LIV KROONA

ORAL CONTACT ALLERGY TO CARVONE

With a focus on oral lichen

MALMÖ
UNIVERSITY

ORAL CONTACT ALLERGY TO CARVONE

Malmö University, Faculty of Odontology
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Malmö University, 2018
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Till mamma och pappa

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ABSTRACT

This thesis examines carvone (l-carvone), a mint flavour, and contact allergy to carvone with a focus on oral lichen. Carvone is a constituent of spearmint oil and is used to flavour toothpaste and food. Like many flavours and fragrances, carvone may cause contact allergy, but the prevalence is low, between 1.6 and 2.8%. Affected patients often have perioral or oral signs. A couple of studies have shown that patients with oral lichen planus or oral lichenoid lesions often have contact allergy to carvone but it is not known if these lichenoid lesions are a manifestation of contact allergy or part of the auto-immune disease, oral lichen planus.

In paper I, the amount of carvone was measured in 66 toothpastes and the ingredient lists were studied. Carvone was detected in all toothpastes with flavour, even fruit flavoured toothpaste, in up to 0.35%. The measured concentrations were all within the safe use level estimated to not induce contact allergy, but carvone concentrations over 0.1% are high enough to elicit a reaction in already allergic individuals. The regulation of carvone as a constituent in toothpaste was discussed.

Registry data (age, sex, referring information and patch tests results) on patients with a positive patch test reaction to carvone was studied in paper II. Data was retrieved from 1996 to 2016 and was compared with other patch tested groups not allergic to carvone. A matched comparison was also made between carvone-positive and carvone-negative patients tested with the same test series. Patients with contact allergy to carvone had a high mean age and were often women. According to the referrals, they often had oral signs and

57% had oral lichenoid lesions. In the matched comparison, oral (lichenoid) lesions were more common in carvone-positive patients.

A provocation test (or use test) with carvone in toothpaste was performed in contact allergic subjects in paper III. Subjects with a positive patch test to carvone used toothpaste with 1 % carvone during a month. Subject with oral lichen and healthy controls also participated in the study. The oral mucosa and the perioral area were examined three times during the test. The subjects' oral health-related quality of life was also assessed with a questionnaire (S-OHIP-49) before and after the use test. Carvone allergic patients exposed to toothpaste with carvone reacted with perioral eczema and/or increased oral lichenoid lesions. They also had reduced quality of life ratings after the use test. It is concluded that the clinical appearance of contact allergy to flavour ingredients may mimic oral lichen planus.

In paper IV, mucosal tissue samples from the subjects in paper III were investigated with. The inflammatory pattern and immune expression were analysed in allergic subjects and subject with oral lichen planus. No major differences were found between the groups; only Langerhans cells were more prevalent in oral lichen planus.

For most of us, carvone is a harmless flavour despite the life-long exposure from toothpaste. However, for individuals with oral lichen there is an increased risk to acquire contact allergy to carvone. Patients with oral lichen and contact allergy to carvone may get aggravated symptoms when exposed to carvone. Contact allergic reactions to carvone may imitate the clinical features of oral lichen planus and affected patients are potentially left undiagnosed with contact allergy to carvone. Clinicians treating patients with OLP should be made aware of this contact allergen and other soluble allergens.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Avhandlingen undersöker karvon (l-karvon), ett mintsmakande aromämne, och hur kontaktallergi mot karvon yttrar sig. Karvon finns framför allt i grönmynta/spearmint, men aromämnet kan även framställas ur limonen, ett citrondoftande parfymämne. Karvon är ett svagt kontaktallergen och allergiförekomsten är bara några procent i lapptestade populationer. Kontaktallergi mot karvon har i enstaka studier visat sig vara vanligare hos individer med lichenoida munslemhinneförändringar som oral lichen planus. Avsikten med avhandlingen har varit att studera detta samband vidare.

I studie I analyserades mängden karvon i 66 tandkrämer inköpta i Sverige. Även innehållsförteckningarna studerades. Lagstiftningen och innehållsmärkningen av karvon diskuterades. Karvonmängden i tandkräm varierar stort, 0,35–0,00005 %, men återfinns i alla tandkrämer med någon form av smak, även fruktsmak. De uppmätta mängderna är under det tillåtna gränsvärdet för att inte kunna ge kontaktallergi men några av tandkrämerna innehåller tillräckligt mycket karvon för att ge en reaktion hos redan kontaktallergiska individer.

I studie II studerades journal- och databasdata från alla patienter i södra Sverige som testats positivt för kontaktallergi mot karvon mellan 1996-2016. Gruppdata jämfördes med andra lapptestade grupper som inte var karvonallergiska, bl.a. en matchad grupp (ålder, kön, tidpunkt för test och liknande besvär). Karvonallergiska patienter hade hög medelålder och var ofta kvinnor. De hade ofta besvär från munhålan och över hälften hade orala lichenoida

förändringar. I den matchade jämförelsen hade karvonallergiska patienter betydligt mer orala lichenoida förändringar jämfört med patienter utan karvonallergi.

I studie III undersöktes hur allergi mot karvon i tandkräm yttrar sig. Karvonallergiska försökspersoner fick använda tandkräm med 1 % karvon under en månads tid. Även försökspersoner med orala lichenoida förändringar och individer utan allergi eller munslemhinneförändringar deltog. Slemhinnorna och läpparna undersöktes vid tre tillfällen under det månadslånga användartestet. Förutom den kliniska bedömningen svarade försökspersonerna på en livskvalitetsenkät (S-OHIP-49) före och efter användartestet. Karvonallergiska individer exponerade för karvon i tandkräm reagerade med antingen eksem runt munnen eller med förvärrade orala lichenoida förändringar. Även livskvaliteten försämrades hos dessa individer.

I studie IV undersöktes vävnadsprover från kind tagna på försökspersonerna i studie III. Inflammationen och olika markörer för inflammationsceller undersöktes. Det gick inte att finna någon större skillnad i inflammationsgrad eller i förekomst av inflammationsceller mellan individer med karvonallergi och individer med oral lichen planus förutom avseende förekomst av Langerhans celler, vilka var vanligare i vävnader från individer med oral lichen planus. Den lichenoida reaktionen som ses i de undersökta grupperna tolkas därför som ett reaktionsmönster vilket kan ha olika orsaksursprung.

För de flesta individer är karvon ett ofarligt smakämne trots livslång exponering från tandkräm och andra mintsmakande produkter. Individer med orala lichenoida förändringar tycks dock ha en ökad risk för karvonallergi och allergin förvärrar dessutom symptomen från munslemhinnan. Karvonallergi kan både kliniskt och på vävnadsnivå likna oral lichen planus vilket normalt sett inte kopplas samman med kontaktallergi. Karvonallergiska individer riskerar därför att inte bli diagnostiserade för sin allergi. Kliniker som handhar patienter med oral lichen planus bör därför uppmärksammas om denna form av allergi.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals. All papers are appended to the end of the thesis and reprinted with permission from the copyright holders.

- I. Quantification of l-carvone in toothpastes available on the Swedish market.
Kroona L, Warfvinge G, Isaksson M, Ahlgren C, Dahlin J, Sörensen Ö, Bruze M.
Contact Dermatitis 2017; 77: 224-230
- II. Carvone contact allergy in southern Sweden – a 21-year retrospective study.
Kroona L, Isaksson M, Ahlgren C, Dahlin J, Bruze M, Warfvinge G.
Acta Derm Venereol. 2018 Aug 7
- III. Use test with l-Carvone in toothpaste on sensitised individuals
Kroona L, Ahlgren C, Bruze M, Dahlin J, Isaksson M, Warfvinge G.
Manuscript
- IV. Molecular profiling of oral contact reactions to l-carvone and oral lichenoid reactions
Kroona L, Warfvinge G, Prgomet Z.
Manuscript

ABBREVIATIONS AND DEFINITIONS

ac	Acetone
aq	Aqua
CA	Contact allergy to carvone
CA-group	Subjects with a positive patch test to carvone
CA+/OL±/c	Carvone allergic subjects using carvone toothpaste
CA+/OL±/nf	Carvone allergic subjects using non-flavoured toothpaste
CA+/OL+/c	Subjects with carvone allergy and oral lichen using carvone toothpaste
CA-/OL+/c	Subjects with oral lichen using carvone toothpaste
CAOL-C	Tissue from subjects with carvone allergy and oral lichen using carvone toothpaste
CAOL-NF	Tissue from subjects with carvone allergy and oral lichen using non-flavoured toothpaste
DAB	Diaminobensidine
DALUK	Databas för Lagring av Uppgifter om Kontaktallergi (Database of contact allergy)
HIAR	Heat-induced antigen retrieval
HPBK	Peroxidase-blocking solution

HPLC	High-performance liquid chromatography
ICDRG	International Contact Dermatitis Research Group
IFRA	International Fragrance Association
LR	Lichenoid reaction (histological diagnosis)
MHC	Major Histocompatibility Complex
NM-C	Tissue from subjects with normal mucosa using carvone toothpaste
OHIP	Oral Health Impact
OHRQL	Oral Health Related Quality of Life
OL	Oral lichen (when referring to both OLP and OLL)
OLL	Oral lichenoid lesions (clinical diagnosis)
OLP	Oral lichen planus (clinical diagnosis)
OLP-C	Tissue from subjects with oral lichen planus using carvone toothpaste
pet	petrolatum
RIFM	Research Institute for Fragrance Materials
SCCS	Scientific Committee of Consumer Safety (scientific advisory body of the European Commission)
QRA	Quantitative risk assessment (of an allergen)

INTRODUCTION

This thesis investigates the mint-flavour carvone (l-carvone) and how contact allergy to carvone is manifested, with a special focus on oral lichenoid lesions.

Carvone, the main constituent of spearmint oil, can be found in toothpaste and mint flavoured food. Almost any substance may cause contact allergy in humans and carvone is not an exception. Many use mint flavoured toothpaste daily and are probably exposed to low doses of carvone. Still, the frequency of contact allergy is fairly low. The prevalence is 1.6–2.8% in investigated dermatitis populations (1,2) and experimental studies have shown carvone to be a weak allergen (3-5). However, a few studies indicate that patients with oral lichen planus and oral lichenoid lesions seem to be more affected by contact allergy to carvone (6,7).

The introduction covers carvone as a flavouring ingredient and the regulations governing its use. The mechanism and diagnosis of contact allergy is also described. This is followed by describing different forms of oral lichen and how they relate to contact allergy.

The origin of carvone

Fragrances are organic volatile compounds with characteristic odours, while flavours are fragrances used for their aromas (8). Carvone belongs to a group of fragrances called cyclic terpenes, which are often found in essential oils. Carvone exists in two forms, l-carvone and d-carvone, with differing aromas. They are enantiomers; meaning they are mirror images of each other (Figure 1). The d-form of carvone has an herbal aroma of caraway and is the main constituent

in oil from caraway seeds or dill seeds. The l-form of carvone has a minty aroma and is the main constituent of spearmint oil (9,10). In this thesis, the use of the name ‘carvone’ refers to the l-form.

Figure 1. Enantiomers of carvone and d-limonene

Mint or *Mentha* is part of the Lamiaceae family with over 40 species and hundreds of subspecies, varieties and cultivars (11). Carvone is predominantly found in spearmint, *Mentha spicata* or *Mentha viridis* (Figure 2), and depending on subspecies, environmental factors and harvest time, the concentration in oil can be up to 80% (12-16). Carvone has also been found in peppermint oil (*Mentha piperita*) and other *Mentha* species (16-20), but to a much lesser degree and not in all investigations (21,22). Limonene (d-limonene, Figure 1) is also found in spearmint oil where measured concentrations (10 to 20%) are the highest among the *Mentha* species. There is a relationship between these two chemicals: Carvone can be synthetically produced from d-limonene (13) and air-oxidation of limonene generates oxidation products, including carvone (23).

Figure 2. *Mentha viridis* var. *crispata* Schrader (24)

Regulations of carvone as an allergen

To ensure consumer safety and to avoid adverse reactions, the use of fragrances and labelling of cosmetic products are regulated by authorities (25). In Europe the Cosmetics Regulation applies to all cosmetic products sold, including toothpastes (26). Producers are required to have information on the composition of the product, the safety of each ingredient and an evaluation of overall product safety. Regarding contact allergy, certain fragrance allergens must be declared on the ingredient list when concentrations of the individual fragrances exceed 0.001% in leave-on products and 0.01% in rinse-off products. This applies to limonene and 25 other fragrance allergens, but not to carvone or any other mint flavours. Instead, all other fragrance and flavour ingredients, including carvone, are collectively labelled as *parfum* or *aroma*. Carvone is an established contact allergen (3,4) and although it is not required to be listed, the Scientific Committee of Consumer Safety (SCCS) has recommended that several fragrance allergens, including carvone, should be labelled on cosmetic products (5). Besides the Cosmetics Regulation, there are EU rules on flavourings

and chemicals (27-30). Carvone is listed as a flavouring substance and may be used in food (28). When carvone is used as a chemical, i.e. not used as food flavour or cosmetic substance, it should be declared on the ingredient list when the concentration exceeds 0.1% (29). However the main exposure to carvone comes from foodstuffs and particularly oral healthcare products such as toothpaste (31).

Cosmetic ingredients are evaluated by an independent committee of expert, the SCCS, before being adopted into regulations. The assessment data may also come from other sources such as other scientific committees, external experts or the cosmetics industry (32,33). The International Fragrance Association (IFRA) is a common-interest association that represents the interest of the fragrance industry. The IFRA sets guidelines (standards) for the safe use of fragrances in products. Member companies comply with these standards and a Code of Practice in the manufacturing and handling of their products (34). The recommendations are based on evaluations performed by the Research Institute for Fragrance Materials (RIFM) together with an expert panel of independent researchers. RIFM evaluates available scientific data on individual fragrances and conducts a quantitative risk assessment (QRA) for the induction of dermal contact allergies. The assessment is based on allergen threshold levels, measured in humans and/or animal experiments, to avoid contact allergy. It takes into consideration which area of the body the cosmetic product is intended for and the expected daily dose, but also if the fragrance ingredient may be present in vehicles containing irritants, emulsifiers, or penetration-enhancing factors (35-37). For carvone, the safe use level to prevent induction of contact allergy to oral hygienic products has been calculated to be 1.9% (4).

Contact allergy and the immune response

The word allergy can pertain to different types of reactions where the immune system reacts to harmless external substances. The two most common forms derive from type I and type IV hypersensitivity. Type I hypersensitivity, also called IgE-mediated or immediate hypersensitivity reaction, may affect most parts of the body and is often associated with protein allergens causing allergic rhinoconjunctivitis and asthma, e.g. pollen allergy. In contact allergy the immune process is a type IV hypersensitivity reaction defined as a

delayed reaction to a certain allergen. The clinical manifestation of contact allergy on skin is called allergic contact dermatitis and a common example is eczema from nickel contact (38).

An allergen is the agent that causes the allergic disease. To develop an allergic reaction the immune system needs to recognise the allergen as a threat. The process by which the immune system recognises the allergen for the first time is called sensitisation and occurs in all forms of allergy. When a sensitised individual is re-exposed to the allergen the immune system reacts with an allergic reaction, a process called elicitation.

The allergens causing contact allergy, or contact sensitivity, are also known as haptens. They are often small and lipophilic with chemically reactive properties; features that facilitate the penetration of the hapten into the outer layers of the skin or mucosa (39). Haptens are not recognised by the immune system directly; instead they form complexes with skin proteins and induce a local irritation or micro-injury. The irritation initiates an inflammation and epidermal dendritic cells called Langerhans cells recognise the conformed proteins as foreign matter. The protein complex is engulfed and processed by a Langerhans cell and a small part of the protein, a peptide, becomes a part of a cell surface receptor called major histocompatibility complex (MHC). The Langerhans cell travels from the epidermis along lymphatic vessels to a local lymph node. The lymph node contains lymphocytes called T-cells that can recognise peptides, also called antigens, presented on the MHC. The peptide on the Langerhans cell is recognised by a naïve (not previously activated) T-cell that begins to proliferate and release cytokines. All the copies of the activated T-cell now recognise the peptide derived from the hapten and some of them become memory T-cells for future encounters with the hapten.

Upon re-exposure to the hapten the same process as described in the sensitisation phase activates the Langerhans cells. This time, the memory T-cells recognise the peptide on the MHC. They proliferate and release inflammatory cytokines which create an influx of inflammatory cells, often macrophages. Effector T-cells that are cytotoxic attack cells where the hapten is present. The inflammation that now develops is seen in the area of skin that was exposed to the allergen. Clinical signs of the inflammation, or the allergic contact dermatitis, can be seen a few days after exposure to the allergen, often recognised as an eczema (40,41).

Diagnosis of contact allergy – patch testing

Patch testing is the standard procedure for making a diagnosis of contact allergy. The test reproduces the elicitation phase of contact allergy. The test subject is exposed to allergens, although under standardised conditions. If the subject is allergic to a specific substance applied, an eczematous lesion develops in the area of application and the intensity of the reaction is graded.

Patch tests are performed on patients where there is suspicion of contact allergy or where contact allergy needs to be excluded. Suspensions that lead to a test are a history of eczema or worsening of pre-existing lesions. The hallmark lesion is eczema, or dermatitis, but other clinical conditions may also represent contact allergic reactions, e.g. lichenoid, psoriatic or granulomatous reactions.

At specific concentrations, the investigated allergens are dispersed in a vehicle, often white soft paraffin (petrolatum), and are applied in small test chambers. The chambers are then attached with tape on the investigated patient's skin, often on the upper back (Figure 3).

Figure 3. Patch testing. The test chambers are attached on the back. The positions are marked to facilitate future test readings.

There are a variety of test chambers on the market, made in different materials and sizes. The patches are left on the back for 48 hours and are then removed, often by the patient. A reading of the test is performed at least twice where any reactions are recorded. In the studies conducted for this thesis, test readings were performed at day 3 or 4 (D3/4) and at day 7 (D7) (42,43). The patch testing technique has developed and undergone standardisations over the years to improve the certainty of positive test results and to facilitate the comparison of prevalence in different patch test populations. The test reactions are scored according to the criteria of the International Contact Dermatitis Group (ICDRG) as negative, irritant or positive (44). A positive reaction is scored + (weak positive), ++ (strong positive), or +++ (extreme positive). A doubtful reaction (+?) is scored when it is not possible to distinguish between negative and positive reactions (Table 1).

Table 1. Patch test grading according to the criteria of the ICDRG

Symbol	Morphology	Assessment
–	No reaction	Negative reaction
?+	Faint erythema only	Doubtful reaction
+	Erythema, infiltration, discrete papules	Weak positive reaction
++	Erythema, infiltration, papules, vesicles	Strong positive reaction
+++	Intense erythema, infiltration, confluent vesicles	Extreme positive reaction
IR	Various morphologies (soap effect, vesicles, blisters, necrosis)	Irritant reaction

Patch test series and testing with carvone

The patient’s history and clinical signs can give clues as to which allergens should be tested. However, it is not sufficient to patch test only with the suspected allergens as it has been shown that allergies can be discovered despite a negative history. There are thousands of known allergens and it is impossible to test all of them in one test. Instead, a panel with common allergen is used, called the baseline

series. The composition of the baseline series may vary slightly between regions and is often revised to adapt to current knowledge on and changes in exposure to allergens (45). There are also other test series with additional allergens that are used based on the clinical suspicion. They can be aimed at specific professions e.g. hairdressing series, or different products e.g. fragrance series.

Patients with oral signs and symptoms of contact allergy are tested with a specific test series called the dental series (for patients). Carvone is one of the tested substances (in southern Sweden), as carvone exposure comes mainly through oral contact with toothpaste and foodstuffs. Carvone is also included in a test series involving common lip allergens, the cheilitis series, but is not included in the baseline series as it is not a common contact allergen. The recommended test concentration is 5% carvone in petrolatum (46).

Oral lichen

Lichen planus and oral lichen planus

Lichen planus is a common chronic mucocutaneous disease with a T-cell mediated immune response. Affected individuals may have oral lesions, termed oral lichen planus (OLP), or skin involvement. Lesions in other mucosal areas have also been described but are less common (47,48). One or several areas (skin or mucosa) may be involved (49).

OLP is found in less than 2% of the Swedish population, with a predilection for middle-aged and elderly women, although lesions can be found in both sexes and all age groups (50,51). Clinically, OLP often manifests as bilateral white and reticular lesions of the buccal mucosa but virtually any area of the oral mucosa can be affected and may also present as plaque-like, erythematous or ulcerated lesions (Figure 4). The various patterns can be seen in a single patient and the patterns may change over time (49). The clinical symptoms range from mild to severe. White lesions may produce sensitivity to spicy or acidic foods whereas erythematous lesions may be very painful and have an impact on the daily lives of affected patients (52).

Figure 4. Clinical patterns of oral lichen planus. Upper left) Buccal mucosa with white reticular lesions and slight erythema. Upper right) The same individual as in 'upper left', seen with plaque-like lesions on the tongue. Lower left) Erythematous and ulcerated lesion on the buccal mucosa. Lower right) Lesions on gingiva, buccal mucosa and lower lip with reticular, erythematous and plaque-like patterns.

Despite a vast number of studies, the aetiology of OLP is poorly understood. OLP have been linked to viruses and high stress (53,54) but most research points towards OLP being a T-cell mediated immune response against surface epithelium and it is believed by many to be an autoimmune disease (55). With the primary intention of reducing pain and symptoms, current treatment modalities aim to suppress the immune response with corticosteroids (56).

Diagnosis can usually be made on clinical presentation alone, but non-reticular lesions may be difficult to distinguish from other oral lesions when concurrent typical reticular lesions are absent. A biopsy can be taken to rule out other diagnoses e.g. vesiculobullous disorders or epithelial dysplasia (49,57).

Histologically, OLP present as a lichenoid reaction. The histological features are not pathognomonic for OLP but are a tool for making a correct diagnosis. Although there is a spectrum of variation, lichenoid reactions are typically described as having a subepithelial band-like

lymphocytic infiltrate and epithelium with basal cell degeneration (58). The difference between normal mucosa and mucosa with lichenoid reaction is illustrated in Figure 5.

