure 3).

**Figure 3.** Examples of test reactions on day 3 to thin layer chromatograms of oxidized p-phenylenediamine 5 µl in two test patients. Left patient 10 and right patient 13.



### Patch testing

The dilution series of PPD and BB, were applied on the backs of the patients with Finn chambers, diameter: 8 mm. 15 µl of each dilution was used [24] except for 2.9% BB, which was patch tested in petrolatum, 20 mg [25]. Patch tests were removed after 2 days, D2.

Patch tests were evaluated and scored after 3 or 4 days (D3/4) and after one week (D7). The tests were considered positive if there was at least a + reaction registered, according to the criteria of the International Contact Dermatitis Research group (ICDRG), corresponding to the location of the Finn Chamber [26]. The highest initial test concentration of PPD was 0.1 %. The most PPD sensitized patients had their highest test concentration of PPD lowered to 0.01 %. If a patient presented with negative reaction on D3/4 the higher concentrations up to PPD 1.0 % were tested. Thus these reactions were only read once, on D3/4 after patch test application (Table 1).

The site of contact to the spots on the TLC strip, were marked out on the patients' back as well as the edges of the TLC strip. The chromatograms were applied with Scanpor tape and removed on D2. Initially the patients were tested with TLC strips with 5  $\mu$ l and 25  $\mu$ l of an oxidized PPD solution. A patient who presented with a negative reaction to these TLC strips on D3/4 was tested with a 50  $\mu$ l TLC strip which was read on D3/4 only.

The composition of the individual thin layer chromatogram must be taken into account when reading patch test reactions to TLC strips. The amount of possible different allergens, and their distribution area, are unknown. The whole skin area that has been covered with the TLC sheet and where the substances have eluted will be evaluated. This means that an area where there is no visible spot can be positive. The reason can be that the chemical is not detectable in the light used. The chemical, which the patient reacts to, might move too slowly through the silica gel and leave a track of molecules behind that a patient with high reactivity can respond to.

The TLC strips are prepared in duplicates and a copy of the TLC strip serves as test protocol. Possible reactions can be correlated to the exact site of the TLC strip, which can then be used for identification of the correct spot. Infiltration and redness in the area corresponding to where the substances have been eluted will be regarded a one plus reaction. If the reaction also has papules, it will be regarded a two plus reaction. If there are intense redness, papules and even vesicles it will be regarded a three plus reaction.

### Patients

Fourteen female patients, mean age 53 years (range 20-77), were included in the study. They were previously tested with the baseline series at the Occupational and Environmental Dermatology Department in Malmö, because of eczema and suspected contact allergy, and found positive to PPD in the past 10 years. PPD allergy was due to professional exposure (hair dressers) or exposure as consumer of hair dye and/or black henna tattoo.

### Controls

15 consecutively patch tested dermatitis patients negative to PPD, or other hair dye allergens, were patch tested with BB 0.29% w/v in acetone and served as controls.

### Statistics

For comparison of test patients and controls the two sided Fisher's Exact Test was used. A p-value < 0.05 was considered to be significant. Data analysis was performed with the statistical software SPSS version 22.

### Chemical analysis

The purity of the raw material of BB and PPD used for patch testing was investigated by high-performance liquid chromatography (HPLC). The HPLC system consisted of a P4000 quaternary pump, an UV 6000 diode array detector and an AS3000 autosampler (Thermo Finnigan, San José, CA, USA). The system was software-controlled and monitored by Chromeleon 7 (Thermo Scientific Inc, Waltham, MA, U.S.A.). The Hypersil column (4.6 mm i.d. x 250 mm) (Thermo Scientific) was packed with 120 Å, 5  $\mu$ m silica. The detector scanned the eluent in the range 190 to 400 nm and chromatograms recorded at 254 nm were used for detection and measurements. The injection volume was 20  $\mu$ l and the flow rate 1.0 ml/min. Elution was isocratic with a mobile phase consisting of ethanol:heptan:water: n-butylamine 25:74.475:0.5:0.025 % (v/v) which was prepared by mixing 250 ml ethanol, 5 ml water, 25  $\mu$ l n-butylamine then adding heptane up to 1000 ml. Freshly prepared ethanol samples of PPD and BB in 0.10% and 0.04%, respectively, were analyzed with regard to contamination of each other in the raw material.

### Ethics

This study was approved by the Regional Ethical Review Board, Lund, Sweden (2007, No. 327/2007) and conducted in accordance with the ethical standards specified in the Declaration of Helsinki. Informed written consent was obtained from all subjects.

### Results

#### Analytical results

HPLC analysis showed <0.2% BB in the PPD and 0.3% PPD in the BB used.