Figure 5. Haematoxylin-eosin-stained sections of normal mucosa (left) and mucosa with a lichenoid reaction (right), taken from the buccal mucosa. Note a dense inflammatory infiltrate adjacent to the epithelium (right). Scale bar indicate 250 μ m

Oral lichenoid lesions

Oral lichenoid lesions (OLL) are clinically similar to OLP but have a known aetiology or a different clinical presentation. The lesions can be associated with graft versus host disease or hypersensitivity reactions to dental materials or drugs. Additionally, any lichenoid lesion that lack one or more clinical aspects of characteristic OLP is considered to be OLL, e.g. unilateral distribution of reticular (classical) lesions or erythematous lesions without concurrent classical lesions. In such cases, a differential diagnosis, such as leukoplakia or erythroplakia, should be considered and a biopsy taken to rule out epithelial dysplasia or even squamous cell carcinoma (49). The various types of OLL have the same histological features as seen in OLP – a lichenoid reaction. Thus these lesions cannot be diagnosed based on routine-stained sections alone (58,59).

In both OLP and OLL, there is a risk of progression to malignancy. The malignant transformation rate seems to be lower in OLP than in OLL, but because of their similarities both clinically and histologically it is difficult to make a uniform interpretation of available data (60). OLP have a malignant transformation rate of around 1% (61,62)

whereas the measured rates for OLL range between 2.5–5% (61-63). Risk factors that increase the risk of malignant transformation include smoking, alcoholism, hepatitis C virus infection and lesions involving the tongue (62,63).

Manifestations of oral contact allergy

Contact allergy on skin is typically manifested as eczema, but other lesions may also represent skin allergy (64). Oral contact allergy can manifest itself in many different ways, e.g. cheilitis, stomatitis, lichenoid contact lesions, ulcerations and burning mouth syndrome. There are several dental materials and products that can irritate the oral mucosa or cause allergic reactions; both type I and type IV hypersensitivity reactions. The most common agents are dental restorative materials, flavourings and preservatives (65-67). Lichenoid contact lesions, a form of OLL, may develop near amalgam fillings as a contact allergic reaction to mercury (68-71). Contact allergies seem to be more common in patients with oral lichen (6,72,73), who often have positive patch test reactions to metals, acrylates and flavours.

Contact allergy to carvone

There are few reports on oral (or perioral) contact allergy to carvone or spearmint oil, and they mainly consist of case reports or group studies on cheilitis patients. The studies reported differing findings and for the most part they were perioral, i.e. cheilitis or perioral dermatitis. Oral findings were diffusely described as ‘oral involvement’ or ‘oral erosions in a patient with OLP’ (1,74-79). Ahlgren et al. found that patients with oral lichen had a higher frequency of l-carvone sensitisation compared to dermatitis patients, 12.1 % versus 1.2% (6). A registry study by Gunatheesan et al. investigated patients with a positive patch test for spearmint oil (7). 19 of 73 patients were classified as relevant according to clinical presentation and usage of products containing spearmint. 14 of 19 had OLP, where 10 showed signs of improvement when avoiding spearmint products, suggesting that spearmint allergy may cause OLP.

AIMS

- To qualitatively and quantitatively investigate the presence of carvone in commercially available toothpastes on the Swedish market
- To retrospectively estimate the prevalence of contact allergy to carvone in Southern Sweden and to assess the characteristics of this group with regard to other contact allergies and clinical signs with special reference to OLP and OLL
- To investigate how the oral mucosa and perioral area in carvone-sensitised individuals respond to carvone in toothpaste
- To investigate immune cell expression in the oral mucosa in individuals with OLP and in individuals with contact allergy to carvone

MATERIALS AND METHODS

The methods used in the four papers are quite dissimilar from each other, ranging from laboratory analysis of toothpaste to clinical experimental investigations. The common denominator is carvone. In paper I, the carvone content was measured in toothpastes using high-performance liquid chromatography. Paper II is a registry study based on patch test and referral data from patients with contact allergies to carvone. In a clinical experimental study, represented by paper III and IV, allergic subjects used toothpaste containing carvone for the period of a month. Clinical data, questionnaires and tissue samples were analysed. A detailed description of the materials and methods used can be found in the individual papers.

Assessing and quantifying flavours in toothpaste (papers I and III)

High-performance liquid chromatography (HPLC)

The HPLC method is used to separate different chemical components in a mixture and to subsequently identify and quantify the component of interest. The sample is dissolved in a liquid and transferred under pressure through a column with adsorbent material. Because of different flow rates, the components in the mixture separate when passing through the column. The components are detected and quantified by studying the absorbance of UV-light.

The toothpastes analysed in papers I and III were prepared before the analysis by firstly dissolving them in saturated sodium chloride solution, followed by adding n-heptane to extract the carvone. For separation, a straight-phase HPLC SpectraSystem was used with a silica column and a mobile phase with an isocratic solution of

n-heptane with 0.165% butanol or 0.1% propanol. Carvone was detected and quantified by comparing the retention time and UV-absorption with a reference sample.

Toothpastes

The 66 toothpastes analysed in paper I were acquired at different supermarkets and pharmacies. The advertised flavour on the toothpastes was noted and the product labels were studied with reference to any flavouring ingredient. The toothpastes are listed in the 'Results' section.

In paper III, toothpaste in 20 ml syringes with 1% added carvone was studied to see if the carvone content was stable. Toothpaste samples from the top, middle and bottom of the syringes were analysed.

Figure 6. Toothpastes analysed in paper I.

Retrospective study of carvone contact allergy (paper II)

Selection from database and retrieval of data

Information on patch tested individuals was retrieved from a clinical database named DALUK at the Department of Occupational and Environmental Dermatology, Malmö, Sweden (80). All patients who were patch tested with carvone between 1996 and 2016 were included in the study and the retrieved information included sex, age at the time of investigation, type of patch test series in which carvone was included, positive patch test results and the grading of test reaction to carvone. For patients with a positive patch test reaction to carvone, additional information was retrieved from referrals. The following information was collected: referrer's profession, diagnosis, clinical signs and localisation of the signs.

Descriptive and comparative analysis of retrieved data

A descriptive analysis was performed on data taken from the carvone-positive patients. The majority of the patients were tested with either the baseline series or the dental series and patch test data from these two groups was subsequently compared with carvone-negative patients tested with the same series. A matched comparison was also made in which 99 carvone-positive patients tested with the dental series were compared to 99 carvone-negative patients tested with the same series. Patch test data, clinical signs and diagnosis were compared. The matching criteria were sex, age ± 6 years and date of testing ± 3 months. In two cases the criteria were extended to age ± 10 years and date of testing ± 4 months. The selection of patients matching the criteria was randomised. Figure 7 shows the different study groups that were compared.

Figure 7. Graphical depiction of the various study cohorts in the baseline series and the dental series that were subjected to comparative analysis.
C+, carvone-positive patch test; C-, carvone-negative patch test

Patch testing

During the entire investigated period all patients were patch tested with 8 mm Ø Finn Chambers® attached to Scanpore tape® together with the different patch test preparations. For carvone, the test preparation was 5% w/w in petrolatum. The test reading was performed, as described in the introduction, at day 3 or 4 and day 7 according to the ICDRG guidelines (44).

Provocation test with carvone (papers III and IV)

Subjects

Initial selection

Possible subjects with a previous positive patch test reaction to carvone were retrieved from DALUK, the same database used in paper II. Of 147 patients with a positive patch test to carvone, 92 were not diseased and lived in the regional county, Skåne, Sweden. Fourteen of the 92 subjects agreed to attend the study. All subjects were women and the mean age was 69.5 ± 7.6 .

Twenty subjects with oral lichen, either OLP or OLL, were recruited from the department of Oral Surgery and Oral Medicine, Malmö University, Sweden. All were diagnosed histologically, through biopsy, with lichenoid reaction. The mean age was 65.0 ± 10.3 years and 15 of 20 subjects were female.

Three dermatitis patients were also included in the study as a control group. They had no positive patch test to carvone and no oral lesions. The mean age was 68.9 ± 8.2 years and two subjects were female.

In all, 37 subjects were included in the study. All subjects were over 20 years and used toothpaste daily. Subjects who had possibly used any immune-modulating drugs in the 4 weeks prior to the use test were excluded – this included the use of both oral local and systemic corticosteroids. All subjects were contacted consecutively using lists of patients who met the inclusion and exclusion criteria for each group.

Use test groups and groups for evaluation (paper III)

Subjects with either OLP or OLL were treated as one group and their lesions were called *oral lichen* (OL). It was anticipated that some of the carvone-allergic subjects (CA) would have OL and that subjects recruited with OL could be contact allergic to carvone. Thus, the initial groups were slightly rearranged after patch testing (Figure 8A). The contact allergic group (CA-group) increased to 16 subjects, as two subjects with OL had a positive patch test to carvone. Randomly selected, 5 subjects from the CA-group received non-flavoured toothpaste and the rest of the group received toothpaste with carvone. The group with only OL and the control group both received toothpaste with carvone. The use test evaluated two factors: I) the effect of carvone toothpaste (c) compared to a non-flavoured toothpaste (nf) on carvone sensitised subjects regardless of OL lesions (CA+/OL±/c versus CA+/OL±/nf), and II) how subjects with OL with or without carvone sensitivity reacted to the test toothpaste (CA+/OL+/c versus CA-/OL+/c) (Figure 8B).

Figure 8. Study groups and evaluation of use test. A) Initial selection of subjects and the subsequent allocation into use test groups. B) Evaluation of use test: I) Subjects with contact allergy to carvone using toothpaste with or without carvone; II) Subjects with OL using carvone toothpaste with or without carvone contact allergy.

CA: contact allergy to carvone, OL: oral lichen, c: toothpaste containing carvone, nf: non-flavoured toothpaste.

Study groups for analysis of mucosal biopsies (paper IV)

Of the 37 subjects participating in the use test, 28 were selected for further analysis of mucosal biopsies taken after the use test. Eleven subjects were clinically classified as having OLP and had no contact allergy to carvone or to any other clinically relevant allergen. Fourteen participants had contact allergy to carvone and all but one had some form of clinical oral lichen (OL); either OLP or OLL. Subjects with OLP only used carvone toothpaste during the use test and are referred to as (OLP-C). Of the allergic subjects, nine used carvone toothpaste (referred to as CAOL-C) and five used toothpaste with no flavour (referred to as CAOL-NF) of which one had no OL. Three participants with no contact allergy to carvone and no OL served as controls. They all had normal mucosa and used toothpaste with

carvone (referred to as NM-C). Subjects with OLP were compared to carvone allergic participants receiving carvone (OLP-C vs. CAOL-C). Carvone allergic participants receiving carvone toothpaste were compared to carvone allergic participants receiving non-flavoured toothpaste (CAOL-C vs. CAOL-NF).

Study design of use test (paper III)

In all, the subjects were studied for five weeks and an overview can be seen in Figure 9. One week before the use test, the oral cavity was examined and medical history taken for all 37 subjects. Any lichenoid lesions were diagnosed. After the examination the subjects were patch tested. To avoid interference of other sources of carvone, the subjects were instructed to avoid any mint-tasting products for the duration of the study. They were also given a fruit-flavoured toothpaste without carvone to use during the week prior to the use test.

The use test lasted four weeks and subjects were studied three times with a two-week interval. The clinical examiners were blinded to information on subjects' possible contact allergies and what toothpaste they received. The subjects could request an examination and/or terminate the test at any time. The test was also terminated if ulcers in the oral mucosa or eczematous changes on the skin developed.

The subjects were instructed to brush their teeth two times a day with 1ml of toothpaste. The toothpaste was given in two 20 ml syringes at a time. To ensure that the correct amount had been used the level of toothpaste left in the old syringes was examined.

A clinical examination was performed on three occasions where the perioral skin and oral mucosa were examined by an experienced dentist. For each subject, 14 clinical photographs were taken of the same areas. At the start and end of the use test, subjects filled out an Oral health impact (OHIP) questionnaire, and were given a periodontal examination. The gingival pocket depth and bleeding on probing was measured.

Figure 9. Study design and timeline

Patch testing

All subjects were patch tested with carvone from a stock solution of 5% carvone in acetone w/v. Controls were tested with only 5% carvone. All subjects except controls were patch tested with ten serial dilutions of carvone. The dilution factor was half-logarithmic ($1:10^{-1}$) and was made in the range between 5.0% and 0.000158%. The three toothpastes used in the study (a fruit-flavoured toothpaste and a non-flavoured toothpaste with and without carvone) was tested diluted in water at 50% and 5% w/v.

Subjects recruited with OL who had not been previously patch tested were also tested with the Swedish dental series.

The patch tests were performed with 8 mm Ø Finn Chambers® placed on the subjects upper backs and attached with Scanpore tape®. The placement of the different preparations was blinded to the test readers. The test reading was performed, as described in the introduction, at day 3 or 4 and day 7 according to the ICDRG guidelines (44). Subjects and readers were advised to avoid communication with each other.

Toothpaste with added carvone

The use test-toothpaste was produced by adding carvone to a non-flavoured toothpaste provided by Proxident AB. Subjects given non-flavoured toothpaste were given the same toothpaste with no additives. Initially, the concentration was intended to be 1.9% carvone to ensure a sufficient contact allergic reaction in the test

subjects; 1.9% is also the maximum safe use level in toothpaste according to IFRA (4). However, the concentration of carvone was not uniform when analysing different samples from the same mixture. A separation was seen between carvone and toothpaste with carvone being more hydrophobic. A lower concentration, 1% carvone, was used instead which proved to be more stable. To avoid separation over time the toothpastes were mixed just before every use test period and were later analysed with HPLC. All toothpastes were administered in 20ml syringes (Injekt® Luer Solo).

Analysis of mucosal reactions

A clinical scoring system for mucosal disease by Escudier et al. (81) was used to assess any change between the examinations (Table 2). The score was calculated from recordings of 17 different mucosal sites together with a pain score i.e. the subjects' own perceived pain from the oral area, assessed by means of a numerical rating scale. The mucosal sites were measured by: a) mucosal affection (site score) and b) the severity of the lesions (severity score). For each mucosal site an activity score was calculated by multiplying the site score by the severity score ($a \times b$). A total weighted score (S_{tot}) was defined as the sum of the overall site score + overall activity score (S_a) + pain score.

The scoring was performed using the clinical photographs taken at the start and finish of the use tests. The identity of the subject or any subject code was hidden to the observer and test occasion was randomised (start and end). Two observers independently evaluated the images from the use test and then reviewed all images together to agree on a consensus score.

Table 2. Scoring system for mucosal disease by Escudier et al. showing sites examined, numerical and maximum scores as well as calculation of the total weighted score (S_{tot}). Site score: 0, no lesion; 1, lesion present and 2, lesion covering > 50 % of the examined site. Severity score; 0, hyperkeratosis; 1, hyperkeratosis with mild erythema; 2, marked erythema and 3, ulceration.

Site	Site score (a)	Severity score (b)	Activity score (a x b)
Outer lips	0 or 1	0–3	0–3
Inner lips	0 or 1	0–3	0–3
Left buccal mucosa	0, 1 or 2	0–3	0–6
Right buccal mucosa	0, 1 or 2	0–3	0–6
<i>Gingiva</i>			
Lower right	0 or 1	0–3	0–3
Lower central	0 or 1	0–3	0–3
Lower left	0 or 1	0–3	0–3
Upper left	0 or 1	0–3	0–3
Upper central	0 or 1	0–3	0–3
Upper right	0 or 1	0–3	0–3
Dorsum of tongue	0, 1 or 2	0–3	0–6
Lateral tongue, right	0 or 1	0–3	0–3
Lateral tongue, left	0 or 1	0–3	0–3
Floor of mouth	0, 1 or 2	0–3	0–6
Hard palate	0, 1 or 2	0–3	0–6
Soft palate	0, 1 or 2	0–3	0–6
Oropharynx	0, 1 or 2	0–3	0–6
Maximum score	24		72

S_{tot} (max 106) = Overall site score (max 24) + overall activity score (max 72) + pain score (max 10)

OHIP

The OHIP-questionnaire that was filled out before and after the use test assesses oral health-related quality of life (OHRQoL). A Swedish validated version (OHIP-S) (82) was used, comprising 49 questions with a response format in an ordinal rating scale from 0-4 ('none/never', 'hardly ever', 'occasionally', 'often' or 'very often').

The response was calculated as change of the sum of scores between the start and end of the experiment. Both the total OHIP score and the scores for the four domains (appearance, function, pain and psychological treats) were calculated.

The inflammatory response in oral biopsies (paper IV)

At the end of the use test, incisional biopsies were taken in the buccal mucosa. Punch biopsies, 6 mm Ø, were taken from areas with clinically evident inflammation or lichenoid lesion if possible. Immunohistochemistry was performed on 3-µm-thick serial sections of formalin-fixed paraffin-embedded tissues.

Immunohistochemistry

Antibodies and their characteristics are listed in Table 3. For analysis of anti-CD1a, CD3, CD4, CD8, CD20, CD68 and CD138, the tissue sections were deparaffinised and rehydrated prior to heat-induced antigen retrieval (HIAR) in TRIS-EGTA buffer, pH 9.0 at 95°C for 40 min in a Decloaking Chamber™. Endogenous peroxidase was blocked for 5 min with peroxidase blocking reagent (HPBK) followed by 10 min with background punisher. The sections were incubated for 30 min with primary antibodies. The sections were then incubated with a secondary antibody (goat anti-rabbit/horseradish peroxidase) for 30 min at room temperature. Prior to the secondary antibody, the sections incubated with mouse antibodies (CD1a, CD8, CD20, CD68 and CD138) were treated with Mouse Probe for 15 min.

For analysis of anti-CD11c, CD123, and MxA, HIAR was performed in 10 mM citrate buffer, pH 6.0 at 95°C for 40 min. Sections were incubated for 10 min with HPBK followed by background punisher for 10 min at room temperature. Sections were incubated with primary antibody over-night at 4°C followed by Mouse Probe for 15 min prior to incubation with secondary antibody for 30 minutes.

For all sections, the immunoreaction was visualised with diaminobenzidine (DAB) for 5 min. The tissue sections were counterstained with haematoxylin, dehydrated, and mounted.

Table 3. Antibodies used for immunohistochemical staining

Antibody	Clone	Target cells	Source	IHC dilution
CD1a M3571	Monoclonal Mouse anti-Human, clone O10	Langerhans cells	DAKO, Glostrup, Denmark	1:400
CD3 A0452	Polyclonal Rabbit anti-Human	Peripheral T-cells	DAKO, Glostrup, Denmark	1:500
CD4 db133616	Monoclonal Rabbit anti-Human	Helper T-cells	Abcam, Cambridge, UK	1:2000
CD8 M7103	Monoclonal Mouse anti-Human, clone C8/144B	Cytotoxic T-cells	DAKO, Glostrup, Denmark	1:400
CD11c 111M	Monoclonal Mouse anti-Human, clone 5D11	Myeloid dendritic cells Macrophages	Sigma-Aldrich Sweden AB	1:200
CD20cy M0755	Monoclonal Mouse anti-Human, clone L26	B-cells	DAKO, Glostrup, Denmark	1:2000
CD68 M0814	Monoclonal Mouse anti-Human, clone KP1	Macrophages	DAKO, Glostrup, Denmark	1:8000
CD123 554527	Monoclonal Mouse anti-Human, clone 7G3	Plasmacytoid dendritic cells	BD Biosciences, San Jose, CA, USA	1:400
CD138 M7228	Monoclonal Mouse anti-Human, clone M115	Plasma cells (B-cells)	DAKO, Glostrup, Denmark	1:800
MxA MABF938	Monoclonal Mouse anti-Human, clone CL143	Plasmacytoid dendritic cells	Merck Millipore, Darmstadt, Germany	1:4000

Evaluation of immunohistochemistry

Both routine- and immuno-stained sections were scanned using a Nano Zoomer histology scanner with a 40× objective. For each sample, an area of interest, with a surface width of approximately 2 mm, was chosen, preferably with present inflammatory infiltrates. Three high-magnification areas, in 40× magnification, were subsequently chosen in the epithelial-connective tissue junction, one at each border and one centrally in the area of interest (Figure 10). The selected high-magnification areas were used throughout the serial tissue sections for quantification of positive immunostaining.

Figure 10. Overview of a tissue section with the area of interest. High-magnification areas indicated as squares with dashed lines. Scale bar indicate 500 μm .

The immunostaining with CD3, CD4, CD8, CD68 and MxA was evaluated with IHC profiler (83), a tool for automated image analysis. On immunostained sections with CD11c, CD123, CD20, CD138 and MxA, evaluation was made by manually counting immunostained cells. CD1a-positive cells and MxA-positive cells were only counted in the connective tissue.

Cut-off values for the IHC profiler were adjusted to define immunostaining as negative = 0, weak positive = 1, positive = 2 and strong positive = 3. Cut-off values for the quantitatively assessed antibodies (CD1a, CD11c, CD20, CD123, CD138 and MxA) were

set as positive if >5 cells showed immunostaining. Mean values of positively immunostained cells were retrieved from the three areas investigated by the two observers.

Ethical considerations

The use test (paper III) together with mucosal biopsies (paper IV) was approved by the Regional Ethical Review Board in Lund, Sweden. All subjects were informed and gave their signed consent to participate in the study. Papers II-IV, were all conducted in accordance with the ethical standards specified in the Declaration of Helsinki.

Statistical analysis

Data was analysed using SPSS Statistics 24.0 software and Excel™. A two-sided p-value < 0.05 was considered statistically significant.

Paper I

The relation between carvone concentration and limonene on the product label was analysed in two ways: 1. the number of toothpastes with and without limonene according to the labelling was compared with regard to the presence of carvone over and below 0.01% using Fisher's exact test, 2-sided. 2. The differences between measured carvone concentrations in the samples with and without limonene, according to the labelling, respectively, were analysed with the t-test for independent samples.

Paper II

With regard to frequencies of diagnoses and co-reactivity to different allergens, the difference between the carvone-positive and carvone-negative patient groups were analysed with the chi-square test or with Fisher's exact test when expected values were small.

Paper III

The difference between the start and end scores of the mucosal reactions and OHIP was calculated for every group. The two factors were analysed with the Mann-Whitney U-test. In previously recorded allergic subjects, the difference in clinical signs, i.e. S_{tot} , between subjects with positive and negative patch tests when re-tested was analysed with the t-test for independent samples. Controls were not included in the statistical analysis.

Paper IV

The differences in expression of CD1a, CD3, CD4, CD8, CD11c, CD20, CD68, CD123, CD138 and MxA in the studied groups were evaluated using a Mann-Whitney test. The findings from the NM-C group were not used in the statistical analysis.

Carvone used in the papers

Carvone i.e. l-carvone was used for HPLC and patch testing in Paper I-III and came from two suppliers (Table 4).

Table 4. Manufacturers of carvone used in the papers

Chemical	Paper	Application
l-carvone, CAS: 6485-40-1 Acros Organics, Geel, Belgium	I	HPLC, as reference standard
	II	Patch test preparation, in petrolatum
l-carvone; CAS: 6485-40-1 Sigma Aldrich, St Louis, MO, USA	III	Test toothpaste, 1 % carvone
		HPLC, as reference standard Patch test preparation, in acetone

RESULTS

Paper I – Quantification of l-carvone in toothpastes available on the Swedish market

Carvone was detected with concentrations varying between 0.00005 and 0.35% in 64 of the 66 toothpastes analysed. In 30 of the toothpastes the concentration was over 0.01% and in 10 of these the concentration was over 0.1%. Of nine toothpastes intended for children, all had detectable levels of carvone and two of these had concentrations above 0.09% (Figure 11).

Of the 66 toothpastes, 36 were advertised as having some type of mint flavour and 5 were advertised as fruit-flavoured and intended for children. The fruit-flavoured toothpastes all had detectable levels of carvone. Twenty-four of the toothpastes had no specified flavour advertised on the package but all had detectable levels of carvone and were labelled as containing *aroma*. Two toothpastes were advertised as being ‘without flavour’ and had no detectable levels of carvone.

The 64 toothpastes with detectable carvone were all labelled as containing *aroma* and 39 of these had additional flavours on the label with limonene being the most prevalent ($n = 34$) (Table 5). Of the 34 limonene-labelled toothpastes, a significant number contained carvone at concentrations higher than 0.01% compared with toothpastes without limonene on the label (22 of 34 versus 8 of 32; $p=0.0015$). Toothpastes labelled with limonene had a significantly higher concentration of carvone than those without limonene on the label – $0.059\% \pm 0.074\%$ versus $0.023\% \pm 0.067\%$ $p=0.045$ (Figure 12).

Figure 11. Carvone concentration in toothpaste. *) Toothpastes labelled containing Limonene, ♦) Toothpastes intended for children, †) Toothpastes advertised as “without flavour”.

Table 5. Declared flavours.

Flavour		n	Toothpaste
Flavours of the 26 fragrance allergens legislated in the EU			
Benzyl Alcohol		3	Pepsodent Kids 2-6 strawberry
Cinnamal			Pepsodent White system
			Pepsodent White System Enamel Renewal
		4	Colgate Smiles 6+
			Oral-B Pro-Expert All-Around Protection
Citral			Oral-B Pro-Expert Professional Sensitivity
			Oral-B Pro-Expert Sensitive & Gentle Whitening
Eugenol		1	Pepsodent White Naturals
		5	Colgate Smiles 6+
			Oral-B Pro-Expert All-Around Protection
			Oral-B Pro-Expert Professional Sensitivity
			Oral-B Pro-Expert Sensitive & Gentle Whitening
			Pepsodent Super Fluor
Other declared flavours		34	See figure 11
Limonene		64	-
Aroma			
Anise Alcohol		1	Sensodyne Pro-Enali
Citrus Medica Limonum (Lemon)		1	Pepsodent White Naturals
Peel Oil			
Mentha Piperita (Peppermint) Oil		1	Pepsodent White Naturals
Mentha Arvensis Leaf extract		1	Pepsodent White Naturals
Menthol		2	Colgate MaxFresh
			Colgate Smiles 6+

Figure 12. Boxplot (Q1/median/Q3, whiskers: min-max) showing mean values of carvone for All toothpastes divided into two groups, without or with limonene. Higher concentrations of carvone were found in toothpastes labelled with limonene ($p = 0.045$).

Paper II – Carvone contact allergy in southern Sweden – a 21-year retrospective study

A positive patch test reaction to carvone was recorded in 147 (3.5%) of 4221 tested patients. The mean age at testing was 66.2 ± 11 years and the male-to-female ratio was 1 : 5.1. For the carvone-negative group the male-to-female ratio was 1 : 2.6. The test series that gave a positive patch test result for carvone are listed in Table 6.

Table 6. Different test series in which carvone was included and the number of patients with a positive reaction to carvone (with OL) for each series.

Test series	Time period	Number of tested	Carvone-positive ^a	with OL (%) ^{ab}
Dental series (dental patients)	1996-2016	1938	99	67 (68)
Baseline series	1997-1998	1355	14	1 (5)
Cheilitis series	2000-2016	500	9	1 (11)
Dental series (dental personnel)	1998-2016	460	8	1 (13)
Research series (oral lichen)	2006-2008	259	15	11 (73)
Plant series	1996	31	2	0
Cosmetic series	1996	23	0	0
Personal series	n.a.	n.a.	6	2 (33)

a: some patients have been tested with more than one test series; b: number of carvone-positive patients with simultaneous oral lichen (OL); n.a.: not applicable

Descriptive analysis of the carvone positive patients

Referring information

Referring and clinical information was only available for 145/147 patients. Carvone-positive patients were primarily referred by dentists (n = 99), over half of whom were oral surgeons (n = 52). Physicians referred 36 of the patients and 10 patients were participants in research projects on oral lichen. Patients referred by dentists were primarily tested with the dental series and when referred by physicians, the patients were tested with a variety of test series depending on the localisation of clinical signs.

Recorded diagnoses and clinical signs were often localised intra-orally and to a lesser degree peri-orally. Eczema on other parts of the body was present in 34 patients and 19 of these had no oral or peri-oral involvement. In the referrals, it was not always possible to distinguish whether the referrer meant oral lichen planus or oral lichenoid lesions. Consequently all oral lesions diagnosed with some form of description of lichen were grouped together and called ‘oral lichen’ (OL). OL was diagnosed in 82 of the patients. Eleven patients had lichen planus on the skin or genital area.

Table 7 summarises the distribution of clinical signs and diagnoses as well as the patch test series used.

Table 7. Diagnoses and clinical signs in carvone-positive patients (n = 145) and the proportion of the major patch test series used (dental, baseline or cheilitis).

Clinical diagnosis or symptom	Number of patients	Patch test series ^a		
		<i>Dental</i>	<i>Baseline</i>	<i>Cheilitis</i>
Diagnosis:				
OL (OLP or OLL)	82	68	1	1
Dermal or genital lichen planus	11	5	1	0
Clinical signs:				
Oral signs	106	87	2	2
Perioral signs	20	8	1	8
Dermal (no oral or perioral signs)	19	4	11	0

a: some of the patients have been tested with more than one test series.
OL: oral lichen; OLP: oral lichen planus; OLL: oral lichenoid lesions

Patch test reactions to carvone

Of the 147 patients with a positive test reaction to carvone, 110 showed a positive reaction at day 3 and 111 showed a reaction at day 7. Thirty-one patients showed only a positive reaction at day 7. Additionally, six patients showed an even later reaction, from 10 days up to a month after the test application. Of these, two were re-tested and both showed a reaction on day 3.

The distribution of the different patch test reaction grades among the 147 patients was 78 ‘+’, 59 ‘++’ and 10 ‘+++’. There were also 83 patients with a doubtful ‘(+)’ reaction.

Other contact allergies

Positive patch test reactions to other allergens were present in 111 of the 147 patients, with a mean number of 4.1 ± 4.9 other test reactions. Gold, nickel and mercury were the most common allergens giving a concomitant reaction. Since the 147 patients were tested with different test series, some allergens, such as fragrances and acrylates, were only tested on part of the group. Common concomitant reactions for those tested were to spearmint oil, fragrance mix I and balsam of Peru. Some of the patients were tested with their personal hygienic products and 11 of 16 patients tested with toothpaste displayed a

positive reaction. The toothpastes were all of different brands and no brand was singled out as giving more or stronger patch test reactions. Table 8 displays the most common concomitant reactions and their frequencies.

Table 8. Common positive patch test reactions among the 147 carvone-positive patients. a) Substances that the entire group were tested with, b) substances that only part of the group were tested with, c) test with patients' personal toothpaste.

	Test substance	Positive reactions/ number of tested	% of the entire group, n = 147
a)	Gold sodium thiosulfate	52/147	35.4
	Nickel sulfate	23/146	15.6
	Mercury	20/147	13.6
	Potassium dichromate	15/147	10.2
	Cobalt chloride	12/146	8.2
	Palladium chloride	11/147	7.5
	Colophonium	11/147	7.5
	Formaldehyde	6/147	4.1
			% of tested
b)	Spearmint oil	55/81	67.9
	Fragrance Mix I	23/88	26.1
	Balsam of Peru	19/117	16.2
	Cinnamal	9/93	8.8
	Cinnamyl alcohol	6/68	8.1
	Peppermint oil	5/80	6.3
	Acrylates ^a	9/125	6.1
	Limonene ^b	4/90	4.4
c)	Toothpaste (various brands)	11/16	68.8

a: Different acrylates grouped together, b: The preparation of limonene has varied over the investigated years of which some preparations were oxidised.

Comparative analysis – carvone-positive versus carvone-negative patients

When studying patch test results from the baseline series and the dental series, the carvone-positive group had significantly higher frequencies of positive patch test reactions to several contact allergens (Table 9). However, in the matched comparison there was no difference in the frequency of patch test reactions between the carvone-positive and carvone-negative groups. A difference was seen in OL, where 67 of 99 carvone-positive patients had OL compared to 28 of 99 patients in the carvone-negative group (Table 10).

Paper III – Use test with l-carvone in toothpaste on sensitised individuals

HPLC

Sixteen syringes were analysed with a mean storage time of 102 days. The average concentration of carvone in the syringes was $0.97 \pm 0.12\%$ and the storage time did not affect the concentration. The concentration of carvone fluctuated slightly in the syringes. The mean concentration of carvone from the top of the syringes was lower than average (0.89%) and the mean concentration from the bottom sections was slightly higher than average (1.06%).

Initial examination and patch test

As two subjects recruited with OL also had a positive patch test to carvone, they were included in the CA-group (n =16). In subjects with OL only, 14 of 18 had a clinical diagnosis of OLP and the rest had OLL. Of the carvone allergic subjects, 13 of 16 had OL where eight were diagnosed as OLP and five as OLL. Ten of the carvone allergic subjects stated that they were using mint-flavoured toothpaste. Positive patch test reactions were only observed in 8 of the 16 allergic subjects. Two subjects had late reactions, at day 19 and day 21. The reaction strength from 5% carvone varied between '+' and '++'. The lowest concentration giving a '+' reaction was 0.05% carvone. Five subjects had a positive reaction to 50% test toothpaste with carvone diluted in water. No reactions were observed to the other toothpastes (Table 11).

Table 9. Positive patch test reactions in a) the baseline series 1997-1998 and b) the dental series 1996-2016 with the number of carvone-positive (with OL) demonstrated.

	a) Baseline series		b) Dental series	
	All tested	Carvone-positive (OL)	All tested	Carvone-positive (OL)
Number of tested	1355	14	1938	99
Patch test reactions to:		(1)		(67)
Gold sodium thiosulfate	298	8**	542	37*
Palladium chloride	39	2	129	5
Nickel sulfate	283	2	364	15
Potassium dichromate	78	2	78	9*
Cobalt chloride	110	4*	134	7
Balsam of Peru	72	2	165	18**
Fragrance mix I	90	2	68	6
Thiuram mix	24	2*	48	5
Ethylenediamine	41	2	28	4(*)
				(3)

Allergens are displayed when ≥ 2 patients had a concomitant positive patch test for carvone. Patients with OL (oral lichen) displayed in brackets. HEMA: Hydroxyethyl methacrylate; EGDMA: Ethylene glycol dimethacrylate. Significant values are shown for the comparison between carvone-positive and carvone-negative groups.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, (*) $p = 0.0510$

Table 10. Positive patch test reactions in the dental series 1996-2016 in carvone-positive patients and a matched carvone-negative group of patients (with OL).

Dental series – matched groups				
	Carvone- positive	(OL)	Carvone- negative	(OL)
Number of tested	99	(67)***	99	(28)
Atopy	20	(13)	20	(1)
Patch test reactions to:				
Gold sodium thiosulfate	37	(30)	38	(12)
Palladium chloride	5	(3)	5	(0)
Nickel sulfate	15	(7)	16	(4)
Potassium dichromate	9	(5)	2	(0)
Cobalt chloride	7	(4)	2	(0)
Mercury	18	(16)	12	(9)
Colophonium	6	(3)	3	(1)
HEMA	5	(3)	2	(1)
EGDMA	4	(3)	0	(0)

Patients with OL (oral lichen) displayed in brackets. HEMA: Hydroxyethyl methacrylate; EGDMA: Ethylene glycol dimethacrylate. Significant values are shown for the comparison between the carvone-positive and the carvone-negative group. *** $p < 0.001$

Table 11. Subjects with contact allergy to carvone (CA-group) – Patch test result and use test result. OL: oral lichen; OLP: oral lichen planus; OLL: oral lichenoid lesions; pet: petrolatum; ac: acetone; MEC: Minimum eliciting concentration; aa: water; Stot: Escudier index, total weighted score; pain: numerical rate scale on pain.

			PATCH TEST			USE TEST				
No	Subject (sex and age)	Toothpaste flavour before patch test	Previous patch test reaction (5 % carvone pet)	Patch test reaction (5 % carvone ac)	MEC and reaction strength (carvone in ac)	Patch test reaction test toothpaste 1% carvone (50 % ac)	Test toothpaste	Use test termination in advance (days in advance)	Mucosal score and difference S _{st} (pain)	Clinical signs on photos
1	Female, 58	OLP	Not tested	++	5 % ++	-	Carvone	Yes (12)	+2 (+2)	Weakly increased OL activity
2	Female, 80	OLP	Not tested	++	0.5 % ++	+	Carvone	No	+10 (0)	Increased OL activity
3	Female, 72	OLL	++	++	1.58 % ++	+	Carvone	Yes (14)	+12 (+6)	Perioral eczema Weak signs of OL
4	Female, 77	OLP	++	++ Late reaction d21	+ Late reaction	++ Late reaction	Carvone	Yes (13)	+7 (+5)	Scaling red lips Increased OL activity
5	Female, 72	OLP	+++	++ Late reaction d19	0.05 % +(+) Late reaction	+(+) Late reaction	Carvone	Yes (3)	+16 (+2)	Increased OL activity
6	Female, 76	OLL	+	(+)	0.5 % +	-	Carvone	No	+7 (-1)	Increased OL lesions and activity
7	Female, 67	OLP	++	+	5 % +	+	Carvone	No	-	Excluded
8	Female, 51	No	+	-	-	-	Carvone	No	-1 (-1)	
9	Female, 53	OLL	+	-	-	-	Carvone	No	+9 (+3)	Increased OL activity
10	Female, 75	OLL	+	-	-	-	Carvone	No	+1 (+1)	
11	Female, 68	OLP	+	-	-	-	Carvone	No	+2 (0)	
12	Female, 73	OLL	++	+(+)	0.5 % +	(+)	Non-flavoured	No	+2 (0)	
13	Female, 67	OLP	+	-	-	-	Non-flavoured	No	0 (0)	
14	Female, 75	No	++	-	-	-	Non-flavoured	No	+1 (0)	
15	Female, 68	No	++	-	-	-	Non-flavoured	No	0 (0)	
16	Female, 71	OLP	+++	-	-	-	Non-flavoured	Yes (4)	-2 (+2)	

Use test

Two subjects received anti-inflammatory medication for other reasons than the experiment and were excluded from the mucosal analysis (Figure 8B in ‘Materials and methods’). Three additional subjects were excluded from the OHIP analysis because of incomplete questionnaires. Five subjects, all contact allergic to carvone, terminated the use test in advance. Four of these subjects used carvone toothpaste and had discomforting reactions and/or clinical signs. One allergic subject used non-flavoured toothpaste and stopped due to a concomitant episode of erythema nodosum not related to the test (Table 11).

Analysis of mucosal reactions and periodontal status

When examining the before and after photos, 7 of the 10 allergic subjects who used carvone toothpaste showed increased clinical signs (Figure 13). The signs presented as aggravated oral lichenoid lesions (7 of 7) and eczematous reactions on the peri-oral skin (2 of 7). Of the seven subjects showing oral signs, four had OLP and three had OLL and the clinical diagnosis (OLP or OLL) did not change after the use test (Table 11).

Figure 13. Perioral and intraoral clinical changes before (A) and after (B) use test. Subject no. 2 A-B. Exacerbation of lichenoid lesions (here noted at the left buccal mucosa and on the inside of the lower lip). Subject no. 3 A-B. Perioral eczematous lesions and weak oral lichenoid lesions (not shown). Subject no.6 A-B. Exacerbation of bilateral lichenoid lesions (here noted at the right buccal mucosa).

Mean values of mucosal scores and pain scores for the different study groups are shown in Table 12. Contact allergic subjects using carvone toothpaste (CA+/OL±/c) displayed a significant worsening of mucosal health status compared to contact allergic subjects receiving non-flavoured tooth-paste (CA+/OL±/nf) (S_a , $p = 0.028$; S_{tot} , $p = 0.019$). Additionally, contact allergic subjects with oral lichen using carvone toothpaste (CA+/OL+/c) had a worsening of the mucosal health status compared to subjects with OL only using carvone (CA-/OL+/c), (S_a , $p = 0.003$; pain score $p = 0.015$; S_{tot} , $p \leq 0.001$).

There was no difference in gingival pocket depth and bleeding on probing between the start and end of the use test or between any of the groups.

Table 12. Use test – mucosal scoring for the different groupings.

SD: standard deviation, CA: carvone contact allergy; OL: oral lichen; c: carvone toothpaste; nf: non-flavoured toothpaste; Sa: Escudier index, overall activity score; pain score: numerical rate scale on pain; Stot: Escudier index, total weighted score.

Groups	Toothpaste	Subjects	Use test – start (SD)			Use test – end (SD)		
			S _a	Pain score	S _{tot}	S _a	Pain score	S _{tot}
CA ⁺ /OL [±] /c	carvone	10	3.6 (5.5)	2.6 (2.4)	11.5 (11.1)	7.4 (7.2)	4.3 (3.0)	18.0 (13.7)
CA ⁺ /OL [±] /nf	non-flavoured	5	4.0 (5.9)	2 (2.1)	9.0 (10.2)	3.4 (5.5)	2.2 (2.3)	9.2 (9.6)
CA ⁺ /OL ⁺ /c	carvone	9	4.0 (5.7)	2.8 (2.4)	12.7 (11.1)	8.2 (7.1)	4.8 (2.8)	20.0 (12.9)
CA ⁺ /OL ⁺ /c	carvone	17	5.7 (4.6)	2.8 (2.1)	14.3 (8.5)	5.9 (4.5)	2.8 (2.1)	14.5 (8.4)
Controls	carvone	3	0.0 (0.0)	0.0 (0.0)	0.3 (0.6)	0.0 (0.0)	0.3 (0.6)	0.7 (1.2)

Table 13. Use test – OHIP (Oral health impact profile) scoring for the different groupings. SD: standard deviation, CA: carvone contact allergy, OL: oral lichen, c: carvone toothpaste, nf: non-flavoured toothpaste.

Groups	Toothpaste	Subjects	OHIP start (SD)	OHIP end (SD)	Mean difference
CA ⁺ /OL [±] /c	carvone	10	33.9 (19.6)	42.3 (30.8)	8.4
CA ⁺ /OL [±] /nf	non-flavoured	4	33.0 (14.0)	26.5 (18.0)	-6.8
CA ⁺ /OL ⁺ /c	carvone	9	35.9 (19.7)	45.7 (30.6)	9.8
CA ⁺ /OL ⁺ /c	carvone	15	29.7 (10.9)	24.7 (14.2)	-5.0
Controls	carvone	3	44.3 (22.0)	39.7 (14.8)	-4.7

OHIP

Mean values of OHIP-scores for the different groups are shown in Table 13. The allergic subjects receiving carvone had an increase in OHIP-score, i.e. they had a negative change in their quality of life.

The OHIP domain 'pain' was increased in contact allergic subjects exposed to carvone (CA+/OL±/c) compared to contact allergic subjects receiving non-flavoured toothpaste (OHIP-pain, $p = 0.036$). Contact allergic subjects with oral lichen and (CA+/OL+/c) also had an increase in OHIP scores compared to subjects with only oral lichen OL (CA-/OL+/c) (OHIP, $p = 0.014$; OHIP-pain, $p = 0.024$; OHIP-psyc, $p = 0.023$).

Paper IV – Molecular profiling of oral contact reactions to l-carvone and oral lichenoid reactions

Histological findings

In the NM-C group, no substantial inflammation was seen and the epithelium was of normal thickness with occasional hyperkeratosis. Distinct features of lichenoid reaction were seen in all cases from the OLP-C group with subepithelial band-like lymphocytic infiltrates and marked thinning of the epithelial thickness. In the CAOL-C group, a lichenoid reaction was seen in 7 of 9 cases. However, the lymphocytic infiltrates were less band-like but rather arranged as rounded clusters, often around vessels. In the CAOL-NF group, weak lichenoid reaction or unspecific chronic inflammation was seen in 3 of 5 cases (Figure 14).

Figure 14. Haematoxylin-eosin-stained sections of study groups. A) Control sample showing normal appearing mucosa. B-D) examples of lichenoid reaction in B)OLP-C, C)CAOL-C and D)CAOL-NF. Scale bar indicate 500 μm .