#### Patch test results of dilution series

The results are summarized in Table 1. Thirteen, out of 14 previously PPD-positive patients, reacted to PPD in at least 1 concentration and 7 reacted to BB in at least 1 concentration. Reactions to PPD were to concentrations in the range 1.0-0.0010 % w/v and to BB in the range 2.9-0.029%. Of those patients who reacted to both PPD and BB, 6 patients had reactions to 0.10 % PPD or lower and one patient to PPD 1.0%. Those who did not react to BB reacted to PPD 1.0 % only and one of those patients did not react to PPD at all (6 of 6 versus 1 of 8;  $p=0.0047$ , Fisher's exact test, two-sided).

Among the test patients 5/14 reacted to BB 0.29% or lower and none of the 15 controls reacted to BB 0.29% (5/14 versus 0/15;  $p=0.017$ ).

### Patch test results of TLC strips of oxidized PPD

The results are summarized in Table 1 and Figure 2. Figure 2 shows the reactions to the spots of the TLC strips with all three volumes (5, 25 and 50  $\mu$ l) used of oxidized PPD. In Table 1 the reactions are shown separately. Seven patients reacted to one or more of the TLC spots. In total, of the 13 patients who were PPD-positive on patch testing with dilution series, 7/13 (54%) had a positive reaction to BB and the same 7/13 (54%) had a positive reaction to one or more of the TLC spots. Eight patients, the ones that did not have a reaction to the thin layer chromatograms with 5  $\mu$ l or 25  $\mu$ l on D3/4 were also tested with thin layer chromatograms of 50  $\mu$ l. Patient number 9 was only tested with thin layer chromatogram with 5  $\mu$ l because of her high reactivity.

Of the seven patients positive to the thin layer chromatogram of oxidized PPD, 6 reacted to spot number 4, 2 reacted to spot number 3, 5 reacted to spot number 2 and 1 reacted to the spot of application, spot number 1. They reacted to the 4 spots in different combinations. One reacted to all 4 spots. (Table 1 and Figure 3). Figure 3 shows an overview of the different reactivity patterns. Figure 4 shows two examples of the patients' reactions to 5  $\mu$ l oxidized PPD.

**Figure 4.** Thin layer chromatogram. From the left BB 0.29 % in acetone, PPD 0.1 % in acetone and oxidized PPD.



### Discussion

The marketed oxidative hair dye products may contain various combinations of more than 100 different precursors and couplers [3]. When ingredients in oxidative hair dyes on the Swedish market were examined, as many as 98% were found to contain potent skin sensitizers, but only 16% were found to contain PPD, according to the labelling [4]. Nevertheless, PPD appears to be a good marker for hair dye contact allergy identifying the majority of positive reactions to other known hair dye allergens [10]. When PPD is patch tested it is applied to the skin without the presence of the many ingredients in a hair dye. Such ingredients in the cream and developer of the typical oxidative hair dye, are greases, couplers and precursors.

The aim of epicutaneous testing is to provoke the patient's skin with the substances that they have been previously exposed to, and to elicit a contact allergic reaction if the patient is sensitized. When an individual has a positive reaction to a substance and a simultaneous reaction to another tested chemical, this might be due to concomitant sensitization to multiple allergens. Besides, it can be due to so called cross reactivity. Cross reactivity is when an individual by getting sensitized to a substance also acquires immunological reactivity to another substance. These two substances might be similar in structure and thus not distinguished by the immune system or they can form the same hapten after metabolism. Cross reactivity to PPD might be an explanation to why PPD serves well as a marker for hair dye allergy, but little is known about this cross reaction pattern.

Investigating the reactions to substances formed in permanent oxidative hair dyes, is of importance for developing preventive measures, such as recommendations for protective gloves for handling of hair dyes. In the present study we explore the partly unknown allergens which are formed, when the dye and developer have been mixed, and applied on the hair. To approach this, a simplified oxidized PPD model was developed, in order to investigate a part of the hair-dyeing process.

The oxidized PPD model used, enables study of possible contact allergic responses to the substances formed in the hair dye by the reaction between PPD and hydrogen peroxide. Patch testing with the oxidized PPD solution separated on thin layer chromatograms allows to differentiate between separate reactions to several substances, as opposed to testing with the solution as such. Moreover, the oxidized PPD used in the present study is closer to real life exposure, than patch testing with unoxidized PPD. Although, simplified in comparison to a hair dye it gives relevant findings in the investigation of PPD contact allergy.