Characterisation of infiltrating cells

The band-like infiltrates in the OLP-C group were predominantly stained for T-cell markers CD3, CD4 and CD8 followed by Type I interferon marker MxA, macrophage markers CD68 and CD11c and B-cell marker CD20. Langerhans cell marker CD1a, plasmacytoid DC marker CD123 and plasma cell marker CD138 were less present. A similar pattern was observed in study group CAOL-C. However, a difference was seen in the expression of Langerhans cell marker CD1a and plasma cell marker CD138 (Figure 15 and 16). The OLP-C group showed an increased expression of CD1a compared to CAOL-C ($p = 0.038$). OLP-C group also displayed more CD138 positive cells but the difference was not statistically significant ($p = 0.067$). When compared to CAOL-C, the CAOL-NF group had a decrease in the expression of all markers except CD1a and CD138.

Figure 15. Representative sections from the different study groups. Haematoxylin and eosin (A-D) and IHC staining for CD4 (E-H), CD8 (I-L), CD20 (M-P) and CD138 (Q-T). Objective magnification 40x.

Figure 16. Representative sections from the different study groups. IHC staining for CD1a (A-D), CD11c (E-H), CD68 (I-L), CD123 (M-P) and MxA (Q-T). Objective magnification 40x.

DISCUSSION

Exposure to carvone in toothpaste

All flavoured toothpastes analysed in paper I contained carvone in varying amounts. For the consumer, neither advertising nor labelling reveal the amount of carvone in the products but limonene on the label may indicate higher carvone content. Ingredient listing of individual fragrance allergens enables consumers with an identified allergy to avoid exposure and it is also important when investigating a suspected sensitisation as the information on the product may help in giving the correct diagnosis without undue delay. For the carvone sensitised individual, choosing non-flavoured toothpaste is the only guaranteed way to avoid elicitation.

Patients with OL presumably use the same amount of toothpaste as any healthy individual, but they nonetheless seem to be more susceptible to sensitisation to carvone. One has to question whether the risk assessments performed on carvone have taken these lesions into consideration. The SCCS has proposed that carvone and several other fragrance allergens should be ingredient listed (5). They also point out several factors that are important for the induction and elicitation of contact allergy: dose per unit area, the vehicle effects, presence of skin irritants or other allergens, duration and frequency of exposure, the anatomical site of exposure, the condition of skin and occlusion. Several of these factors raise concerns related to exposure to carvone in toothpaste in the case of OL patients. Toothpastes are often used on a daily basis as long as the user has teeth, which could mean an entire lifetime. Toothpastes often contain surfactants that act as irritants and facilitate the penetration of potential allergens. The affected mucosa in OL patients has a thinner barrier

and an ongoing sub-epithelial inflammation. Additionally, they may use strong corticosteroids for prolonged periods, which may also contribute towards the thinning of the barrier. Taken together, this may facilitate the penetration of different allergens. Besides carvone, OL patients are often sensitised to clinically relevant allergens, such as gold, mercury and other components used in dental restorations (6,84), leading to the assumption that OL patients have an increased risk of oral sensitisation.

With the current cosmetics regulation, the responsibility for risk reduction lies mainly with the consumers and requires that they have knowledge of the allergens listed. Additionally, several allergens are not required to be listed on cosmetic products, even if the same substances are required to be listed in other consumer goods (30), this includes carvone. Increased transparency between manufacturer and consumers is desirable. With proper labelling, allergic consumers should be able to make an informed choice of products.

Defining oral contact allergy to carvone

The oral and perioral allergic reactions seen in paper III were similar to those reported in the literature (1,6,7,73-79). Contact cheilitis and perioral contact dermatitis developed where there were no previous lesions. For the oral lesions, the interpretation is more difficult. All allergic subjects who reacted intraorally to the toothpaste already had some form of oral lichen and the reaction was seen as aggravated lichenoid lesions. Additionally, the analysis of the mucosal biopsies showed more similarities than differences between the lesions of oral lichen planus and the lesions from the allergic patients exposed to carvone. Unfortunately, there was a lack of recruited allergic subjects without any oral lesions at the start of the use test. Thus, we cannot say what a true oral contact reaction to carvone, without influence of oral lichen, looks like. However, the results could indicate that it is preferably individuals with oral lichen that react orally to carvone. Firstly, because individuals with oral lichen have an increased risk to be sensitised to agents present in the oral cavity (6,73). Secondly, although not yet verified, because it seems that the oral lesions in sensitised individuals are more susceptible to penetration of allergens and that the chronic inflammation facilitates the immune reaction. Looking at similar findings on skin there are no reports on

cutaneous lichen planus patients having more contact allergies, but some studies have shown that patients with endogenous dermatitis or other inflammatory lesions may have more concomitant contact allergies (85-87). However, the inverse relationship is seen in psoriatic patients who seem to be less susceptible to sensitisation (88). Hand eczema is a common multifactorial disease predisposed by both environmental and endogenous factors. A common cause of hand eczema is contact with skin irritants, such as water, soap and mild toxic agents, but contact allergy may also be an additional causative factor. Because hand eczema often is due to a combination of factors, eliminating exposure of a contact allergen may or may not lead to healing (89). This viewpoint could perhaps be applied on patients with oral lichenoid lesions with contact allergy to carvone; the lesions are multifactorial and avoiding carvone may not lead to complete resolution of the oral lesions.

Diagnosis and prevention of contact allergy to carvone

There is no doubt that contact allergy to carvone is more prevalent in patients with oral lichen and extra attention should be given to this patient group. Should all patients with oral lichen be patch tested with the dental series? This is a costly matter, not only for the departments performing the tests, but also for the patients. Positive patch tests that are not clinically relevant may lead to unnecessary treatments, such as replacement of dental restorations. The problem is that the clinical appearance of contact allergy to carvone in many cases is indistinguishable from what is seen in oral lichen planus. Other clinical signs, such as cheilitis, or sudden aggravation of existing lesions may give a clue.

In paper III, 14 subjects with a previous positive patch test to carvone were recruited. When re-tested, only 6 produced a positive patch test reaction whereof 2 had very late reactions. Thus, the reactivity to carvone in sensitised individuals appears to reduce with time. A clinical correlation to the patch test reaction could not be confirmed. Although there was a tendency towards increased clinical signs in those with a positive patch test, the difference was not statistically significant. The scoring system (81) that was used to evaluate mucosal reactions in paper III was able to detect differences between the studied groups and the detected changes correlated

with changes in subjective symptoms and OHIP scores. On the other hand, the size of the scoring values did not correlate very well to the intensity of subjective symptoms or OHIP scores within the individual patients. The scoring system was originally developed for use in intervention studies on oral lichen planus with the intention to have a more standardised outcome measure. In use tests on skin, it is often desired to define an absolute score in order to define a positive change. In contrast, our study shows that the method by Escudier et al. is efficient in evaluating mucosal change but less well suited to assign absolute scoring values. We also found that analysis of randomised photographs (start and end) helped minimising bias of the evaluation caused by drift in diagnostic criteria over time.

Patients with oral lichen should be informed about the increased risk of sensitisation to carvone. Besides flavours, toothpastes contain surfactants that may enhance the penetration of possible allergens or aggravate oral lesions. Recommending a toothpaste without surfactants and flavour, or avoiding toothpastes with strong flavours, could be a preventive measure.

CONCLUSIONS

- Carvone is present in flavoured toothpastes in various amounts
- A majority of patients with contact allergy to carvone have oral symptoms and over half of them have oral lichen in some form
- Among patch tested patients with oral signs, carvone-allergic patients have more oral lichen than those without carvone contact allergy
- In subjects with contact allergy to carvone, carvone in toothpaste induces aggravated oral lichenoid lesions and sometimes perioral eczema
- The inflammatory patterns in oral lichen planus and oral contact reactions to carvone are very similar
- Compared to tissue samples from subjects with OLP, there is a decreased number of CD1a positive cells in oral contact reactions to carvone

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REFERENCES

1. Paulsen E, Andersen KE, Carlsen L, Egsgaard H. Carvone: an overlooked contact allergen cross-reacting with sesquiterpene lactones? *Contact Dermatitis* 1993; 29: 138-143.
2. Spiewak R, Samochocki Z, Grubska-Suchanek E et al. ESCD Abstracts: Posters, P075: Gallates, as well as hydroperoxides of limonene and linalool, are more frequent and relevant sensitizers than any cosmetic ingredient included in the European Baseline Series. *Contact Dermatitis* 2016; 75 (S1): 87.
3. Nilsson AM, Bergstrom MA, Luthman K et al. An alpha,beta-unsaturated oxime identified as a strong contact allergen. Indications of antigen formation via several pathways. *Food Chem Toxicol* 2005; 43: 1627-1636.
4. International Fragrance Association. L-carvone: 6485-40-1, 2009: <http://www.ifraorg.org/en-us/standards>. Updated 2009. Accessed 2016 Aug 15.
5. SCCS (Scientific Committee on Consumer Safety). 1459/11 Opinion on fragrance allergens in cosmetic products. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_102.pdf; Updated 26-27 June 20122016.
6. Ahlgren C, Axell T, Möller H et al. Contact allergies to potential allergens in patients with oral lichen lesions. *Clin Oral Investig* 2013; 18: 227-237.
7. Gunatheesan S, Tam MM, Tate B et al. Retrospective study of oral lichen planus and allergy to spearmint oil. *Australas J Dermatol* 2012; 53: 224-228.
8. Surburg H, Panten J. Ch 1. Introduction. In: Common Fragrance and Flavor Materials, 6th edition, Surburg H, Panten J (eds.): Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA, 2016: 1-6.

9. Surburg H, Panten J. Ch 2. Individual fragrance and flavor materials. In: Common Fragrance and Flavor Materials, 6th edition, Surburg H, Panten J (eds.): Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA, 2016: 7-192.
10. Surburg H, Panten J. Ch 3. Natural raw materials in the flavor and fragrance industry. In: Common Fragrance and Flavor Materials, 6th edition, Surburg H, Panten J (eds.): Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA, 2016: 193-264.
11. Salehi B, Stojanovic-Radic Z, Matejic J et al. Plants of Genus *Mentha*: From Farm to Food Factory. *Plants (Basel)* 2018; 7: 10.3390/plants7030070.
12. Chauhan SS, Prakash O, Padalia RC et al. Chemical diversity in *Mentha spicata*: antioxidant and potato sprout inhibition activity of its essential oils. *Nat Prod Commun* 2011; 6: 1373-1378.
13. de Carvalho CCCR, da Fonseca MMR. Carvone: Why and how should one bother to produce this terpene. *Food Chem* 2006; 95: 413-422.
14. de Groot AC, Schmidt E. Essential Oils, Part III: Chemical Composition. *Dermatitis* 2016; 27: 161-169.
15. Zheljaskov VD, Cantrell CL, Astatkie T, Hristov A. Yield, Content, and Composition of Peppermint and Spearmints as a Function of Harvesting Time and Drying. *J Agric Food Chem* 2010; 58: 11400-11407.
16. Hussain AI, Anwar F, Nigam PS et al. Seasonal variation in content, chemical composition and antimicrobial and cytotoxic activities of essential oils from four *Mentha* species. *J Sci Food Agric* 2010; 90: 1827-1836.
17. Chalchat J, Garry R, Michet A. Variation of the Chemical Composition of Essential Oil of *Mentha piperita* L. during the Growing Time. *J Essent Oil Res* 1997; 9: 463-465.
18. Gulluce M, Sahin F, Sokmen M et al. Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *longifolia*. *Food Chem* 2007; 103: 1449-1456.
19. Rohloff J, Dragland S, Mordal R, Iversen TH. Effect of harvest time and drying method on biomass production, essential oil yield, and quality of peppermint (*Mentha x piperita* L.). *J Agric Food Chem* 2005; 53: 4143-4148.
20. Singh A. Essential oil composition and chemoarrays of menthol mint (*Mentha arvensis* L.) cultivars. *Flavour Fragrance J* 2005; 20: 302-305.
21. Pandey AK, Rai MK, Acharya D. Chemical Composition and Antimycotic Activity of the Essential Oils of Corn Mint (*Mentha arvensis*) and Lemon Grass (*Cymbopogon flexuosus*) Against Human Pathogenic Fungi. *Pharm Biol* 2003; 41: 421-425.

22. Sokovic MD, Vukojevic J, Marin PD et al. Chemical composition of essential oils of *Thymus* and *Mentha* species and their antifungal activities. *Molecules* 2009; 14: 238-249.
23. Karlberg AT, Magnusson K, Nilsson U. Air oxidation of d-limonene (the citrus solvent) creates potent allergens. *Contact Dermatitis* 1992; 26: 332-340.
24. Köhler FE. Krauseminze. *mentha viridis* var. *crispata* schrader. List of Köhler images, public domain, <https://commons.wikimedia.org/w/index.php?curid=255375>. In: *Köhler's Medizinal-Pflanzen*, Pabst G (eds.): Gera-Untermhaus, Germany, 1883-1914.
25. Risk and Policy Analysts Ltd. (Firm), (ed.) Comparative Study on Cosmetics Legislation in the EU and Other Principal Markets with Special Attention to so-Called Borderline Products: Final Report Study Prepared for European Commission, Directorate-General Enterprise. Loddon, Norfolk, UK: Risk and Policy Analysts Limited: 2004. Dr Jan Vernon, Business Development Director, RPA and Tobe A. Nwaogu, Researcher, RPA, (eds.).
26. Council of the European Union, European Parliament. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *OJ L* 2009; 52: 59-209.
27. European Commission. Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. *OJ L* 2008; 51: 34-50.
28. European Commission. Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. *OJ L* 2012; 55: 1-161.
29. European Union. Regulation (EC) no 1907/2006 of the european parliament and of the council of 18 december 2006 concerning the registration, evaluation, authorisation and restriction of chemicals (REACH), establishing a european chemicals agency, amending directive 1999/45/EC and repealing council regulation (EEC) no 793/93 and commission regulation (EC) no 1488/94 as well as council directive 76/769/EEC and commission directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC (text with EEA relevance), 2006. Available at: <http://data.europa.eu/eli/reg/2006/1907/2016-06-21>.

30. European Commission. Commission Regulation (EU) 2015/1221 of 24 July 2015 amending Regulation (EC) No 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures, for the purposes of its adaptation to technical and scientific progress. *OJ L* 2015; 58: 10-23.
31. EFSA Scientific Committee. Scientific Opinion on the safety assessment of carvone, considering all sources of exposure. *EFSA Journal* 2014; 12: 3806.
32. Basketter D, White IR. Legislative Aspects of Cosmetic Safety in the European Union: The Case of Contact Allergy. *Cosmetics* 2016; 3.
33. SCCS (Scientific Committee on Consumer Safety). SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 9th revision, 29 September 2015, SCCS/1564/15. . http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_190.pdf. Updated 25 April 2016.
34. International Fragrance Association. IFRA code of practice, 2015: <http://www.ifraorg.org/en-us/code-of-practice>. Updated 2015. Accessed 2018 Sept 20.
35. Api AM, Belsito D, Bruze M et al. Criteria for the Research Institute for Fragrance Materials, Inc. (RIFM) safety evaluation process for fragrance ingredients. *Food Chem Toxicol* 2015; 82 Suppl: S1-S19.
36. Api AM, Basketter DA, Cadby PA et al. Dermal sensitization quantitative risk assessment (QRA) for fragrance ingredients. *Regul Toxicol Pharmacol* 2008; 52: 3-23.
37. Api AM, Vey M. Implementation of the dermal sensitization Quantitative Risk Assessment (QRA) for fragrance ingredients. *Regul Toxicol Pharmacol* 2008; 52: 53-61.
38. Johansson SG, Bieber T, Dahl R et al. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol* 2004; 113: 832-836.
39. Basketter D, Dooms-Goossens A, Karlberg A, Lepoittevin J. The chemistry of contact allergy: why is a molecule allergenic? *Contact Derm* 1995; 32: 65-73.
40. Fonacier LS, Dreskin SC, Leung DY. Allergic skin diseases. *J Allergy Clin Immunol* 2010; 125: S138-49.
41. Saint-Mezard P, Rosieres A, Krasteva M et al. Allergic contact dermatitis. *Eur J Dermatol* 2004; 14: 284-295.
42. Lachapelle J, Maibach HI. Ch 3. the methodology of patch testing. In: Patch Testing and Prick Testing: A Practical Guide; with 42 Tables Berlin, Heidelberg, Springer-Verlag, 2003: 27-54.

43. Johansen JD, Aalto-Korte K, Agner T et al. European Society of Contact Dermatitis guideline for diagnostic patch testing – recommendations on best practice. *Contact Dermatitis* 2015; 73: 195-221.
44. Fregert S, Group ICDR, Group NACD, (eds.) Manual of Contact Dermatitis. 2nd edition, Copenhagen: Munksgaard: 1981. Fregert S., (ed.) Manual of Contact Dermatitis on Behalf of the International Contact Dermatitis Research Group and the North American Contact Dermatitis Group.
45. Bruze M, Conde-Salazar L, Goossens A et al. Thoughts on sensitizers in a standard patch test series. *Contact Derm* 1999; 41: 241-250.
46. Groot ACd, (ed.) Patch Testing : Test Concentrations and Vehicles for 3700 Chemicals. 2. ed. edition, Amsterdam ; London: Elsevier: 1994.
47. Boyd AS, Neldner KH. Lichen planus. *J Am Acad Dermatol* 1991; 25: 593-619.
48. Eisen D. The evaluation of cutaneous, genital, scalp, nail, esophageal, and ocular involvement in patients with oral lichen planus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1999; 88: 431-436.
49. van der Waal I. Oral lichen planus and oral lichenoid lesions; a critical appraisal with emphasis on the diagnostic aspects. *Med Oral Patol Oral Cir Bucal* 2009; 14: E310-4.
50. Axell T. A prevalence study of oral mucosal lesions in an adult Swedish population. *Odontol Revy Suppl* 1976; 36: 1-103.
51. Salonen L, Axell T, Hellden L. Occurrence of oral mucosal lesions, the influence of tobacco habits and an estimate of treatment time in an adult Swedish population. *J Oral Pathol Med* 1990; 19: 170-176.
52. Cheng S, Kirtschig G, Cooper S et al. Interventions for erosive lichen planus affecting mucosal sites. *Cochrane Database Syst Rev* 2012; (2):CD008092. doi: CD008092.
53. Lodi G, Pellicano R, Carrozzo M. Hepatitis C virus infection and lichen planus: a systematic review with meta-analysis. *Oral Dis* 2010; 16: 601-612.
54. Koray M, Dulger O, Ak G et al. The evaluation of anxiety and salivary cortisol levels in patients with oral lichen planus. *Oral Dis* 2003; 9: 298-301.
55. Kurago ZB. Etiology and pathogenesis of oral lichen planus: an overview. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2016; 122: 72-80.
56. Ismail SB, Kumar SK, Zain RB. Oral lichen planus and lichenoid reactions: etiopathogenesis, diagnosis, management and malignant transformation. *J Oral Sci* 2007; 49: 89-106.
57. Scully C, Carrozzo M. Oral mucosal disease: Lichen planus. *Br J Oral Maxillofac Surg* 2008; 46: 15-21.

58. Andreasen JO. Oral lichen planus. 1. A clinical evaluation of 115 cases. *Oral Surg Oral Med Oral Pathol* 1968; 25: 31-42.
59. Thornhill MH, Sankar V, Xu XJ et al. The role of histopathological characteristics in distinguishing amalgam-associated oral lichenoid reactions and oral lichen planus. *J Oral Pathol Med* 2006; 35: 233-240.
60. Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: risk of progression to malignancy. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2018; 125: 612-627.
61. Fitzpatrick SG, Hirsch SA, Gordon SC. The malignant transformation of oral lichen planus and oral lichenoid lesions: A systematic review. *The Journal of the American Dental Association*. 2014;145(1):45-56.
62. Aghbari SMH, Abushouk AI, Attia A et al. Malignant transformation of oral lichen planus and oral lichenoid lesions: A meta-analysis of 20095 patient data. *Oral Oncology*. 2017;68:92-102.
63. Casparis S, Borm JM, Tektas S et al. Oral lichen planus (OLP), oral lichenoid lesions (OLL), oral dysplasia, and oral cancer: retrospective analysis of clinicopathological data from 2002-2011. *Oral Maxillofac Surg* 2015; 19: 149-156.
64. So JK, Hamstra A, Calame A et al. Another Great Imitator: Allergic Contact Dermatitis Differential Diagnosis, Clues to Diagnosis, Histopathology, and Treatment. *Current Treatment Options in Allergy* 2015; 2: 333-348.
65. Bakula A, Lugovic-Mihic L, Situm M et al. Contact allergy in the mouth: diversity of clinical presentations and diagnosis of common allergens relevant to dental practice. *Acta Clin Croat* 2011; 50: 553-561.
66. Gawkrödger DJ. Investigation of reactions to dental materials. *Br J Dermatol* 2005; 153: 479-485.
67. Raap U, Stiesch M, Kapp A. Contact allergy to dental materials. *JDDG: Journal der Deutschen Dermatologischen Gesellschaft* 2012; 10: 391-396.
68. Bolewska J, Hansen HJ, Holmstrup P et al. Oral mucosal lesions related to silver amalgam restorations. *Oral Surg Oral Med Oral Pathol* 1990; 70: 55-58.
69. Bolewska J, Holmstrup P, Møller-Madsen B et al. Amalgam associated mercury accumulations in normal oral mucosa, oral mucosal lesions of lichen planus and contact lesions associated with amalgam. *J Oral Pathol Med* 1990; 19: 39-42.
70. Koch P, Bahmer FA. Oral lesions and symptoms related to metals used in dental restorations: a clinical, allergological, and histologic study. *J Am Acad Dermatol* 1999; 41: 422-430.
71. Bratel J, Hakeberg M, Jontell M. Effect of replacement of dental amalgam on oral lichenoid reactions. *J Dent* 1996; 24: 41-45.

72. Yiannias JA, el-Azhary RA, Hand JH et al. Relevant contact sensitivities in patients with the diagnosis of oral lichen planus. *J Am Acad Dermatol* 2000; 42: 177-182.
73. Larsen KR, Johansen JD, Reibel J et al. Symptomatic oral lesions may be associated with contact allergy to substances in oral hygiene products. *Clin Oral Investig* 2017; 21: 2543-2551.
74. Clayton R, Orton D. Contact allergy to spearmint oil in a patient with oral lichen planus. *Contact Dermatitis* 2004; 51: 314-315.
75. Skrebova N, Brocks K, Karlsmark T. Allergic contact cheilitis from spearmint oil. *Contact Dermatitis* 1998; 39: 35.
76. Worm M, Jeep S, Sterry W, Zuberbier T. Perioral contact dermatitis caused by L-carvone in toothpaste. *Contact Dermatitis* 1998; 38: 338.
77. Francalanci S, Sertoli A, Giorgini S et al. Multicentre study of allergic contact cheilitis from toothpastes. *Contact Dermatitis* 2000; 43: 216-222.
78. Lim SW, Goh CL. Epidemiology of eczematous cheilitis at a tertiary dermatological referral centre in Singapore. *Contact Dermatitis* 2000; 43: 322-326.
79. Corazza M, Levratti A, Virgili A. Allergic contact cheilitis due to carvone in toothpastes. *Contact Dermatitis* 2002; 46: 366-367.
80. Edman B. The usefulness of detailed information to patients with contact allergy. *Contact Dermatitis* 1988; 19: 43-47.
81. Escudier M, Ahmed N, Shirlaw P et al. A scoring system for mucosal disease severity with special reference to oral lichen planus. *Br J Dermatol* 2007; 157: 765-770.
82. Larsson P, List T, Lundstrom I et al. Reliability and validity of a Swedish version of the Oral Health Impact Profile (OHIP-S). *Acta Odontol Scand* 2004; 62: 147-152.
83. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One* 2014; 9: e96801.
84. Kroona L, Isaksson M, Ahlgren C et al. Carvone Contact Allergy in Southern Sweden: A 21-year Retrospective Study. *Acta Derm Venereol* 2018.
85. Uter W, Werfel T, White IR, Johansen JD. Contact Allergy: A Review of Current Problems from a Clinical Perspective. *Int J Environ Res Public Health* 2018; 15: 10.3390/ijerph15061108.
86. Agullo-Perez AD, Hervella-Garces M, Oscoz-Jaime S et al. Perianal Dermatitis. *Dermatitis* 2017; 28: 270-275.

87. Agner T, Aalto-Korte K, Andersen KE et al. Factors associated with combined hand and foot eczema. *J Eur Acad Dermatol Venereol* 2017; 31: 828-832.
88. Classen A, Buhl T, Schubert S et al. The frequency of specific contact allergies is reduced in psoriasis patients. *Br J Dermatol* 2018.
89. Coenraads PJ. Hand eczema is common and multifactorial. *J Invest Dermatol* 2007; 127: 1568-1570.

PAPERS I – IV

I

Quantification of L-carvone in toothpastes available on the Swedish market

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Summary

Background. Toothpastes have widespread use in the population, and contain flavours used to give a pleasant and often minty aroma. Flavours are prevalent allergens in toothpastes, and adverse reactions often present as perioral dermatitis or stomatitis. L-Carvone, a mint flavour found in spearmint oil, is one of these allergens. There are few studies on contact allergy to L-carvone, and some of them have indicated a positive relationship with oral lichenoid lesions.

Objectives. To qualitatively and quantitatively investigate the presence of L-carvone in commercially available toothpastes on the Swedish market.

Methods. L-Carvone in 66 toothpastes was analysed with straight-phase high-performance liquid chromatography. The product labels were studied with regard to limonene.

Results. L-Carvone was found in 64 of 66 toothpastes (concentration: 0.00005–0.35%). In 10 of these, the concentration exceeded 0.1%. Higher concentrations of L-carvone were found if limonene was listed on the label.

Conclusion. The majority of toothpastes on the Swedish market contain L-carvone, but the concentration hardly relates to the advertised flavour or labelled ingredients. It is hitherto unknown whether the found concentrations are sufficient for induction of contact allergy in individuals with healthy oral mucosa or in those with oral lichenoid lesions or other mucosal disease.

Key words: contact allergy; contact dermatitis; flavour; fragrances; high-performance liquid chromatography; L-carvone; limonene; oral lichen planus; oral lichenoid lesions; spearmint; toothpastes.

Toothpaste and other oral hygiene products are used on a daily basis. Besides having active ingredients such as surfactants to remove plaque and fluoride to prevent caries, these products also serve to make the user feel fresh by means of added flavours. Flavours are added not only for the taste and cooling sensation, but also to mask ingredients with an unpleasant character, such as surfactants. They frequently consist of a mixture of various mint flavours, such as peppermint, spearmint, cornmint, and menthol, and the flavour concentration in toothpastes may vary between 0.3% and 2% (1). Flavours and fragrances are among the most common allergens

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Conflicts of interest: Magnus Bruze is a member of the expert panel of RIFM, a group of experts who evaluate the safety of fragrance materials, supported by the manufacturers of fragrances and consumer products containing fragrances. The other authors declare no conflict of interest.

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in cosmetic products, and this also applies to toothpastes. The most frequent adverse reactions to toothpaste are local irritation and contact allergic reactions, usually manifested as perioral dermatitis and/or stomatitis, often in the form of oral lichenoid lesions (2–8). In addition, occasional cases of immediate hypersensitivity reactions have been reported (7, 9, 10). In most countries, producers of cosmetics are required to list the ingredients of their products according to package and labelling rules (11). However, the composition of fragrance and flavour ingredients is usually not specified, and, instead, the collective names 'parfum' and 'aroma' are used. In the EU, 26 fragrances are presently regulated and labelled individually according to the Cosmetics Regulation when concentrations exceed 0.001% in leave-on products and 0.01% in rinse-off products; however, mint flavours are not specifically included in this regulation (12).

L-Carvone, a mint flavour, is the main constituent of spearmint oil (*Mentha spicata*), constituting up to 70% of the oil, depending on harvest time, environmental factors, and variety of subspecies (13–17). Trace amounts of L-carvone can be found in peppermint oil (*Mentha piperita*) and other *Mentha* species (14, 18–21), but there are also studies in which L-carvone was not detected (22, 23). L-Carvone is, moreover, one of several substances found in oxidized D-limonene (24), and there are methods to produce L-carvone from D-limonene synthetically (16, 25). Although L-carvone is considered to be an established contact allergen (26), there are limited data on adverse reactions to it. A few published cases and studies have reported oral or perioral allergic contact reactions to L-carvone or spearmint oil (2, 27–31), and one report described a case of perioral contact urticaria (32). In a retrospective study by Gunatheesan et al. (33), an association was found between spearmint allergy and oral lichen planus, and the authors suggested that spearmint allergy may cause oral lichen planus or oral lichenoid lesions. In another study, Ahlgren et al. (34) found that the frequency of contact allergies was higher in patients with oral lichenoid lesions than in patients with dermatitis. Patients with oral lichenoid lesions had a higher frequency of contact allergy to gold and mercury but, interestingly, ~12% also had contact allergy to L-carvone. From another perspective, in a group of patients with positive patch test reactions to L-carvone, almost half had oral lichenoid lesions (35).

Even though there are only a few studies on adverse reactions to L-carvone, the reports mentioned above clearly indicate that, for some patients, L-carvone plays a role, either by causing disease or by aggravating already existing problems. To prevent discomfort, patients with known contact allergy to L-carvone may use toothpaste

without flavour, but there are few mint-free alternatives on the market. In addition, the labelling of cosmetics is not always complete and reliable (36), and these patients would therefore benefit from information on the content of L-carvone in toothpaste and in other oral hygiene products. The purpose of this study was to investigate the presence of L-carvone, qualitatively and quantitatively, in 66 commercially available toothpastes on the Swedish market.

Materials and Methods

A total of 66 commercially available toothpastes were obtained from local pharmacies and supermarkets in Malmö, Sweden. The packages and the product labels were studied, and the presence on the label of limonene (i.e. D-limonene) and other flavours was noted.

The following chemicals were used for the analysis: L-carvone [L-(–)-carvone] 99% (Acros Organics, Geel, Belgium; CAS no. 6485-40-1), sodium chloride (Acros Organics), n-heptane of high-performance liquid chromatography (HPLC) Grade (Fisher Scientific UK, Loughborough, UK), 1-butanol (VWR, Fontenay-sous-Bois, France), and 1-propanol (Sigma-Aldrich, St Louis, MO, USA).

Three samples per toothpaste were analysed. A sample of 0.5 g of toothpaste was accurately weighed and dissolved in 2.5 ml of saturated sodium chloride solution in a 12-ml test-tube with a Teflon-lined screw cap. L-Carvone was extracted by adding 3.0 ml of n-heptane, after which the mixture was thoroughly mixed and manually shaken for 2 min. The mixture was left to separate until the n-heptane phase was completely clear. Some of the toothpastes were hard to dissolve to obtain a clear n-heptane phase, and these were carefully centrifuged (225 × g, 2.5 min) to speed up the separation process. The clear solution was analysed with HPLC.

The protocol was developed at the Department of Occupational and Environmental Dermatology, Malmö University Hospital, Sweden. An HPLC SpectraSystem consisting of a P4000 quaternary pump, an AS3000 autosampler and a UV6000 diode array detector (ThermoFinnigan, San José, CA, USA) was used for the analysis. The detector was used for scanning wavelengths of 190–800 nm. The modules were controlled with DIONEX CHROMELEON™ 7 software (Thermo Scientific, Thermo Fisher Scientific, Sunnyvale, CA, USA). A 4.6 × 250-mm² silica column [5 µm, 100 Å Luna Silica (2); Phenomenix, Værore, Denmark] was used for sample separation.

Samples were analysed by the use of isocratic elution with a mobile phase consisting of n-heptane with addition of butanol 0.165% vol/vol or propanol 0.1%

vol/vol. The mobile phase containing propanol was used if additional substances coeluted with L-carvone in the butanol-containing mobile phase. The flow rate was 2 ml/min. L-Carvone was monitored at 228 nm for quantification, and the detected peaks were confirmed as L-carvone by comparison of spectrum and retention time of standards. The equilibration time was 2–4 h. The chromatogram was compared with the reference analysis for quantification. A calibration/reference curve was made before each run in the range between 0% and 0.005% L-carvone by using at least three different concentrations. The detection limit was determined to be 0.00001%.

The numbers of toothpastes with and without limonene according to the labelling were compared with regard to the presence of L-carvone exceeding 0.01% by use of Fisher's exact test, two-sided. The differences between measured L-carvone concentrations in the samples with and without limonene, according to the labelling, were analysed with the *t*-test for independent samples. *p*-Values were considered to be significant when <0.05.

Results

The retention time for the HPLC-analysed samples was 8 h 15 min to 8 h 50 min. Recovery was determined to be 94% ($n = 4$) by extraction of 1% L-carvone added to a toothpaste without flavour. Example chromatograms and ultraviolet spectra for an L-carvone reference sample and one toothpaste sample are shown in Fig. S1.

Detectable levels of L-carvone between 0.00005% and 0.35% were found in 64 of the 66 toothpastes. The mean relative standard deviation of the measurements was 6.1%. In 30 of the toothpastes, the concentration was >0.01%, and in 10 of these the concentration was >0.1%. Nine toothpastes intended for children were included in the study, and the two with the highest concentrations had 0.097% and 0.15% L-carvone, respectively. The mean concentrations of L-carvone in the toothpastes are shown in Fig. 1a, and a detailed list with brand names is shown in Fig. S2.

Thirty-six of the toothpastes were advertised as having some type of mint flavour, and 24 toothpastes had no specified flavour advertised on the package. Five toothpastes were advertised as fruit-flavoured and intended for children, and one of these had a mix of both mint and fruit flavour. Two toothpastes were advertised as being 'without flavour'. The toothpastes with only fruit flavour still had detectable amounts of L-carvone (0.0096%, 0.0061%, 0.00030%, and 0.000075%). There were also several toothpastes stated to have mint flavour that contained very low concentrations of L-carvone. L-Carvone

Fig. 1. Boxplot (Q1/median/Q3; whiskers, minimum–maximum) showing mean concentrations of L-carvone for: (a) all toothpastes investigated; and (b) all toothpastes divided into two groups, without or with limonene. Higher concentrations of L-carvone were found in toothpastes labelled as containing limonene ($p = 0.045$).

was below the detectable level in the two toothpastes advertised as being 'without flavour'.

Limonene was declared on the product label for 34 of the 66 toothpastes. A significant number contained L-carvone at a concentration of >0.01% as compared with those without limonene (22 of 34 versus 8 of 32; $p = 0.0015$). The difference in the concentration of L-carvone in products with and without limonene was significant ($p = 0.045$); products with limonene contained $0.059\% \pm 0.074\%$ L-carvone, and products without limonene contained $0.023\% \pm 0.067\%$ L-carvone (Fig. 1b). There were few other declared flavour ingredients. Nine of the toothpastes contained one or more of the EU-legislated 26 fragrance allergens. These and non-legislated flavours declared on the product label are shown in Table 1.

Discussion

This study shows that L-carvone is present in nearly all toothpastes on the Swedish market. The limited clinical data on contact allergy to L-carvone make it challenging to estimate the threshold concentration needed to sensitize or elicit an allergic reaction, and it is not yet known whether the measured concentrations in this study are sufficient to cause clinical reactions.

The toothpastes selected for this study do not include all of the toothpastes available for sale in Sweden, but can be regarded as representative of the market, as all of

Table 1. Declared flavour ingredients

	Flavour	n	Toothpaste
Flavours of the 26 fragrance allergens legislated in the EU	Benzyl alcohol	3	Pepsodent Kids 2–6 strawberry
			Pepsodent White system
			Pepsodent White System Enamel Renewal
	Cinnamal	4	Colgate Smiles 6+
			Oral-B Pro-Expert All-Around Protection
			Oral-B Pro-Expert Professional Sensitivity
	Citral	1	Oral-B Pro-Expert Sensitive and Gentle Whitening
	Eugenol	5	Pepsodent White Naturals
			Colgate Smiles 6+
			Oral-B Pro-Expert All-Around Protection
			Oral-B Pro-Expert Professional Sensitivity
			Oral-B Pro-Expert Sensitive and Gentle Whitening
Other declared flavours	Limonene	34	Pepsodent Super Fluor
			See Fig. S2
	Aroma	64	–
	Anise alcohol	1	Sensodyne Pro-Enalij
	Citrus medica limonum (lemon) peel oil	1	Pepsodent White Naturals
	<i>Mentha piperita</i> (peppermint) oil	1	Pepsodent White Naturals
	<i>Mentha arvensis</i> leaf extract	1	Pepsodent White Naturals
	Menthol	2	Colgate MaxFresh
			Colgate Smiles 6+

the major brands were included. In Sweden, manufacturers of toothpaste, but not their different products, are registered, and a rough estimate is that the toothpastes included in this study represent ~80% of the total market. Besides the two toothpastes without flavour, all of the toothpastes analysed contained L-carvone, although only a few had concentrations of >0.1% (n = 10). Several toothpastes had no flavour specified on their packages, and, in these, L-carvone concentrations ranged from low to high (data not shown). Fruit-flavoured toothpastes had detectable levels of L-carvone, although at the lower end of the range, being <0.01%. Low levels of L-carvone were also found in the mint-flavoured toothpastes, which most likely had lower amounts of flavour added or contained types of mint other than spearmint, that is, less L-carvone.

The occurrence of limonene in the list of ingredients was more frequent in toothpastes with a high L-carvone concentration, but no relationship was seen between fruit flavour and the presence of limonene. Spearmint contains the most limonene of the different *Mentha* species, that is, between 10% and 20% (14, 17, 37), but L-carvone is also one of several compounds formed from autoxidized limonene (24). One explanation for the higher L-carvone concentrations in toothpastes with limonene may be that limonene-labelled toothpastes have L-carvone from two sources, that is, from intentional additions of spearmint

and from limonene oxidation. However, it has previously been shown that only 4% of limonene in pet. is transformed into L-carvone by oxidation in 3 months (38), and the proportion of L-carvone derived from oxidized limonene in toothpaste is therefore most probably very low. Additionally, there were other flavours declared in the tables of contents, some of them from the 26 fragrances legislated in the EU (Table 1), but these did not correlate with L-carvone concentrations (data not shown), and were far less prevalent than declared limonene. It is well known that cinnamal in toothpaste may give oral allergic reactions (39), but, as for benzyl alcohol, citral, and eugenol, there are no scientific reports on toothpaste-related adverse reactions, although these fragrances are known sensitizers (26).

Besides scientific evidence of L-carvone being a contact allergen, both the EU and the International Fragrance Association (IFRA), a trade organization, consider L-carvone to be a skin sensitizer (40–43). According to the European Chemical Regulations, L-carvone should be declared on the label when the concentration in chemical mixtures is ≥0.1%. On the other hand, toothpastes are considered to be cosmetic products in Europe, and there are currently no labelling requirements for L-carvone in the European Cosmetics Regulation (44, 45). However, the European Commission's scientific advisory body, the Scientific Committee of Consumer Safety,

has recommended that several other fragrance sensitizers should be labelled in addition to the 26 fragrances mentioned above, one of these being L-carvone (41).

The Research Institute for Fragrance Materials evaluates available scientific data on the possible sensitization capacity of fragrances, and these evaluations are published and forwarded to the IFRA. If a substance has sensitization potential, the IFRA generates a standard for this fragrance ingredient to ensure the safe use of fragrances in cosmetics. The standards are based on an exposure-based quantitative risk assessment (QRA) that takes into consideration which area of the body the cosmetic product is intended for and the expected daily dose, but also whether the fragrance ingredient may be present in matrixes containing irritants, emulsifiers, or penetration-enhancing factors. To calculate safe exposure levels, data from several studies are used, mainly experimental sensitization studies (46–48). According to the standards of L-carvone, the safe-use level to prevent the induction of contact allergy to oral hygienic products has been calculated to be 1.9% (43), which is well above the levels of L-carvone found in the present study. However, the safe-use levels in the QRA are calculated to avoid sensitization but not elicitation. Elicitation most often requires a lower concentration of the allergen, and patch testing with toothpaste may yield positive reactions in sensitized individuals (own clinical observations, 29, 30). However, it has also been shown that repeated exposure to low concentrations may induce sensitization (49). The total amount of L-carvone in a Finn Chamber® (diameter, 8 mm; ~20 µl) with 5% L-carvone in pet. used for patch testing corresponds to the quantity in the recommended amount of toothpaste (1 ml) with 0.1% L-carvone, both containing ~1 mg of L-carvone. In addition, toothpaste is probably the major exposure source of L-carvone, besides chewing gum and other mint-flavoured confectionery. Hence, it cannot be excluded that the concentrations found in the present study are sufficient to elicit contact stomatitis in sensitized individuals. The majority of toothpastes also have ingredients such as surfactants that can cause irritation by themselves (50). Sodium lauryl sulfate (SLS), a common surfactant in toothpastes, has been used in clinical studies on contact dermatitis to enhance the penetration of allergens (51, 52), and it is entirely possible that SLS may have the same effect on allergens in toothpaste.

Although the prevalence of clinical disease resulting from toothpaste exposure seems to be low, certain groups of patients appear to be more susceptible to sensitization, and patients with oral lichenoid lesions have a higher frequency of contact allergy to L-carvone (34). In addition, patch-tested individuals with contact allergy to L-carvone

frequently have oral lichenoid lesions (35), but it is not clear whether lichenoid lesions make the mucosa more prone to sensitization, or whether the lesions themselves are manifestations of contact allergy. The prevalence of oral lichenoid lesions and oral lichen planus in Sweden has been estimated to be 1.9% (53), and, in the study by Ahlgren et al. mentioned above (34), 12% of a group of patients with oral lichenoid lesions had contact allergy to L-carvone. For comparison, the proportions of sensitization to L-carvone among dermatitis patients in two studies were 1.6% and 2.8%, respectively (54, 55). L-Carvone is included in the dental series (5% pet.; Chemotechnique Diagnostics, Vellinge, Sweden) for patch testing, but patients with oral lichenoid lesions are not routinely tested, even though oral contact reactions are often expressed as lichenoid lesions (56). Consequently, one could suspect that a substantial number of individuals with oral lichenoid lesions have unrecognized contact allergy to L-carvone but, as of now, the clinical implications of this are unknown.

In summary, the present study shows that L-carvone is present in the majority of toothpastes on the Swedish market. The concentrations vary substantially, differing by a factor of 7000 between the highest and the lowest concentrations. In addition, the consumer cannot estimate the amount of L-carvone on the basis of the advertised flavour or the ingredient listing. Interestingly, toothpastes containing limonene have higher concentrations of L-carvone than those without limonene. Currently, it is difficult to determine the clinical relevance of the L-carvone concentrations found in the toothpastes, and further research is needed. A repeated open application test has therefore been initiated to study how L-carvone affects the oral mucosa in sensitized individuals with and without oral lichenoid lesions.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (A) Chromatogram of a reference standard of 0.002% L-carvone (top) and a sample of toothpaste (bottom) at 228 nm. L-Carvone elutes at ~8.8 min. Early peaks are seen in the toothpaste sample at 2 min. Similar peaks were seen in other samples and were not further analysed. (B) UV spectra of the same samples between 190 and 300 nm. Red curve: reference standard. Black curve: toothpaste.

Figure S2. L-Carvone concentrations in toothpaste. *, Toothpastes labelled as containing limonene. ◇, Toothpastes intended for children. †, Toothpastes advertised as 'without flavour'.