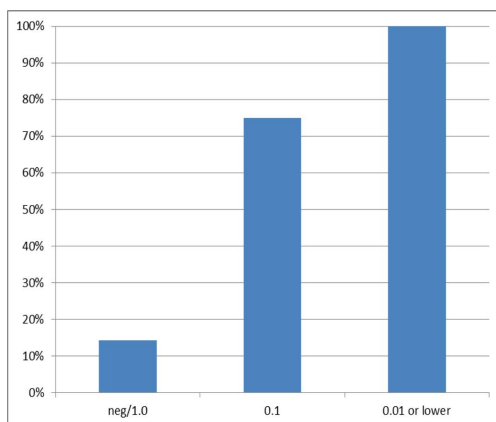
As shown by the results, the PPD-sensitized patients in the present study react in different patterns to the spots, suggesting the presence of several allergens. The 4 TLC spots may

consist of one or several substances each. Thus the patients may theoretically test positive to a spot that contains one or more possible sensitizing compounds, i.e. oxidation products and other derivatives of PPD. Also the presence of sensitizing contaminants must be considered.

The test patients were also patch tested with BB, a trimer of PPD formed through oxidation in the absence of hair dye couplers [14,15]. In a previous study where 43 PPD-positive patients were patch-tested with BB, seven (16%) were positive to either 0.1% or 1.0 % BB [15]. These results show a lower prevalence compared to the present study, where 6 patients of 14 (43%) reacted to BB 0.29% or lower.

We chose to test BB in equimolar concentrations to PPD, hence the highest concentration tested was 2.9%, equimolar to 1% PPD. In total 7 (50%) individuals hypersensitive to PPD tested positive to BB. Figure 4 shows a thin layer chromatogram where BB, PPD and oxidized PPD are eluted for comparison. This chromatogram is eluted in the same system and for the same time as oxidized PPD. The main BB spot is close in colour, brown, and location, although slightly above, spot number 2 of oxidized PPD (Figure 4). The main spot of PPD is also similar in colour and slightly lower than spot number 2. The proximity might imply that PPD may contain BB and vice versa.

The association between the patients' reactions to BB and their reactions to the thin layer chromatograms also indicates the possibility of impurities of BB in PPD and the opposite. It could also indicate cross reactivity between the two substances. The analytical HPLC analysis showed, however, only <0.2% BB in the freshly prepared PPD solution and 0.3% PPD in the BB used. It is unlikely that the contamination demonstrated has any significance for the patch test results to PPD and BB. The concentration of BB in PPD may increase depending on time, solvent and temperature.



**Figure 5.** Diagram showing relationship between positive concentration to PPD and positive reaction to BB in any concentration tested. On the y-axis the percentage of patients positive to BB is shown and on the x-axis the patients are grouped according to the lowest positive PPD concentration (%).

The relationship between positive reaction to PPD and positive reaction to BB is shown in figure 5. 1/7 patients who tested negative to PPD or positive to only 1.0 % PPD was positive to BB. 3/4 patients who tested positive down to 0.1% PPD were positive to BB and 3/3 patients who tested positive to 0.01% PPD or lower were positive to BB. These figures suggest the possibility of cross reactivity between the two substances.

Observing the patterns of test responses to the tested TLC strips does not only provide us with valuable clues about what happens to PPD in a mixed hair dye cream. It does give information about the unoxidized PPD in patch tests on the skin, where it is oxidized by air oxygen and where it may be metabolized on the skin by bacteria [27] and furthermore in the skin [11,28]. Additionally, after skin penetration, metabolism of PPD may take place within the rest of the body [28].

There may thus be several reasons for the different reaction patterns found when patch-testing with TLC strips. Differences in reactivity to PPD, and the other involved allergens, can partly be explained by individual differences in skin metabolism [29,30]. These differences might explain: why some PPD-sensitized test patients had negative reactions to the TLC strips, others reacted to one or two TLC spots, one previously PPD-positive patient tested negative and why the patient with the strongest reactions to PPD reacted to all 4 TLC spots. The concentrations and amounts of the allergens present on the thin layer chromatogram are not known. More knowledge will be gained when the substances found in the spots are identified and the patients tested with defined doses of these.

## Conclusion

The present study demonstrates the presence of multiple PPD associated allergens. This was done by in vivo-patch testing of PPD-sensitized patients, with an oxidized PPD solution, consisting of PPD and hydrogen peroxide, separated with TLC. Additional studies are needed to identify these allergens. To verify their role as contact allergens by patch test, and to further study the complex hair dye process regarding contact allergens.

## Acknowledgements

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**Conflict of interest**

None declared.