References

- Lippert F. An introduction to toothpaste – its purpose, history and ingredients. *Monogr Oral Sci* 2013; **23**: 1–14.
- Andersen KE. Contact allergy to toothpaste flavors. *Contact Dermatitis* 1978; **4**: 195–198.
- Morton C A, Garioch J, Todd P et al. Contact sensitivity to menthol and peppermint in patients with intra-oral symptoms. *Contact Dermatitis* 1995; **32**: 281–284.
- Francalanci S, Sertoli A, Giorgini S et al. Multicentre study of allergic contact cheilitis from toothpastes. *Contact Dermatitis* 2000; **43**: 216–222.
- Tong D C, Leaper M R, Colquhoun A N, Rich A M. An unusual presentation of oropharyngeal mucosal plasmacytosis related to toothpaste. *J Laryngol Otol* 2008; **122**: 1112–1114.
- Poon T S, Freeman S. Cheilitis caused by contact allergy to anethole in spearmint flavoured toothpaste. *Australas J Dermatol* 2006; **47**: 300–301.
- Sainio E L, Kanerva L. Contact allergens in toothpastes and a review of their hypersensitivity. *Contact Dermatitis* 1995; **33**: 100–105.
- Zirwas M J, Otto S. Toothpaste allergy diagnosis and management. *J Clin Aesthet Dermatol* 2010; **3**: 42–47.
- Paiva M, Piedade S, Gaspar A. Toothpaste-induced anaphylaxis caused by mint (*Mentha*) allergy. *Allergy* 2010; **65**: 1201–1202.
- Andersson M, Hindsén M. Rhinitis because of toothpaste and other menthol-containing products. *Allergy* 2007; **62**: 336–337.
- Risk and Policy Analysts Ltd. *Comparative Study on Cosmetics Legislation in the EU and Other Principal Markets with Special Attention to so-called Borderline Products: Final Report. Study Prepared for European Commission, Directorate-General Enterprise*. Lodon, Risk and Policy Analysts Ltd, 2004.
- Council of the European Union, European Parliament. Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *OJ L*, 2009; 52: 59–209.
- Zheljazkov V D, Cantrell C L, Astatkie T, Hristov A. Yield, content, and composition of peppermint and spearmints as a function of harvesting time and drying. *J Agric Food Chem* 2010; **58**: 11400–11407.
- Hussain A I, Anwar F, Nigam P S et al. Seasonal variation in content, chemical composition and antimicrobial and cytotoxic activities of essential oils from four *Mentha* species. *J Sci Food Agric* 2010; **90**: 1827–1836.
- Chauhan S S, Prakash O, Padalia R C et al. Chemical diversity in *Mentha spicata*: antioxidant and potato sprout inhibition activity of its essential oils. *Nat Prod Commun* 2011; **6**: 1373–1378.
- de Carvalho C C R, da Fonseca M M R. Carvone: why and how should one bother to produce this terpene. *Food Chem* 2006; **95**: 413–422.
- Surburg H, Panten J (eds.). Natural raw materials in the flavor and fragrance industry. In: *Common Fragrance and Flavor Materials*, Chapter 3, 6th edition: Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA, 2016; pp. 193–264.
- Rohloff J, Dragland S, Mordal R, Iversen T H. Effect of harvest time and drying method on biomass production, essential oil yield, and quality of peppermint (*Mentha x piperita* L.). *J Agric Food Chem* 2005; **53**: 4143–4148.
- Chalchat J, Garry R, Michet A. Variation of the chemical composition of essential oil of *Mentha piperita* L. during the growing time. *J Essent Oil Res* 1997; **9**: 463–465.
- Gulluce M, Sahin F, Sokmen M et al. Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *longifolia*. *Food Chem* 2007; **103**: 1449–1456.
- Singh A. Essential oil composition and chemocarrays of menthol mint (*Mentha arvensis* L.) cultivars. *Flavour Fragrance J* 2005; **20**: 302–305.
- Sokovic M D, Vukojevic J, Marin P D et al. Chemical composition of essential oils of Thymus and *Mentha* species and their antifungal activities. *Molecules* 2009; **14**: 238–249.
- Pandey A K, Rai M K, Acharya D. Chemical composition and antimicrobial activity of the essential oils of corn mint (*Mentha arvensis*) and lemon grass (*Cymbopogon flexuosus*) against human pathogenic fungi. *Pharm Biol* 2003; **41**: 421–425.
- Karlberg A T, Magnusson K, Nilsson U. Air oxidation of d-limonene (the citrus solvent) creates potent allergens. *Contact Dermatitis* 1992; **26**: 332–340.
- Surburg H, Panten J (eds.). Individual fragrance and flavor materials. In: *Common Fragrance and Flavor Materials*, Chapter 2, 6th edition: Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA, 2016; pp. 7–192.
- Uter W, Johansen J D, Börje A et al. Categorization of fragrance contact allergens for prioritization of preventive measures: clinical and experimental data and consideration of structure–activity relationships. *Contact Dermatitis* 2013; **69**: 196–230.
- Clayton R, Orton D. Contact allergy to spearmint oil in a patient with oral lichen planus. *Contact Dermatitis* 2004; **51**: 314–315.
- Skrebova N, Brocks K, Karlsmark T. Allergic contact cheilitis from spearmint oil. *Contact Dermatitis* 1998; **39**: 35–36.
- Corazza M, Levrat A, Virgili A. Allergic contact cheilitis due to carvone in toothpastes. *Contact Dermatitis* 2002; **46**: 366–367.
- Worm M, Jeep S, Sterry W, Zuberbier T. Perioral contact dermatitis caused by L-carvone in toothpaste. *Contact Dermatitis* 1998; **38**: 338.
- Lim S W, Goh C L. Epidemiology of eczematous cheilitis at a tertiary dermatological referral centre in Singapore. *Contact Dermatitis* 2000; **43**: 322–326.
- Hansson C, Bergendorff O, Wallengren J. Contact urticaria caused by carvone in toothpaste. *Contact Dermatitis* 2011; **65**: 362–364.
- Gunatheesan S, Tam M M, Tate B et al. Retrospective study of oral lichen planus and allergy to spearmint oil. *Australas J Dermatol* 2012; **53**: 224–228.
- Ahlgren C, Axel T, Möller H et al. Contact allergies to potential allergens in patients with oral lichen lesions. *Clin Oral Investig* 2013; **18**: 227–237.
- Kroona L, Ahlgren C, Bruze M et al. 411: carvone contact allergy in southern Sweden – a 15-year retrospective study. Oral communications, abstracts of the 12th Congress of the European Society of Contact Dermatitis (ESCD), Barcelona, Spain, 25–28 June 2014. *Contact Dermatitis* 2014; **70** (Suppl. 1): 31–40.
- Horev L, Isaksson M, Engfeldt M et al. Preservatives in cosmetics in the Israeli market conform well to the EU legislation. *J Eur Acad Dermatol Venerol* 2015; **29**: 761–766.
- Tisserand R, Young R. Essential oil profiles. In: *Essential Oil Safety: A Guide for Health Care Professionals*, Chapter 13, 2nd edition: Edinburgh, Churchill Livingstone/Elsevier, 2014; pp. 427–428.
- Nilsson U, Magnusson K, Karlberg O, Karlberg A T. Are contact allergens stable

- in patch test preparations? Investigation of the degradation of d-limonene hydroperoxides in petrolatum. *Contact Dermatitis* 1999; **40**: 127–132.
- 39 Isaac-Renton M, Li M K, Parsons L M. Cinnamon spice and everything not nice: many features of intraoral allergy to cinnamic aldehyde. *Dermatitis* 2015; **26**: 116–121.
- 40 European Commission. Commission Regulation (EU) 2015/1221 of 24 July 2015 amending Regulation (EC) No. 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures, for the purposes of its adaptation to technical and scientific progress. *OJ L*, 2015; 58: 10–23.
- 41 SCCS (Scientific Committee on Consumer Safety). 1459/11 opinion on fragrance allergens in cosmetic products, 26–27 June 2012. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_102.pdf (last accessed 01 March 2017).
- 42 EFSA Scientific Committee. Scientific opinion on the safety assessment of carvone, considering all sources of exposure. *EFSA J* 2014; **12**:3806 [74 pp.].
- 43 International Fragrance Association. IFRA standards booklet, l-Carvone: 6485-40-1, July 16 2008. Updated October 2009. Available at: www.ifraorg.org (last accessed August 12 2016).
- 44 Kiaschka U. Dangerous cosmetics – criteria for classification, labelling and packaging (EC 1272/2008) applied to personal care products. *Environ Sci Eur* 2012; **24**: 1–10.
- 45 European Union. Regulation (EC) no. 1907/2006 of the European parliament and of the Council of 18 December 2006 concerning the registration, evaluation, authorisation and restriction of chemicals (REACH), establishing a European chemicals agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) no. 793/93 and Commission Regulation (EC) no. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC (text with EEA relevance), 2006. Available at: <http://data.europa.eu/eli/reg/2006/1907/2016-06-21> (retrieved August 2016).
- 46 Api A M, Basketter D A, Cadby P A et al. Dermal sensitization quantitative risk assessment (QRA) for fragrance ingredients. *Regul Toxicol Pharmacol* 2008; **52**: 3–23.
- 47 Api A M, Vey M. Implementation of the dermal sensitization Quantitative Risk Assessment (QRA) for fragrance ingredients. *Regul Toxicol Pharmacol* 2008; **52**: 53–61.
- 48 Api A M, Belsito D, Bruze M et al. Criteria for the Research Institute for Fragrance Materials, Inc. (RIFM) safety evaluation process for fragrance ingredients. *Food Chem Toxicol* 2015; **82** (Suppl): S1–S19.
- 49 Friedmann P S. The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans. *Br J Dermatol* 2007; **157**: 1093–1102.
- 50 van Loveren C (ed.). *Toothpastes*. Vol. **23**: Basel, S. Karger, 2013.
- 51 McLelland J, Shuster S, Matthews J N. 'Irritants' increase the response to an allergen in allergic contact dermatitis. *Arch Dermatol* 1991; **127**: 1016–1019.
- 52 Hauksson I, Pontén A, Gruvberger B et al. Skincare products containing low concentrations of formaldehyde detected by the chromotropic acid method cannot be safely used in formaldehyde-allergic patients. *Br J Dermatol* 2016; **174**: 371–379.
- 53 Axell T. A prevalence study of oral mucosal lesions in an adult Swedish population. *Odontol Revy Suppl* 1976; **36**: 1–103.
- 54 Spiewak R, Samochocki Z, Grubska-Suchanek E et al. P075: Gallates, as well as hydroperoxides of limonene and linalool, are more frequent and relevant sensitizers than any cosmetic ingredient included in the European Baseline Series. Posters, 13th Congress of the European Society of Contact Dermatitis (ESCD), Manchester, United Kingdom, 14–17 September 2016. *Contact Dermatitis* 2016; **75** (Suppl. 1): 60–106.
- 55 Paulsen E, Andersen K E, Carlsen L, Esgaard H. Carvone: an overlooked contact allergen cross-reacting with sesquiterpene lactones? *Contact Dermatitis* 1993; **29**: 138–143.
- 56 Raap U, Stiesch M, Kapp A. Contact allergy to dental materials. *J Dtsch Dermatol Ges* 2012; **10**: 391–396.

II

Carvone Contact Allergy in Southern Sweden: A 21-year Retrospective Study

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Carvone (l-carvone), a mint flavour in spearmint oil, is considered a mild skin sensitizer. Carvone-sensitization may be linked to oral/perioral signs and oral lichen planus, but studies are sparse. The prevalence of patch test reactions to carvone and relevant findings from the positive group were investigated. Records for patch-tested patients at the Malmö clinic, for the period 1996 to 2016, were studied. Carvone-positive and carvone-negative patients were compared regarding patch test data from baseline series and dental series. Dental series-tested carvone-positive patients were also compared with a matched group. A total of 147 out of 4,221 referred patients had a positive patch test to carvone. Sensitized patients had higher mean age and were primarily women; 73% had oral signs and 57% had oral lichen. Concomitant patch test reactions to gold, nickel and mercury were common. In the matched group-comparison carvone-positive patients had a higher frequency of oral lichen, but no difference was found in sensitization to gold and mercury.

Key words: l-carvone CAS: 6485-40-1; spearmint; patch test; allergens; contact allergy; oral lichen planus; oral lichenoid lesions.

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The monoterpene carvone (l-carvone; Fig. S1a¹) is the chief component of spearmint oil and is widely used as a mint-flavouring agent (1). The flavour is typically found in oral healthcare products and foodstuffs, such as chewing gum and sweets. In addition to isolation from natural oils, carvone can be synthesized from d-limonene (Fig. S1b¹) (2) and it is also one of several oxidation products found in auto-oxidized d-limonene (3).

Oral healthcare products are a major source of exposure, and carvone is a constituent of most toothpastes (4, 5). Carvone is considered a weak sensitizer (6) and the prevalence of contact allergy in patch-tested cohorts has been estimated to be 1.6–2.8% (7, 8). A few papers on patients' adverse reactions to carvone or spearmint oil have reported both perioral and intraoral lesions from oral

SIGNIFICANCE

Carvone is a mint flavour found in most oral healthcare products. This study investigates the prevalence of contact allergy to carvone and describes common features of affected patients. In a cohort of 4,221 tested patients 3.5% had contact allergy to carvone. These patients often had oral signs and variants of oral lichenoid lesions, which otherwise only affects a few percent of the population. These data show that soluble allergens such as flavours and fragrances can cause oral contact allergy.

healthcare products, with intraoral lesions described as stomatitis or erosions associated with oral lichen planus (OLP) (9–13). Studies on patients with OLP or oral lichenoid lesions (OLL) have shown an association with carvone or spearmint contact allergy (14–16). However, these reports have not made a clear distinction between OLP, which is considered to be an autoimmune disease, and OLL, such as contact reactions to dental materials or other lichenoid lesions not fulfilling the criteria of OLP (17, 18).

The aim of this retrospective study was to estimate the prevalence of individuals with a positive patch test to carvone in Southern Sweden and to assess the characteristics of this group with regard to other contact allergies and clinical signs with special reference to OLP and OLL.

METHODS

Study population

Data were obtained from a clinical database comprising all patients subjected to patch testing at the Department of Occupational and Environmental Dermatology, Skåne University Hospital in Malmö, Sweden (19). All patients patch-tested with carvone during a 21-year period between 1996 and 2016 were included in the study.

Data recording

Information retrieved from the database included age at time of investigation, sex, type of patch test series in which carvone was included, grading of carvone test reaction and other contact allergies. From patients with a positive carvone patch test, information was also collected regarding the referrer's profession, diagnosis, clinical signs, and localization of signs related to the investigation. It was not possible to deduce from the referrals or patient records whether the patients had OLP or OLL. Therefore, all variants of lichenoid lesions will henceforth be denoted as oral lichen (OL) unless otherwise specified.

¹<https://www.medicaljournals.se/acta/content/abstract/10.2340/00015555-3009>

Descriptive and comparative analysis

During the investigated period carvone was used in various patch test series. A descriptive analysis was performed on the collected data of carvone-positive patients regardless of which series they were tested with.

In order to compare the group data of the carvone-positive patients with a more general patch test population, data from the 2 test series with the largest collections of carvone-positive patients, the baseline series (extended) and the dental series (dental patients), were investigated further (Fig. S2¹). Carvone was included in the Malmö extended baseline series during 1997 to 1998 for research purposes and prevalence assessment. In this paper, “baseline series” will subsequently refer to the extended baseline series. Patch test data from the baseline series were analysed by comparing carvone-positive patients with carvone-negative patients. For the dental series, which included carvone during the whole investigated period, a similar comparison was made between carvone-positive and carvone-negative patients.

In addition, carvone-positive patients within the dental series were compared with a matched group of carvone-negative patients (Fig. S2¹). The presence of OL, atopy and patch test data were compared. Patients were considered atopic if they had present or previous atopic dermatitis, allergic rhinitis and/or allergic asthma. The matching criteria were sex, age ± 6 years and date of testing ± 3 months except for 2 cases where the criteria were extended to age ± 10 years and date of testing to ± 4 months. Patients were chosen by using a random number generator on sets of patients meeting the required criteria.

The study was performed in accordance with the Declaration of Helsinki.

Patch testing

The patch test method for carvone and other investigated allergens has been consistent throughout the investigated period. Patch testing was carried out using 8-mm diameter Finn chambers (Epitest Ltd Oy, Tuusula, Finland or SmartPractice, Phoenix, AZ, USA) attached to Scanpor tape[®] (Norgeplaster A/S, Oslo, Norway).

Finn chambers with 20 mg of petrolatum test preparation were applied for 48 h and discarded by the patients themselves. For liquid preparations 15 μ l were used, applied to the chambers with a micropipette. Reactions were read on days 3 or 4 and on day 7. Occasional late reactions beyond 7 days were also recorded. Test reactions were graded by an experienced dermatologist according to the International Contact Dermatitis Research Group guidelines (20).

Test preparations of carvone (l-carvone, Acros Organics, Geel, Belgium, CAS: 6485-40-1) were prepared at the Department of Occupational and Environmental Dermatology, Malmö University Hospital, Sweden at a concentration of 5% in petrolatum.

Statistical analysis

The frequencies of the patients' different diagnoses, clinical signs and co-reactivity to different allergens were analysed with the χ^2 test, or with Fisher's exact test when expected values were small. A 2-sided p -value < 0.05 was considered statistically significant.

RESULTS

Of 4,221 referred and patch-tested patients, 147 (3.5%) had a positive test reaction to carvone. The positive reactions were most commonly detected by the dental series, followed by the baseline series and cheilitis series (Table I). Complete records were available for 145 patients,

Table I. Different test series in which carvone was included and the number of patients with a positive reaction to carvone (with oral lichen (OL) for each series

Test series	Time period	Tested <i>n</i>	Carvone- positive ^a <i>n</i>	With OL ^{a,b} <i>n</i> (%)
Dental series (dental patients)	1996–2016	1,938	99	67 (68)
Baseline series	1997–1998	1,355	14	1 (5)
Cheilitis series	2000–2016	500	9	1 (11)
Dental series (dental personnel)	1998–2016	460	8	1 (13)
Research series (oral lichen)	2006–2008	259	15	11 (73)
Plant series	1996	31	2	0
Cosmetic series	1996	23	0	0
Personal series	n.a.	n.a.	6	2 (33)

^aSome patients have been tested with more than one test series; ^bnumber of carvone-positive patients with simultaneous OL.
n.a.: not applicable.

whereas 2 only had information on patch test reactions. The mean age at testing was 66.2 ± 11 years. The male-to-female ratio in the carvone-positive group was 1:5.1 compared with 1:2.6 in the carvone-negative group.

Referring information

Based on the 145 patients with clinical data, the carvone-positive patients were primarily referred by dentists ($n=99$; 68%), a majority of whom were oral surgeons ($n=52$; 36%). Physicians referred 36 (25%) of the patients and 10 (7%) patients were enlisted as research subjects in projects on oral lichen. Patients referred by dentists were mainly tested with the dental series (87 of 99), whereas patients referred by physicians were tested with a variety of patch test series.

Diagnoses and clinical signs recorded in the referrals were predominantly localized intraorally ($n=106$; 73%), whereas the perioral area was affected to a lesser degree ($n=20$; 14%). Eczema except in the perioral area was present in 34 (23%) patients. Of these, 19 (13%) displayed dermal signs only, i.e. no oral or perioral involvement, often in the form of hand eczema. The main referring inquiry was “contact allergy to dental materials?”. Common words used to describe the clinical presentation were “redness”, “swelling” or “eczematous”. The presence of OL was recorded in 82 (57%) of the patients. OL often coincided with intraoral signs, where 81 of 106 patients had OL, whereas only 5 of the 20 patients with signs from the perioral area had OL. Lichen planus affecting the skin or genital area was recorded in 11 (8%) patients. Patients with OL or intraoral signs were predominantly tested with the dental series, whereas most patients with dermal signs were tested with the baseline series. Most patients with perioral signs were tested with either the dental series or the cheilitis series (Table II).

Patch test reactions

The number of positive test reactions to carvone at day 3 and at day 7, respectively, were essentially the same (110 vs. 111) and 31 patients (22%) tested positive only at day 7. Six patients had late reactions, from 10 days

Table II. Diagnoses and clinical signs in carvone-positive patients ($n=145$) and the proportion of the major patch test series used (dental, baseline or cheilitis)

Clinical diagnosis or symptom	Patients <i>n</i>	Patch test series ^a		
		Dental <i>n</i>	Baseline <i>n</i>	Cheilitis <i>n</i>
Diagnosis				
OL (OLP or OLL)	82	68	1	1
Dermal or genital lichen planus	11	5	1	0
Clinical signs				
Oral signs	106	87	2	2
Perioral signs	20	8	1	8
Dermal (no oral or perioral signs)	19	4	11	0

^aSome of the patients have been tested with more than one test series.
OL: oral lichen; OLP: oral lichen planus; OLL: oral lichenoid lesions.

up to one month after the test application. Two of these patients were re-tested and both showed a reaction on day 3. The test reaction strength (highest strength observed) was “+” in 78 (53%), “++” in 59 (40%) and “+++” in 10 (7%) patients. Besides the 147 patients with a positive reaction, 83 patients had a doubtful reaction to carvone, but no irritant reactions were noted.

Other contact allergies

A majority of the 147 patients had reactions to other allergens ($n=111$, 76%), most commonly to gold, nickel and mercury (Table III(a)). Patients with OL more often had positive reactions to mercury than patients without OL ($p=0.009$). The mean number of positive reactions to various allergens was 4.1 ± 4.9 . The allergens tested were, however, not the same among the carvone-positive patients, since different test series had been used and allergens such as fragrances and acrylates were only included in one or few of the test series. Allergens that gave frequent reactions, but were not tested in the whole group, were spearmint oil, fragrance mix I and balsam

Table III. Common positive patch test reactions among the 147 carvone-positive patients

Test substance	Positive reactions/ number of tested	%
(a) Substances that the entire group were tested with ($n=147$)		
Gold sodium thiosulphate	52/147	35.4
Nickel sulphate	23/146	15.6
Mercury	20/147	13.6
Potassium dichromate	15/147	10.2
Cobalt chloride	12/146	8.2
Palladium chloride	11/147	7.5
Colophonium	11/147	7.5
Formaldehyde	6/147	4.1
(b) Substances that only part of the group were tested with		
Spearmint oil	55/81	67.9
Fragrance Mix I	23/88	26.1
Balsam of Peru	19/117	16.2
Cinnamal	9/93	8.8
Cinnamyl alcohol	6/68	8.1
Peppermint oil	5/80	6.3
Acrylates ^a	9/125	6.1
Limonene ^b	4/90	4.4
(c) Test with patients' personal toothpaste		
Toothpaste (various brands)	11/16	68.8

^aDifferent acrylates grouped together; ^bthe preparation of limonene has varied over the investigated years of which some preparations were oxidized.

of Peru (Table III(b)). Sixteen patients were tested with their private toothpaste and 11 of these displayed a positive reaction (Table III(c)). The tested toothpastes were, for the most part, diluted in water, often at 50% w/v, but several dilutions were often tested, ranging from 5% to 50%, and they varied over the investigated years. Four patients were also tested with undiluted toothpaste. Irritant reactions were noted in 4 patients, of whom 3 were tested with 50% toothpaste and 1 was tested with undiluted toothpaste. No specific brand was singled out as giving more frequent reactions.

Comparative analysis of contact allergies within the baseline and dental test series: carvone-positive vs. carvone-negative patients

Carvone-positive patients had significantly higher frequencies of several contact allergies compared with the carvone-negative patients when investigating both series (Table SI¹). For carvone-positive patients tested with the dental series, positive test reactions to gold and mercury were highly allocated to patients with OL (Table SI¹).

Positive test reactions to other allergens in the dental series, however, did not differ when comparing the 99 carvone-positive patients to the matched group of carvone-negative patients, but the frequency of OL was significantly higher in the group of carvone-positive patients: 67/99 vs. 28/99 ($p<0.0001$; Table IV).

DISCUSSION

The majority of patients with a positive patch test reaction to carvone were referred for oral or perioral signs, indicating that the main exposure to carvone was from oral healthcare products (5). In addition, OL was prevalent in the studied group, with over half of the patients being affected; an association also seen in previous studies on patients with OL (14–16). Patients with OL

Table IV. Positive patch test reactions in the dental series 1996 to 2016 in carvone-positive patients and a matched carvone-negative group of patients (with oral lichen (OL))

	Dental series – matched groups	
	Carvone-positive (OL) <i>n</i> (%)	Carvone-negative (OL) <i>n</i> (%)
Number of tested	99 (67)***	99 (28)
Atopy	20 (13)	20 (1)
Patch test reactions to:		
Gold sodium thiosulphate	37 (30)	38 (12)
Palladium chloride	5 (3)	5 (0)
Nickel sulphate	15 (7)	16 (4)
Potassium dichromate	9 (5)	2 (0)
Cobalt chloride	7 (4)	2 (0)
Mercury	18 (16)	12 (9)
Colophonium	6 (3)	3 (1)
HEMA	5 (3)	2 (1)
EGDMA	4 (3)	0 (0)

*** $p<0.001$.

HEMA: hydroxyethyl methacrylate; EGDMA: ethylene glycol dimethacrylate. Significant values are shown for the comparison between the carvone-positive and the carvone-negative group.

were, for the most part, referred by dentists, and patients with dermal signs, such as eczema, were primarily referred by physicians. However, the data may be biased as dentists and physicians are primed to search for clinical signs in different areas. The clinical signs seem also to have raised a suspicion of contact allergy against dental restorative materials rather than rinse-off products, such as dental healthcare products.

A previous study by Paulsen et al. (7) investigated carvone sensitization in patients patch-tested with the baseline series, and found 15 out of 541 (2.8%) consecutively tested patients. The proportion was higher than in the present study, in which 14 patients were positive to carvone out of 1,355 patients (1.0%) tested with the baseline series. When considering all investigated carvone-allergic patients in our study, regardless of test series, the proportion was 3.5% (147 of 4,221), but compared with Paulsen's study in which no clinical relevance was seen in a majority of the patients the presently investigated group seems to have been skewed towards patients with oral or perioral signs. In addition, the mean age was higher in the present study and there was a greater predominance of women among carvone-positive patients, indicating that the sampling may have affected the outcome. The strength of the patch test reactions was similar between the 2 studies, with approximately 50% having a "++" reaction or higher. Paulsen found an association between carvone and Compositae allergy (sesquiterpene lactones), and the reaction to carvone was stronger in patients with both allergies. Of all series investigated in the present study, only the baseline series had sesquiterpene lactone mix included and 11 of 1,355 tested patients had a positive patch test reaction to this. Of the carvone-positive patients, only one patient reacted to sesquiterpene lactone mix.

A positive patch test to carvone on day 7 only was observed in 31 of the 147 patients. This demonstrates the importance of performing more than one patch test reading, since over 20% of the carvone-positive patients would otherwise have been left undiagnosed. Furthermore, 2 of the 6 patients with a late patch test reaction to carvone, day 10 or later, had positive test reactions on day 3 when re-tested. This indicates a possible active sensitization at the initial patch test. However, we have recently observed that carvone-positive patients may show positive reactions as late as 21 days upon re-testing, raising the possibility that a late appearing patch test reaction to carvone may not need to be a sign of active sensitization (to be published).

Overall, the 147 carvone-positive patients showed a high rate of additional contact allergies, especially against metals and fragrance substances (Table III). The high prevalence of spearmint oil sensitization is not surprising, since carvone is the main constituent of spearmint oil (1). Interestingly, a majority of the carvone-positive patients tested with their own toothpaste had a

positive test reaction. The toothpastes were of various brands and we do not know what toothpaste constituent the patients reacted to, but most toothpastes contain carvone, though at variable concentrations, as our previous work has shown (4). Sensitization to other flavours found in oral healthcare products, such as peppermint, cinnamal, cinnamyl alcohol and limonene, were not common in the investigated group, but a considerable number of patients had reactions to fragrance mix I and balsam of Peru, which contain flavour substances such as cinnamal and cinnamyl alcohol. Spearmint oil also contains 10–15% limonene (d-limonene) (1), a common fragrant constituent in cosmetic products (21) and also present in toothpastes (4). During a large part of the investigated time period the test preparation of limonene was not intentionally oxidized and it is essentially the oxidation products in limonene that are sensitizers, e.g. carvone (3). Of the carvone-positive patients only 4 of 90 tested with limonene had a positive reaction (Table IIIb) and 2 of these were tested with oxidized limonene. There is also a possibility that patients may have been sensitized to carvone from oxidized limonene, either orally or via the skin, but in a study by Matura et al. only a few patients sensitized to oxidized limonene reacted to carvone in patch tests (22).

It has been shown previously that patch-tested patients with OL often have contact allergies both related to their dental restorations and to carvone (15, 23). The present study also demonstrates a relationship between contact allergy to carvone and metals used in dental restorations. Of the 147 carvone-positive patients studied 55 (35.4%) had a concomitant patch test reaction to gold, and these patients also often had a strong test reaction to carvone. The frequency was comparable to observations on patients with dental gold restorations (24, 25). However, we do not have data regarding the presence of dental materials in the studied group, but the mean number of restorations was probably high, as dental restorations increase with age and the mean age in the group was over 65 years. The 147 investigated patients also had a slightly higher frequency of patch test reactions to mercury (13.6%) compared with studies on dental series tested or patients with OL (15, 26).

Patch test reactions to gold and mercury were more common in carvone-positive patients, when comparing them with all carvone-negative patients tested within the baseline or dental series and the frequency of test reactions was even higher in carvone-positive patients with OL (Table SI[†]). In contrast to what was observed in the unmatched comparative analysis there was no difference in the proportions of contact reactions between the 2 matched groups of carvone-positive and carvone-negative patients tested with the dental series (Table IV). The observed high ratio of women and high mean age in the carvone-positive groups were corrected in the matched comparison. Still, OL was strikingly over-represented

in carvone-positive patients and thus this relationship is neither connected with concomitant contact allergy to gold nor to mercury. Furthermore, regardless of reactivity to carvone, patients with OL in both groups had a high proportion of reactions to mercury.

The oral mucosa differs from the skin, in that it seems to be more tolerant to sensitization and elicitation (27, 28). Still, one must assume that the sensitization route of carvone is primarily through the oral mucosa. Factors that disturb the mucosal environment, such as OL make the oral mucosa more sensitive to external elements and could possibly increase the risk of sensitization (29). Previous studies on oral contact allergy have primarily focused on dental materials, such as acrylates and metals, and only sporadic studies have approached allergy to soluble agents in oral healthcare products, food and beverages. This is especially evident when it comes to lichenoid reactions, although it recently has been shown that contact allergy to fragrances is common among patients with OL (16). The present study demonstrates that contact allergy to carvone is closely related to oral signs and OL, and that oral exposure to carvone is a probable sensitization route.

The authors have no conflicts of interest to declare.

REFERENCES

1. Surburg H, Panten J. Ch 3. Natural Raw materials in the flavor and fragrance industry. In: Surburg H, Panten J, editors. Common fragrance and flavor materials, 6th edn. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2016: p. 193–264.
2. Surburg H, Panten J. Ch 2. Individual fragrance and flavor materials. In: Surburg H, Panten J, editors. Common fragrance and flavor materials, 6th edn. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2016: p. 7–192.
3. Karlberg AT, Magnusson K, Nilsson U. Air oxidation of d-limonene (the citrus solvent) creates potent allergens. *Contact Dermatitis* 1992; 26: 332–340.
4. Kroona L, Warfvinge G, Isaksson M, Ahlgren C, Dahlin J, Sörensen Ö, et al. Quantification of l-carvone in toothpastes available on the Swedish market. *Contact Dermatitis* 2017; 77: 224–230.
5. EFSA Scientific Committee. Scientific Opinion on the safety assessment of carvone, considering all sources of exposure. *EFSA J* 2014; 12: 3806.
6. International Fragrance Association. I-Carvone: 6485-40-1. IFRA Standards booklet 2009 Oct [retrieved 2016 Aug 15]. Available from: <http://www.ifraorg.org/en-us/standards>.
7. Paulsen E, Andersen KE, Carlsen L, Egsgaard H. Carvone: an overlooked contact allergen cross-reacting with sesquiterpene lactones? *Contact Dermatitis* 1993; 29: 138–143.
8. Spiewak R, Samochocki Z, Grubska-Suchanek E, Czarnobilska E, Pasnicki M, Czarnecka-Operacz M, et al. ESCD Abstracts: Posters, P075: Gallates, as well as hydroperoxides of limonene and linalool, are more frequent and relevant sensitizers than any cosmetic ingredient included in the European Baseline Series. *Contact Dermatitis* 2016; 75 (S1): 87.
9. Andersen KE. Contact allergy to toothpaste flavors. *Contact Dermatitis* 1978; 4: 195–198.
10. Bonamonte D, Mundo L, Daddabbo M, Foti C. Allergic contact dermatitis from *Mentha spicata* (spearmint). *Contact Dermatitis* 2001; 45: 298.
11. Clayton R, Orton D. Contact allergy to spearmint oil in a patient with oral lichen planus. *Contact Dermatitis* 2004; 51: 314–315.
12. Francalanci S, Sertoli A, Giorgini S, Pigatto P, Santucci B, Valsecchi R. Multicentre study of allergic contact cheilitis from toothpastes. *Contact Dermatitis* 2000; 43: 216–222.
13. Skrebova N, Brocks K, Karlsmark T. Allergic contact cheilitis from spearmint oil. *Contact Dermatitis* 1998; 39: 35.
14. Gunatheesan S, Tam MM, Tate B, Tversky J, Nixon R. Retrospective study of oral lichen planus and allergy to spearmint oil. *Australas J Dermatol* 2012; 53: 224–228.
15. Ahlgren C, Axell T, Möller H, Isaksson M, Liedholm R, Bruze M. Contact allergies to potential allergens in patients with oral lichen lesions. *Clin Oral Invest* 2013; 18: 227–237.
16. Larsen KR, Johansen JD, Reibel J, Zachariae C, Pedersen AML. Symptomatic oral lesions may be associated with contact allergy to substances in oral hygiene products. *Clin Oral Invest* 2017; 21: 2543–2551.
17. van der Meij EH, van der Waal I. Lack of clinicopathologic correlation in the diagnosis of oral lichen planus based on the presently available diagnostic criteria and suggestions for modifications. *J Oral Pathol Med* 2003; 32: 507–512.
18. van der Waal I. Oral lichen planus and oral lichenoid lesions; a critical appraisal with emphasis on the diagnostic aspects. *Med Oral Patol Oral Cir Bucal* 2009; 14: E310–314.
19. Edman B. The usefulness of detailed information to patients with contact allergy. *Contact Dermatitis* 1988; 19: 43–47.
20. Fregert S, editor. Manual of Contact Dermatitis on behalf of the International Contact Dermatitis Research Group and the North American Contact Dermatitis Group, 2nd edn. Copenhagen: Munksgaard 1981: p. 139.
21. Yazar K, Johnsson S, Lind ML, Boman A, Lidén C. Preservatives and fragrances in selected consumer-available cosmetics and detergents. *Contact Dermatitis* 2011; 64: 265–272.
22. Matura M, Goossens A, Bordalo O, Garcia-Bravo B, Magnusson K, Wrangsjö K, et al. Patch testing with oxidized R-(+)-limonene and its hydroperoxide fraction. *Contact Dermatitis* 2003; 49: 15–21.
23. Scalf LA, Fowler Jr JF, Morgan KW, Looney SW. Dental metal allergy in patients with oral, cutaneous, and genital lichenoid reactions. *Am J Contact Dermatitis* 2001; 12: 146–150.
24. Schaffran RM, Storrs FJ, Schalock P. Prevalence of gold sensitivity in asymptomatic individuals with gold dental restorations. *Am J Contact Dermat* 1999; 10: 201–206.
25. Ahlgren C, Bruze M, Möller H, Gruvberger B, Axell T, Liedholm R, et al. Contact allergy to gold in patients with oral lichen lesions. *Acta Derm Venereol* 2012; 92: 138–143.
26. Khamaysi Z, Bergman R, Weltfriend S. Positive patch test reactions to allergens of the dental series and the relation to the clinical presentations. *Contact Dermatitis* 2006; 55: 216–218.
27. Lowney ED. Immunologic unresponsiveness to a contact sensitizer in man. *J Invest Dermatol* 1968; 51: 411–417.
28. Ahlfors EE, Lyberg T. Contact sensitivity reactions in the oral mucosa. *Acta Odontol Scand* 2001; 59: 248–254.
29. Tlaskalova-Hogenova H, Tuckova L, Lodinova-Zadnikova R, Stepankova R, Cukrowska B, Funda DP, et al. Mucosal immunity: its role in defense and allergy. *Int Arch Allergy Immunol* 2002; 128: 77–89.

Fig. S1. Representation of chemical structures of: (a) l-carvone and (b) d-limonene.

Fig. S2. Graphical depiction of the various study cohorts in the baseline series and the dental series that were subjected to comparative analysis. C+: carvone-positive patch test; C-: carvone-negative patch test.

Table S1. Positive patch test reactions in the baseline series 1997 to 1998 and the dental series 1996 to 2016 with the number of carvone-positive (with oral lichen (OL)) demonstrated

	Baseline series		Dental series	
	All tested <i>n</i>	Carvone- positive (OL) <i>n</i> (%)	All tested <i>n</i>	Carvone- positive (OL) <i>n</i> (%)
Number of tested	1,355	14 (1)	1,938	99 (67)
Patch test reactions to:				
Gold sodium thiosulphate	298	8** (1)	542	37* (30)**
Palladium chloride	39	2	129	5 (3)
Nickel sulphate	283	2	364	15 (7)
Potassium dichromate	78	2	78	9* (5)
Cobalt chloride	110	4*	134	7 (4)
Balsam of Peru	72	2	165	18** (16)***
Fragrance mix I	90	2	68	6 (3)
Thiuram mix	24	2*	48	5 (3)
Ethylene diamine	41	2	28	4(*) (3)

Significant values are shown for the comparison between carvone-positive and carvone-negative groups. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; (*)*p* = 0.0510.
Allergens are displayed when ≥ 2 patients had a concomitant positive patch test for carvone.
HEMA: hydroxyethyl methacrylate; EGDMA: ethylene glycol dimethacrylate.

III

ABSTRACT

Background. Carvone (l-carvone), a mint-tasting flavour often found in toothpaste, is considered a weak contact allergen. Contact allergy to carvone among dermatitis patients is 1.6–2.8% but a higher prevalence (12%) has been observed in patients with oral lichen planus (OLP) or oral lichenoid lesions (OLL). Patients with contact allergy to carvone often have also OLP/OLL.

Objective. To investigate how carvone affects the oral mucosa and perioral area in sensitised individuals through a use test with toothpaste containing carvone.

Methods. 14 allergic subjects with a previous positive patch test to carvone, 20 subjects with OLP/OLL and 3 healthy controls were patch tested with toothpaste and carvone at different dilutions. During the month-long use test, Subjects used toothpaste with 1% carvone twice daily and were examined fortnightly. 5 allergic subjects received non-flavoured toothpaste and all other subject received the same toothpaste with carvone added. Clinical signs were assessed with a mucosal scoring system by Escudier et al. and the subjects' quality of life (QoL) were assessed before and after the use test with a questionnaire, oral health impact profile.

Results. 2 additional subjects, recruited with OLP/OLL, tested positive to carvone. A majority of the allergic subjects had OLP/OLL upon their first visit (13/16). A positive patch tests to carvone were recorded in 8 of 16 allergic subjects whereof two had late reactions (day 19 and 21). Allergic subjects reacted to carvone toothpaste with aggravated OLL (7/10) and two displayed perioral eczematous reactions. The mucosal score increased in allergic subjects receiving carvone toothpaste compared to those receiving non-flavoured toothpaste. Additionally, allergic subjects with OLP/OLL had an increased mucosal score compared to subjects with OLP/OLL only. The difference of QoL, before and after the use test, showed the same pattern as the mucosal scores.

Conclusion. When exposed to carvone, allergic individuals react with increased OLL and/or perioral eczema. These lichenoid lesions mimic OLP and allergic individuals would therefore be at risk of not being investigated with regard to flavour contact allergy.

INTRODUCTION

Contact allergy to the mint-tasting flavour carvone (l-carvone) appears to be frequent in patients with oral lichenoid lesions (1). However, there is lack of knowledge how contact allergy to carvone manifests in the oral mucosa.

Carvone is the main constituent of spearmint oil and one of the oxidation products of air-exposed limonene (d-limonene) (2). Most toothpastes on the Swedish market contain carvone with concentrations varying between 0.00005 to 0.35% and the use of toothpaste is widespread(3). The frequency of positive patch tests to carvone varies between 1.6 and 2.8% among routine dermatitis patients (4,5). We recently showed that 57% of patients with a positive patch test to carvone also have oral lichenoid lesions and that referring clinicians often suspect adverse reactions to dental restorative materials rather than to soluble allergens (6).

Contact allergic reactions in the oral mucosa are less well defined, both clinically and histologically, than cutaneous lesions and may manifest in various ways. Lichenoid contact reactions from dental restorative materials, such as mercury in amalgam fillings, are the most recognised manifestations but plasma cell gingivitis from cinnamal and more generalised descriptions of stomatitis to various substances have also been reported (7-10).

Lichenoid contact reactions fall under the group of oral lichenoid lesions (OLL) – oral lesions similar to oral lichen planus (OLP). OLP, the oral counterpart of the skin disease lichen ruber planus, is a T-cell driven autoimmune disease and mostly affects middle-aged and older women. The lesions are typically distributed bilaterally on the buccal mucosa or tongue with the hallmark appearance being white striations, but any oral region may be affected and lesions may also present as erythematous or ulcerated (11,12). On both histological and clinical level, lesions of OLL are more or less identical to OLP, but have either a known aetiology, such as contact allergy, or have an atypical distribution in the oral cavity i.e. unilateral lesions (13,14).

Case reports have described manifestations of contact allergy to carvone and spearmint as either contact cheilitis or OLP (8,15-18). Patients with OLP and OLL have a higher prevalence of positive patch tests to carvone (1), but it is not known if they are more easily sensitised to carvone due to their lesions or if their lesions are manifestations of contact allergy to carvone. The aim of this study was to investigate how the oral mucosa and perioral area in sensitised individuals responds to carvone. Use tests or repeated open application tests (ROAT) have been used within the research field for several decades and aim to investigate whether the normal-use exposure to an allergen in sensitised individuals will give dermatitis or not (19). We here report, to the best of our knowledge, the first intraoral use test with toothpaste containing carvone.

MATERIALS AND METHODS

Initial selection of subjects

Subjects with known contact allergy to carvone were recruited from a database listing patients with a positive patch test to 5% carvone in petrolatum at the Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden. Of 147 such patients retrieved from the database, 92 patients were possible candidates for the study i.e. not deceased and living in the regional county. Consecutively contacted from the patient list, 14 (of 92) subjects were recruited. All were women and the mean age was 69.5 ± 7.6 .

Twenty subjects with OLP or OLL were recruited from the department of Oral Surgery and Oral Medicine, Malmö University, Sweden. They all had a histologically verified oral lichenoid reaction. In this study, subjects with OLP or OLL are treated as one group and their lesions are collectively called *oral lichen* (OL). The mean age of the OL-group was 65.0 ± 10.3 . 15 subjects were female.

In addition, three dermatitis patients with neither a positive patch test against carvone nor OL were recruited from the Department of Occupational and Environmental Dermatology as controls without contact allergy or OL. The control group's mean age was 68.9 ± 8.2 . Two subjects were female.

Subjects included were over 20 years old, were using toothpaste and were not using systemic or local oral corticosteroids nor any other immune-modulating drugs 4 weeks prior to the start of the study. For all three groups, subjects were recruited consecutively from lists meeting the inclusion and exclusion criteria.

The regional ethical review board in Lund, Sweden approved the study and the subjects agreed by signing an informed consent, all in accordance with the 1964 Helsinki declaration and its later amendments.

Materials used in patch and use tests

Carvone (l-carvone; CAS: 6485-40-1; Sigma Aldrich, St Louis, MO, USA) from the same batch was used in all patch test preparations and in the test toothpaste. For patch testing, a stock solution of 5.0% w/v carvone in acetone (CAS: 67-64-1; Merck KGaA; Darmstadt, Germany) was prepared. A serial dilution with a half-logarithmic dilution factor ($1:10^{-1}$) was made in the range between 5.0 % and 0.000158 % – 10 dilutions.