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## Paper II





# Two sensitizing oxidation products of *p*-phenylenediamine patch tested in patients allergic to *p*-phenylenediamine

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## Summary

**Background.** The results from a previous study indicated the presence of several possible sensitizers formed during oxidation of the potent sensitizer *p*-phenylenediamine (PPD) to which PPD-sensitized patients might react, in various patterns.

**Objectives.** To extract and analyse a yellow spot from a thin-layer chromatogram with oxidized PPD, to which 6 of 14 (43%) PPD-positive patients had reacted in a previous study, in order to identify potential sensitizer(s) and to patch test this/these substance(s) in the 14 PPD-positive patients.

**Methods.** The yellow spot was extracted from a thin-layer chromatogram of oxidized PPD, and two substances, suspected to be allergens, were identified by analysis with gas chromatography mass spectrometry (GCMS). The 14 PPD-positive patients, who had been previously tested with the thin-layer chromatogram of oxidized PPD, participated in the investigation, and were tested with dilutions of the two substances.

**Results.** GCMS analysis identified 4-nitroaniline and 4,4'-azodianiline in the yellow spot. Of the 14 PPD-positive test patients, 5 (36%) reacted to 4-nitroaniline and 9 (64%) reacted to 4,4'-azodianiline.

**Conclusion.** The results show that 4-nitroaniline and 4,4'-azodianiline, formed during oxidation of PPD, are potent sensitizers. PPD-sensitized patients react to a high extent to concentrations equimolar to PPD of 4-nitroaniline and 4,4'-azodianiline.

**Key words:** 4,4'-azodianiline; 4-nitroaniline; allergic contact dermatitis; gas chromatography; hair dye; oxidation products; PPD; thin-layer chromatogram.

*p*-Phenylenediamine (PPD) and toluene-2,5-diamine are still the most commonly used precursors in permanent

hair dyes (1). Contact allergy is a problem for both of these substances, and, as hair dyeing is becoming more common, there is a need to both understand how these substances act as allergens and to find less sensitizing substances. Recently, a new hair dye substance, 2-methoxymethyl-*p*-phenylenediamine, has been developed with less sensitizing properties (2), but a number of PPD-sensitized patients still seem to cross-react, making the understanding of PPD contact allergy even more important (3). In hair dyes, PPD reacts with hydrogen peroxide and couplers to give the final colour. PPD is also known as an ingredient in products in so-called 'temporary black henna tattoos', and is used because it intensifies the dark shade of the tattoo. Usually, no couplers are present in the black henna tattoos, so the dark shade results from a dark-coloured oxidation product

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derived from PPD itself. Some products, mainly from Asian countries, for dyeing hair, beards, moustaches and eyelashes are in powder form, and contain henna, PPD, and an oxidizing agent, for example barium peroxide (4). In such products, oxidation starts when the products are mixed with water. When the products are stored in powder form, the conditions are favourable for the formation of lower amounts of PPD oxidation products.

It is assumed that PPD in itself is not immunogenic but that oxidation of PPD gives *p*-benzoquinonediimine (5–7) and other reactive substances that have the potential to act as haptens. PPD is also known to be enzymatically converted by *N*-acetyltransferase 1 to substances that are not considered to be allergenic (8, 9). To what extent PPD-allergic patients may react to the different oxidation products formed is not known, and it is also not known to what extent different patterns of reactivity to the formed products may explain the different cross-reaction patterns that are found in patients sensitized to PPD (10–19).

In a previous study, we patch tested PPD-positive patients with PPD that had been oxidized with hydrogen peroxide. The oxidation products were separated on thin-layer chromatograms that had a plastic support, making it possible to patch test with the thin-layer chromatography (TLC) strips (19). On the TLC strips, the PPD oxidation products were visible as four different spots (Fig. 1, left). The patients reacted in various patterns to the spots. These results indicate the presence of allergens among the PPD oxidation products for which further investigation is of great interest.

In the present study, based on the reaction pattern of the previously tested patients, the spot to which most patients reacted was chosen for chemical analysis. The identified substances were patch tested in the patients previously found to be PPD-positive and in controls.

## Materials and Methods

### Chemicals

The following chemicals were used: acetone [ $\geq 99.5\%$  (Scharlau Chemie SA, Sentmenat, Spain) and  $99.9\%$  (VWR, Fontenay-sous-Bois, France)], 4,4'-azodianiline (95%; Acros Organics, Geel, Belgium), distilled water (Millipore; Q-Guard 1, Molsheim, France), ethyl acetate (99.9%; VWR), helium (Alphagaz 2 quality; Air Liquide, Malmö, Sweden), hydrogen peroxide (30% wt/wt; Merck KGaA, Darmstadt, Germany), 4-nitroaniline ( $\geq 99\%$ ; Sigma Aldrich, Steinheim, Germany), methanol (99.8%; BDH Prolabo, Leuven, Belgium), petrolatum (Vaselinum album, Snow White Quality E; Apoteket Produktion & Laboratorier, Göteborg, Sweden), and PPD ( $> 99\%$ ; Sigma-Aldrich, St Louis, MO, USA).



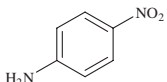
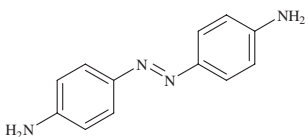
**Fig. 1.** Thin-layer chromatogram with oxidized *p*-phenylenediamine (PPD) and reference substances. Solutions were applied on four spots at the bottom line, and eluted with acetone. The four applied solutions are, from the left, oxidized PPD (1), 4-nitroaniline (2), 4,4'-azodianiline (3), and an extract of spot no. 4 from the thin-layer chromatogram (4). Oxidized PPD, solution no. 1, gave four visible spots, of which the second from the bottom may be PPD and polymerized PPD, such as Bandrowski's base.

### Sample preparation

Five TLC sheets of oxidized PPD were prepared for analysis in the same way as those that had been used for patch testing of patients (19). TLC plastic roll ( $500 \times 20 \text{ cm}^2$ ) with silica gel 60F254 (Merck KGaA) was used. Spot no. 4 on the TLC sheets (Fig. 1) was then scraped into a beaker, and extracted with acetone and then with methanol. These extracts were combined, filtered, and evaporated at  $30^\circ\text{C}$  with a rotary evaporator. The residue was dissolved in 1.0 ml of ethyl acetate, and this solution was used for chemical analysis.

### Gas chromatography mass spectrometry (GCMS)

Separation of components in the samples was performed with an Agilent 6890N gas chromatograph (Agilent

	<b>4-Nitroaniline</b> CAS no.: 100-01-6 MW: 138 Log P <sub>o/w</sub> : 1.01
	<b>4,4'-Azodianiline</b> CAS no.: 538-41-0 MW: 212 Log P <sub>o/w</sub> : 2.86

**Fig. 2.** Chemical structures, Chemical Abstract Services (CAS) numbers and physical properties of 4-nitroaniline and 4,4'-azodianiline.

Technologies, Palo Alto, CA, USA) equipped with an HP-MSI capillary column (Agilent Technologies) with a length of 30 m, an internal diameter of 0.250 mm, and a film thickness of 0.25  $\mu$ m. Helium was the carrier gas, with a flow rate of 1.0 ml/min. The injection was splitless, and the inlet was heated to 250°C. The injection volume was 1  $\mu$ l. The temperature program was isothermal at 70°C for 3 min, an increase at 8°C/min to a final temperature of 300°C, and maintenance at this isothermal temperature for 10 min. The gas chromatograph was connected to a Jeol GCmate II mass spectrometer (Jeol Datum Ltd, Tokyo, Japan). Electron ionization mass spectra were recorded with  $m/z$  from 50 to 600, with scan duration of 0.3 seconds and an interscan delay of 0.2 seconds. The temperature of the ion source was 250°C, and the GCMS interface temperature was 250°C. The electron energy was 70 eV. The National Institute of Standards and Technology (Gaithersburg, MD, USA) library of mass spectra was used for identification.

The GCMS analysis showed that the substances isolated from thin-layer chromatogram spot no. 4 were 4-nitroaniline and 4,4'-azodianiline (Fig. 2).

#### Patch test preparation

Dilution series for patch testing were prepared from 4-nitroaniline and 4,4'-azodianiline. In the present study, the two substances were tested in concentrations equimolar to the PPD concentrations used in the dilution series in the previous study (19), except for the highest concentrations. For concentrations and vehicles, see Table 1.

The highest test concentration recommended for 4-nitroaniline is 1%, according to de Groot (20). 4-Nitroaniline 1.0% wt/vol was soluble in ethanol/acetone 40:60, so this solvent was used. The following dilutions, all in acetone, were 0.13% wt/vol 4-nitroaniline, which is equimolar to a solution of 0.10% PPD. From this solution, four 10-fold serial dilutions were made (Table 1).

For the highest test concentration of 4,4'-azodianiline (1.0% wt/wt), the vehicle was pet., because the substance has too low solubility in liquids for patch testing to be feasible. The following dilutions were all prepared in acetone 0.20% wt/vol, and from this solution four 10-fold serial dilutions (Table 1) were made. 4,4'-Azodianiline 0.20% wt/vol is equimolar to a solution of 0.10% PPD.

#### Patch testing

The 14 test patients were tested with the dilution series of 4-nitroaniline and 4,4'-azodianiline. Twenty milligrams (21) of the pet. preparation and 15  $\mu$ l (22) of each dilution were applied on the backs of the patients by the use of Finn Chambers® with a diameter of 8 mm (Epitest OY, Tuusula, Finland) on Scanpor® tape (Norgeplaster A/s, Vennessla, Norway), and removed after 2 days. Patch tests were read and scored at day (D)3 or D4 and D7, and considered to be positive if at least a + reaction according to the criteria of the ICDRG (23) was present.

#### Patients

Fourteen female patients, with a mean age of 53 years (range 20–77 years), were included in the study. They had, in the past 10 years, been tested with the baseline series in Malmö, because of eczema and suspected contact allergy, and found to be positive for PPD. They had also been patch tested with other series, depending on the investigation for their original dermatitis. PPD allergy resulted from professional exposure (hairdressers) or exposure as consumer of hair dye and/or black henna tattoo. The 14 female patients had participated in the previously described PPD study, and had been tested with dilution series of PPD and Bandrowski's base, as well as with TLC strips of oxidized PPD (19). Patch test reactions to PPD and to TLC spot no. 4 are shown in Table 1. Patient no. 2 (Table 1) fulfilled the inclusion criteria, but did not show any positive reactions to any of our test preparations, either in the previously presented study or in the present study.

#### Controls

Fifteen consecutively patch tested dermatitis patients negative for PPD were patch tested with 4-nitroaniline 1.0%

**Table 1.** Results from patch testing with *p*-phenylenediamine, thin-layer chromatography (TLC) spot no. 4 [adapted from Ref. 19], 4-nitroaniline, and 4,4'-azodianiline

Test substance	Concentration (%)	Patient no.													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
<i>p</i> -Phenylenediamine*	1.0	+	–	NT	NT	++	+++	++	+++	NT	NT	+	+++	NT	+
	0.10	–	–	++ <sup>†</sup>	+	–	–	–	+++	++	NT	+	–	NT	–
	0.010	–	–	–	–	–	–	–	–	–	+	–	–	++	–
	0.0010	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	0.00010	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	0.000010	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TLC spot no. 4	50 µl <sup>‡</sup>	–	–	NT	+	–	NT	–	NT	NT	NT	–	–	NT	–
	25 µl <sup>‡</sup>	–	–	++ <sup>†</sup>	–	–	+ <sup>†</sup>	–	–	NT	+ <sup>†</sup>	–	–	+++	–
	5 µl <sup>‡</sup>	–	–	–	–	–	–	–	++	–	–	–	–	+++	–
4-Nitroaniline <sup>§</sup>	1.0	–	–	–	–	–	NT	–	–	NT	+	–	–	NT	+
	0.13	–	–	–	–	–	++	–	–	NT	–	–	–	NT	–
	0.013	–	–	–	–	–	–	–	–	++	–	–	–	+	–
	0.0013	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	0.00013	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	0.000013	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4,4'-Azodianiline <sup>¶</sup>	1.0	–	–	NT	+	–	NT	–	NT	NT	NT	–	NT	NT	NT
	0.20	–	–	++ <sup>†</sup>	+	–	+++	–	++	NT	++	–	+	NT	+
	0.020	–	–	+	–	–	+++	–	+	NT	–	–	–	+++	–
	0.0020	–	–	+ <sup>†</sup>	–	–	+	–	–	+	–	–	–	+++	–
	0.00020	–	–	–	–	–	–	–	–	+	–	–	–	+	–
	0.000020	–	–	–	–	–	–	–	–	–	–	–	–	–	–

NT, not tested.

\*Vehicle is acetone and concentration is % wt/vol.

<sup>†</sup>Test reaction stronger on D7 than on D3/4. The strongest reaction is noted in the table.<sup>‡</sup>No. of microlitres of oxidized PPD solution on the tested TLC strip.<sup>§</sup>Vehicle is acetone and concentration is % wt/vol, except that, for 1.0% wt/vol of 4-nitroaniline, vehicle is ethanol/acetone 40:60.<sup>¶</sup>Vehicle is acetone and concentration is % wt/vol, except for 1.0% wt/wt of 4,4'-azodianiline prepared in pet.

wt/vol and 4,4'-azodianiline 0.020% wt/vol, and served as controls.

## Statistics

For comparison of test reactions in patients and controls, a two-sided Fisher exact test was used. A *p*-value of <0.05 was considered to be significant. Data analysis was performed with the statistical software SPSS version 22.

## Ethics

This study was approved by the Regional Ethical Review Board, Lund, Sweden (2007, No. 327/2007), and conducted in accordance with the ethical standards specified in the Declaration of Helsinki. Informed written consent was obtained from all subjects.

## Results

### Patch test reactions

The test reactions to the identified substances in TLC spot no. 4 are shown in Table 1, and the previous reactions

to PPD and TLC spot no. 4 are also included for comparison. Five patients reacted to 4-nitroaniline and 9 patients reacted to 4,4'-azodianiline. Of the 6 patients who reacted to spot no. 4, 4 reacted to 4-nitroaniline and all 6 reacted to 4,4'-azodianiline. Of the 8 patients who did not react to spot no. 4, 1 reacted to 4-nitroaniline and 3 reacted to 4,4'-azodianiline. The lowest 4-nitroaniline concentration that elicited a reaction was 0.0013% (*n* = 1). One patient reacted to 4-nitroaniline 0.013%, 1 reacted to 4-nitroaniline 0.13% and 3 reacted to 4-nitroaniline 1.0% as the lowest concentration. The lowest 4,4'-azodianiline concentration that elicited a reaction was 0.00020% (*n* = 2).

### Controls

Of the 15 controls, none reacted to 4-nitroaniline 1.0% (0/15 versus 5/14, *p* = 0.017) and none reacted to 4,4'-azodianiline 0.020% (0/15 versus 5/14, *p* = 0.017).

## Discussion

When an individual, in a patch test situation, reacts to a complex solution consisting of several different

substances, it is not known to which substance(s) the individual reacts. One way to investigate this is to separate the substances with TLC, and then patch test with the TLC strip, enabling identification of the allergens (24, 25).

PPD is oxidized when it is used for hair dyeing in the complex hair dye solution, forming oxidation products to which the individual might then react. To investigate this, in our previous study the TLC technique was used to test patients who were already PPD-sensitized. It was shown that PPD-sensitized patients react in various patterns to the oxidation products of PPD divided into four visible TLC spots. The TLC spots may consist of one or several substances each.

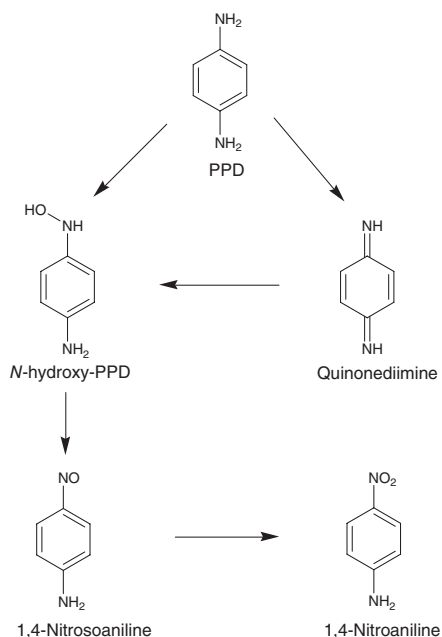
After identification of the substance(s) found in a spot in a thin-layer chromatogram to which an individual has reacted, the possible allergen must be patch tested in the individual to prove that it is actually the culprit allergen. In order to avoid a possible false-positive reaction, the substance must also be patch tested in controls.

In the present study, we used GCMS to analyse the spot to which the majority of the patients reacted, and found two substances. The substances are 4-nitroaniline and 4,4'-azodianiline (Fig. 2). In Fig. 1, a comparative thin-layer chromatogram with oxidized PPD, 4-nitroaniline, 4,4'-azodianiline and an extract of TLC spot no. 4 is shown.

Of the 6 patients who reacted to TLC spot no. 4, all 6 reacted to 4,4'-azodianiline and 4 reacted to 4-nitroaniline. Interestingly, of the 8 patients who did not react to TLC spot no. 4, 3 reacted to 4,4'-azodianiline and 1 reacted to 4-nitroaniline. An explanation for this might be that the concentrations of these substances in the TLC spot were too low to elicit a reaction. There were no reactions in the controls.

Our results show two sensitizers that are formed during oxidation of PPD: 4-nitroaniline and 4,4'-azodianiline. These substances that have actually been found by our group in a powder hair dye product in which the oxidizing agent was present (26). Not all of the participating PPD-sensitized patients reacted to these two substances. Different sensitization patterns found in the tested patients may be explained by factors such as differences in the exposure levels of PPD, varying enzymatic properties of the skin (27), or the existence of several sensitizers involved in contact allergy to PPD. If different potent sensitizers are involved in the sensitization of patients, they might show varying patterns of reactivity and cross-reactivity.

4-Nitroaniline is mainly used industrially as a precursor to PPD, and is commonly used in the synthesis of dyes (28). It is a starting material for the synthesis of Para Red, one of the first azo dyes, which has been used since 1880



**Fig. 3.** Steps involved in the formation of 4-nitroaniline from *p*-phenylenediamine (PPD) by autooxidation and/or oxidative metabolism.

(29, 30). 4-Nitroaniline has been evaluated as a possible degradation product of Disperse Orange I (11), which is known to be a common sensitizer in the textile dye mix (12). The oxidation pathway of PPD to 4-nitroaniline is shown in Fig. 3.

4,4'-Azodianiline is an azo compound with the characteristic nitrogen–nitrogen double bond, called an azo group. 4,4'-Azodianiline has been found as an impurity in the PPD trimer Bandrowski's base, to which many of the 4,4'-azodianiline PPD-allergic patients show simultaneous contact allergies (31). The oxidation pathway of PPD to 4,4'-azodianiline is shown in Fig. 4.

PPD is not considered to be directly protein-reactive; that is, it cannot bind covalently to skin proteins to form hapten–protein complexes in order to become immunogenic and sensitize. PPD may thus be classified as a pre-hapten; it must form other chemical compounds to be able to haptenate, for example through oxidation (32). When used in permanent hair dyes, PPD is oxidized in the hair dye formula on the skin, and it can be further metabolically oxidized in the skin. PPD might thus form several possible haptens. This might partly explain the association between PPD and other groups of allergens,

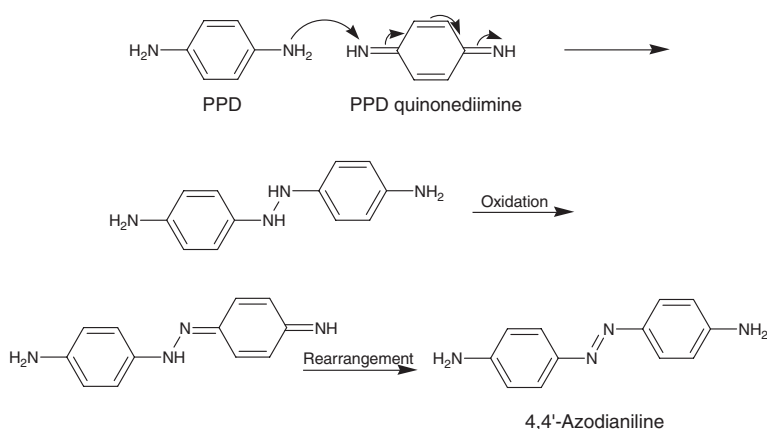


Fig. 4. Possible reaction pathway from *p*-phenylenediamine (PPD) to 4,4'-azodianiline.

such as black rubber mix and textile dyes. In the skin, acetylation to monoacetyl PPD and diacetyl PPD can occur. These acetylated derivatives are metabolites that are less prone to haptenate (32).

With regard to the identified sensitizers 4-nitroaniline and 4,4'-azodianiline, it is impossible to determine whether the simultaneous reactions found are attributable to cross-reactions or concomitant primary sensitization to these chemicals. However, individual comparisons of the degrees of reactivity to PPD on the one hand and 4-nitroaniline and 4,4'-azodianiline on the other may indicate whether the two latter substances are primary or secondary sensitizers (Table 1). For PPD and 4-nitroaniline, there is a tendency for there to be a higher degree of test reactivity (positive reactions to lower test concentrations when equimolar concentrations are compared) to PPD than to 4-nitroaniline. This may indicate cross-reactivity. For PPD and 4,4'-azodianiline, the opposite is true, with a tendency for a higher degree of test reactivity to 4,4'-azodianiline, which may indicate that 4,4'-azodianiline is a primary sensitizer in PPD-based hairdyes. The higher degree of test reactivity could also be attributable to better skin penetration and splitting of the molecule into two PPD molecules by azoreduction.

To investigate cross-reactivity, animal studies such as guinea-pig maximization tests need to be performed. This will hopefully shed further light on the cross-reactivity patterns in PPD sensitivity (17, 33). Identification of the possible allergens of the other spots of the thin-layer chromatograms to which the patients reacted will hopefully provide further understanding of the reason for the different reaction patterns in PPD-sensitized patients, and perhaps even further clues as to why the cross-reactivity patterns differ.

The identification of haptens involved in contact allergy is of importance to enable evaluations of occupational and non-occupational exposure. It is also needed to enable preventive advice and measures, for both primary and secondary prevention.

In order to understand PPD as a sensitizer, more studies need to be performed.

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