A fruit-flavoured toothpaste without sodium lauryl sulphate or carvone was provided by Zendium® via CCS Healthcare AB, Borlänge, Sweden and was used by all subjects one week before the use test started.

The test toothpaste was prepared by adding 1 % carvone to a non-flavoured toothpaste without preservatives or sodium lauryl sulphate provided by Proxident AB, Falun, Sweden. The mixture was vigorously stirred to ensure that the toothpaste base and carvone did not separate.

The toothpaste was distributed to the subjects in 20 ml syringes (Injekt® Luer Solo; B.Braun Melsungen AG; Melsungen, Germany).

High-performance liquid chromatography

To estimate any loss of carvone from the syringes during the study due to evaporation and degradation, every batch of carvone toothpaste mixture was analysed with high-performance liquid chromatography (HPLC) after the use test. The method has been described recently (3). Toothpaste from the front (adjacent to the needle adaptor), middle and back of the syringe barrels were analysed. A sample of approximately 0.5 g was taken for each barrel section.

Patch test

A skin patch-test was performed one week prior to the start of the use test. All 37 subjects were patch-tested with the 10 serial dilutions of carvone except controls who were tested with only one dilution, 5.0 % carvone and the rest of the chambers empty.

The fruit-flavoured toothpaste and the test toothpaste with and without 1 % carvone were also tested, all three diluted in water at 50 % w/v and 5 % w/v. Subjects recruited from the department of Oral Surgery and Oral Medicine were also tested with the Swedish dental series since they had not been previously patch tested.

Finn chambers® (SmartPractice, Phoenix, Arizona, USA) with approximately 20 mg of petrolatum preparations were applied on the subjects' upper back with Scanpore™ tape (Norgeplaster A/S, Vennesla, Norway). The placement of the different preparations was randomised on a group level and blinded to the test readers. For preparations in liquid, 15 µl of test solution was applied using a micropipette. After 48 h, the patches were removed by the subjects themselves. It was stressed that communication between the readers and subjects should be avoided. The upper back was read at day 3 or 4 and at day 7 by experienced dermatologists according to the International Contact Dermatitis Research Group guidelines (20).

Study design of use test

One week prior to the start of the use test, all 37 subjects had their oral cavity examined, medical history taken and were patch tested. Any lichenoid lesions were diagnosed clinically according to the criteria by van der Meij and van der Waal (13,14). Additionally, to avoid interference from other mint products during the study, the subjects were given the fruit-flavoured toothpaste without carvone to use during the week preceding the use test and they were instructed to avoid any mint-tasting products for the next five weeks.

The use test period ran over a month's time and the subjects were examined fortnightly (Figure 1) or earlier at their own request. No information about any contact allergies the subjects might have, was available to the clinical examiners during the study. Five randomised subjects

with contact allergy to carvone were given non-flavoured toothpaste. The remaining subjects were all given toothpaste with 1 % carvone. The subjects were instructed to brush their teeth for at least 2 minutes twice daily with 1 ml of toothpaste - approximately 10 g of carvone per occasion. Two 20 ml syringes with toothpaste were given to the subjects at every appointment. To ensure that the correct amount of toothpaste had been used, old syringes were examined or the subjects were asked to indicate how much toothpaste they had used by showing the level on a 20 ml syringe.

A clinical examination was performed on three occasions during the month-long use test, where the perioral skin and the oral mucosa were examined and 14 clinical photographs were taken on each subject, all performed by an experienced dentist. Additionally, the subjects had a periodontal examination measuring gingival pocket depth and bleeding on probing at the start and end of the use test. The use test was terminated ahead of time upon the subjects' request or if ulcers in the oral mucosa or eczematous changes on the skin developed.

Figure 1. Study design

Analysis of mucosal reactions

To assess the mucosal status and any change between the examinations, a clinical scoring system for oral lichen planus by Escudier et al. was used (21). At 17 different mucosal sites, the scoring system measures the extent of mucosal affection (site score, a) and the severity of the lesions (severity score, b). By multiplying the site score and severity score ($a \times b$) an activity score is generated for each site. The test subjects' own perceived pain from the intraoral and perioral area is rated by means of a numerical rating scale (pain score) from 0-10 (22). A total weighted score (S_{tot}) is defined as the sum of the overall site score + overall activity score (S_a) + pain score (table I).

The scoring was performed on clinical photographs taken at the start and end of the experiment with the same camera. Any lesion not relating to the scoring system was also

recorded. The images, mounted in pairs on PowerPoint™ slides, were randomised and blinded both with respect to identity of the subjects and to occasion (start or end). Two independent observers evaluated the images. They initially calibrated their scoring on images of regular OLP patients. Seventeen site values were recorded on 14 pairs of pictures from each subject. The two observers subsequently agreed on a consensus score.

Table I. Scoring system for mucosal disease by Escudier et al. (21) showing sites examined, numerical and maximum scores as well as calculation of the total weighted score (S_{tot}). Site score: 0, no lesion; 1, lesion present and 2, lesion covering > 50 % of the examined site. Severity score; 0, hyperkeratosis; 1, hyperkeratosis with mild erythema; 2, marked erythema and 3, ulceration.

Site	Site score (a)	Severity score (b)	Activity score (a × b)
Outer lips	0 or 1	0–3	0–3
Inner lips	0 or 1	0–3	0–3
Left buccal mucosa	0, 1 or 2	0–3	0–6
Right buccal mucosa	0, 1 or 2	0–3	0–6
<i>Gingiva</i>			
Lower right	0 or 1	0–3	0–3
Lower central	0 or 1	0–3	0–3
Lower left	0 or 1	0–3	0–3
Upper left	0 or 1	0–3	0–3
Upper central	0 or 1	0–3	0–3
Upper right	0 or 1	0–3	0–3
Dorsum of tongue	0, 1 or 2	0–3	0–6
Lateral tongue, right	0 or 1	0–3	0–3
Lateral tongue, left	0 or 1	0–3	0–3
Floor of mouth	0, 1 or 2	0–3	0–6
Hard palate	0, 1 or 2	0–3	0–6
Soft palate	0, 1 or 2	0–3	0–6
Oropharynx	0, 1 or 2	0–3	0–6
Maximum score	24		72
$S_{tot} \text{ (max 106)} = \text{Overall site score (max 24) + overall activity score (S}_a\text{, max 72)}$ + pain score (max 10)			

OHIP

All subjects filled out a questionnaire in order to assess their oral health related quality of life (OHRQoL). The questionnaire, Oral Health Impact Profile (OHIP-49) (23) has been used to measure patient-reported outcomes in several oral conditions, including mucosal diseases such as OLP (24). A Swedish validated version (OHIP-S) (25) was used, comprising 49 questions with a response format in an ordinal rating scale from 0–4 ('none/never', 'hardly ever', 'occasionally', 'often' or 'very often'). The questions can be referred to four different domains: appearance, function, pain and psychological treats (26,27). In the present study, the response

was calculated as change of the sum of scores between the start and end of the experiment. Both the total OHIP score and the scores for the four domains were calculated.

Study groups

Previous studies have indicated that OL and contact allergy to carvone are related and it was therefore anticipated that some of the recruited subjects could be both sensitised to carvone and have OL. As a result, the study groups were slightly rearranged after the patch test (Figure 2). One group (CA-group) contained 16 subjects contact allergic to carvone (with or without OL) where sensitivity to carvone was determined by either the archival patch test result or the patch test performed prior to the use test. Eleven of these allergic subjects received toothpaste with carvone and five received non-flavoured toothpaste. Another group contained 18 subjects with OL and negative patch test reactions to carvone, all of which received toothpaste with carvone. The control group of three subjects remained unchanged and all received toothpaste with carvone.

Figure 2. Initial selection of subjects and the subsequent allocation into use test study groups. a) Subjects with contact allergy to carvone using toothpaste with or without carvone, b) Subjects with OL using carvone toothpaste with or without carvone contact allergy.

CA: contact allergy to carvone, OL: oral lichen, c: toothpaste containing carvone, nf: non-flavoured toothpaste.

Statistical analysis

The use test evaluated two factors: a) the effect of the test toothpaste compared to a non-flavoured toothpaste on carvone sensitised subjects regardless of OL lesions ($CA^+/OL^+/c$ versus $CA^+/OL^+/nf$), and b) how subjects with OL with or without carvone sensitivity reacted to the test toothpaste ($CA^+/OL^+/c$ versus $CA^+/OL^+/c$) (Figure 2 a and b). The difference between start and end scores of the mucosal reactions and OHIP was calculated for every group and the two factors were analysed with the Mann-Whitney U-test. In previously recorded allergic subjects, the difference in clinical signs, i.e. S_{tot} , between subjects with positive and negative patch tests when re-tested was analysed with the t-test for independent samples. Two-sided p -Values <0.05 were considered statistically significant. Controls were not included in the statistical analysis.

RESULTS

HPLC

After the use test, the syringes with toothpastes containing 1% carvone were stored refrigerated and were analysed after a mean storage time of 102 days. The mean concentration of carvone was $0.97 \pm 0.12\%$. There was a slight difference in concentration between the barrel sections with the front having the lowest mean concentration of carvone (0.89%) and the back having the highest mean concentration (1.06%). The storage time did not affect the concentration.

Initial examination and patch test

Two subjects, recruited because of OL, had positive patch test reactions to carvone and were therefore included in the CA-group (n =16). Besides these two subjects, no positive patch test reactions to carvone were recorded in controls or subjects with OL only. A majority of the subjects with OL only had a clinical diagnosis of OLP (14 of 18) and the rest had OLL. Of the carvone allergic subjects, 13 of 16 had OL upon their first visit whereof eight had OLP and five had OLL. A majority in the CA-group stated that they were using flavoured toothpaste (12 of 16), 10 of which were using mint-flavoured toothpaste. In the CA-group, positive patch test reactions were observed in 8 of the 16 subjects, two of which had late reactions – at day 19 and day 21. The late reactions were discovered by the subjects themselves and were inspected by a dermatologist. The reaction strength of the patch test with 5.0% carvone varied between ‘+’ and ‘++’ with a majority having ‘++’ reactions. The lowest concentration giving a ‘+’ reaction was 0.05% carvone. Five subjects reacted to the test toothpaste (with 1% carvone), but no reactions were observed from neither the fruit-flavoured toothpaste nor the toothpaste without flavour (table II).

Use test

Two subjects were excluded from the clinical analysis (Figure 2). These two received anti-inflammatory medication for reasons other than the experiment. Three additional subjects were excluded from the OHIP analysis due to incomplete questionnaires.

Five of the subjects (Table II, subjects 1, 3, 4, 5 and 16) terminated the use test in advance, all of whom were contact allergic to carvone. Four receiving carvone toothpaste had a discomforting reaction and/or clinical signs, and one subject receiving non-flavoured toothpaste stopped the use test due to a concomitant episode of erythema nodosum not related to the test. Data for these subjects was collected at the termination of the use test instead of at week 4.

			PATCH TEST			USE TEST				
Subject (sex and age)	Toothpaste flavour before patch test	Previous patch test reaction (5 % carvone pet)	Patch test reaction (5 % carvone aq)	MEC and reaction strength (carvone in aq)	Patch test reaction test toothpaste 1% carvone (50 % aq)	Test toothpaste	Use test termination in advance (days in advance)	Mucosal score in difference and S _{test} (pain)	Clinical signs on photos	
No	OL									
1	Female, 58	Mint	Not tested	++	5 % ++	-	Carvone	Yes (12)	+2 (+2)	Weakly increased OL activity
2	Female, 80	Mint	Not tested	++	0.5 % ++	+	Carvone	No	+10 (0)	Increased OL activity
3	Female, 72	Mint	++	++	1.58 % ++	++ Late reaction	Carvone	Yes (14)	+12 (+6)	Perioral eczema Weak signs of OL
4	Female, 77	Non-flavoured	++	++ Late reaction d21	0.158 % + Late reaction	++ Late reaction	Carvone	Yes (13)	+7 (+5)	Scaling red lips Increased OL activity
5	Female, 72	Non-flavoured	+++	++ Late reaction d19	0.05 % ++(+ Late reaction	++(+ Late reaction	Carvone	Yes (3)	+16 (+2)	Increased OL activity
6	Female, 76	Mint	+	(+)	0.5 % +	-	Carvone	No	+7 (-1)	Increased OL lesions and activity
7	Female, 67	Mint	++	+	5 % +	+	Carvone	No	- -	Excluded
8	Female, 51	Fennel	+	-	-	-	Carvone	No	-1 (-1)	
9	Female, 53	Mint	+	-	-	-	Carvone	No	+9 (+3)	Increased OL activity
10	Female, 75	Mint	+	-	-	-	Carvone	No	+1 (+1)	
11	Female, 68	Fruit	+	-	-	-	Carvone	No	+2 (0)	
12	Female, 73	Non-flavoured	++	++(+)	0.5 % +	(+)	Non-flavoured	No	+2 (0)	
13	Female, 67	Mint	+	-	-	-	Non-flavoured	No	0 (0)	
14	Female, 75	Mint	++	-	-	-	Non-flavoured	No	+1 (0)	
15	Female, 68	Mint	++	-	-	-	Non-flavoured	No	0 (0)	
16	Female, 71	Non-flavoured	+++	-	-	-	Non-flavoured	Yes (4)	-2 (+2)	

Table II. Subjects with contact allergy to carvone (CA-group) – Patch test result and use test result. OL: oral lichen planus; OLL: oral lichenoid lesions; pet: petrolatum; ac: acetone; MEC: Minimum eliciting concentration; aq: water; S_{test}: Escudier index; total weighted score; pain: numerical rate scale on pain

Analysis of mucosal reactions and periodontal status

Of the allergic subjects receiving carvone toothpaste, 7 of 10 showed increased clinical signs when examining the before and after photos (Figure 3). The signs were either aggravated oral lichenoid lesions (7 of 7) or eczematous reactions on the perioral skin (2 of 7). Increased clinical signs were seen in subjects who had a positive reaction to carvone in the patch test performed before the use test, but the distribution was not significant ($p = 0.071$). The clinical diagnosis of OL (OLP or OLL) did not change after the use test. Of the seven subjects showing oral signs, four had OLP and three had OLL (Table II).

Figure 3. Perioral and intraoral clinical changes before (A) and after (B) use test. Subject no. 2 A-B. Exacerbation of lichenoid lesions (here noted at the left buccal mucosa and on the inside of the lower lip). Subject no. 3 A-B. Perioral eczematous lesions and weak oral lichenoid lesions (not shown). Subject no.6 A-B. Exacerbation of bilateral lichenoid lesions (here noted at the right buccal mucosa).

The mean values of the scores from the evaluation of clinical pictures, with regard to the different groups are shown in table III. Contact allergic subjects using carvone toothpaste (CA⁺/OL[±]/c) displayed a significant worsening of mucosal health status compared to contact allergic subjects receiving non-flavoured toothpaste (S_a , $p = 0.028$; S_{tot} , $p = 0.019$). Furthermore, allergic subjects with oral lichenoid lesions using carvone toothpaste (CA⁺/OL[±]/c) had a stronger reaction with regard to mucosal health status compared to subjects with OL only (CA⁺/OL[±]/c), (S_a , $p = 0.003$; pain score $p = 0.015$; S_{tot} , $p = 0.001$).

No significant difference in gingival pocket depth or bleeding on probing was seen between the start and end of the use test or between any of the groups (data not shown).

Table III. Use test – mucosal scoring for the different groupings.

SD: standard deviation, CA: carvone contact allergy; OL: oral lichen; c: carvone toothpaste; nf: non-flavoured toothpaste; S_a : Escudier index, overall activity score; pain score: numerical rate scale on pain; S_{tot} : Escudier index, total weighted score.

Groups	Toothpaste	Subjects	Use test – start (SD)			Use test – end (SD)		
			S_a	Pain score	S_{tot}	S_a	Pain score	S_{tot}
CA ⁺ /OL [±] /c	carvone	10	3.6 (5.5)	2.6 (2.4)	11.5 (11.1)	7.4 (7.2)	4.3 (3.0)	18.0 (13.7)
CA ⁺ /OL [±] /nf	non-flavoured	5	4.0 (5.9)	2 (2.1)	9.0 (10.2)	3.4 (5.5)	2.2 (2.3)	9.2 (9.6)
CA ⁺ /OL [±] /c	carvone	9	4.0 (5.7)	2.8 (2.4)	12.7 (11.1)	8.2 (7.1)	4.8 (2.8)	20.0 (12.9)
CA ⁺ /OL [±] /c	carvone	17	5.7 (4.6)	2.8 (2.1)	14.3 (8.5)	5.9 (4.5)	2.8 (2.1)	14.5 (8.4)
Controls	carvone	3	0.0 (0.0)	0.0 (0.0)	0.3 (0.6)	0.0 (0.0)	0.3 (0.6)	0.7 (1.2)

OHIP

The mean values and change of the scores for the OHIP-49, with regard to the different groups, are shown in table IV. Contact allergic subjects exposed to carvone (CA⁺/OL[±]/c) declared a significantly less favourable change in OHIP-pain than contact allergic subjects receiving non-flavoured toothpaste (OHIP-pain, $p = 0.036$). Further, subjects with both OL and carvone allergy (CA⁺/OL[±]/c) had a negative change in OHRQoL compared to subjects with only OL (CA⁺/OL[±]/c), when exposed to carvone (OHIP, $p = 0.014$; OHIP-pain, $p = 0.024$; OHIP-psyc, $p = 0.023$).

Table IV. Use test – OHIP (Oral health impact profile) scoring for the different groupings.

SD: standard deviation, CA: carvone contact allergy, OL: oral lichen, c: carvone toothpaste, nf: non-flavoured toothpaste.

Groups	Toothpaste	Subjects	OHIP start (SD)	OHIP end (SD)	Mean difference
CA ⁺ /OL [±] /c	carvone	10	33.9 (19.6)	42.3 (30.8)	8.4
CA ⁺ /OL [±] /nf	non-flavoured	4	33.0 (14.0)	26.5 (18.0)	-6.8
CA ⁺ /OL [±] /c	carvone	9	35.9 (19.7)	45.7 (30.6)	9.8
CA ⁺ /OL [±] /c	carvone	15	29.7 (10.9)	24.7 (14.2)	-5.0
Controls	carvone	3	44.3 (22.0)	39.7 (14.8)	-4.7

DISCUSSION

In our study, we demonstrate how a use test with toothpaste can be conducted and propose a way to evaluate the mucosal reactions. Sensitised subjects exposed to carvone in toothpaste reacted with perioral eczema and increased mucosal scores i.e. aggravated lichenoid lesions. The OHIP score also increased, especially in the domain for pain.

The sensitisation rate of carvone is low despite the fact that many use mint-flavoured toothpaste daily. However, patients with OL seem to be particularly vulnerable to sensitisation. The mean age of carvone contact allergic patients are high and more women are affected (6). A reason could be that penetration of carvone and subsequent sensitisation are facilitated by having oral lichenoid lesions. In addition, OL is common in the same age group and have the same gender predilection (11). In general, the sensitisation rate and the immune response decreases with higher age but some allergens, often topical medicaments and fragrances, are associated with increased sensitisation rates (28,29). Besides increased and cumulative exposure to these allergens, the epidermal barrier, and possibly also the oral mucosa, becomes impaired with age which facilitates irritation and allergen penetration (30).

The immune reactivity to patch tests generally decreases with age (31) and patch test reactions become weaker and appear later (32). The same features were noted in the present study. Of the recruited subjects with a previous positive patch test to carvone, less than half showed a positive patch test reaction upon re-testing, and two subjects demonstrated very late reactions. For all the re-tested subjects, the mean interval between the two test occasions was 10 years and no difference in the interval was seen between those who tested positive to carvone and those with no reaction at the re-test (data not shown).

Of 92 potential subjects with contact allergy to carvone, only 14 agreed to participate in the study. A high mean age of the possible candidates together with a long test period with several appointments probably influenced the low turnout. A majority of the allergic subjects had OL and since there is a correlation between carvone allergy and OL it is possible that allergic individuals with OL were more interested in attending the study than individuals with no oral complaints.

In use tests, subjects are exposed to repeated doses of allergen and the concentration of the allergen is mimicking the normal-use setting, often much lower than in patch test preparations. In the present study, the toothpaste contained 1% carvone which is almost three times higher than the highest concentration measured in toothpastes on the Swedish market, but the concentration was well below the safe-use level of 1.9% declared by the International Fragrance Association (3,33). Additionally, the toothpaste used did not contain any surfactants, such as sodium lauryl sulphate, which may otherwise increase the allergen response (34). The purpose of the high concentration was to provoke a reaction, rather than imitating normal use, and to ensure that a distinct reaction developed in the subjects. Dermal contact

reactions from patch or use tests most probably differ from the oral counterpart. The scale used to assess the dermal contact reaction takes notes on erythema, papules and vesicles (35). Oral contact reactions may be erythematous but features of papules and vesicles have, to our knowledge, not been reported in the literature. Since lichenoid lesions often are reported as oral contact reactions, a scoring system for measuring OLP seemed appropriate to assess the outcome of the use test. The scoring system does not exclude other diagnoses as long as they express hyperkeratosis, erythema and/or ulceration. As signs and symptoms of OLP often vary over time, one possible flaw with the oral use test and the assessment of it is the difficulty of discriminating true allergic reactions from normal fluctuations of OLP.

In over half of the allergic subjects with OL, the oral lesions were bilateral, imitating classical OLP. Even though these subjects may have both OLP and contact allergy at the same time, the distribution of allergen when brushing with flavoured toothpaste exposes the oral cavity completely. Hence, contact lesions from toothpaste or any other soluble allergen are most probably bilateral and may imitate OLP. As oral contact allergy is traditionally associated with dental restorative materials, i.e. metals and composites, allergic patients with clinical features of OLP are at risk of being undiagnosed, and patch testing for potential soluble antigens might therefore be indicated in cases of therapeutic failure.

REFERENCES

1. Ahlgren C, Axell T, Möller H et al. Contact allergies to potential allergens in patients with oral lichen lesions. *Clin Oral Investig* 2013; **18**: 227-237.
2. Karlberg AT, Magnusson K, Nilsson U. Air oxidation of d-limonene (the citrus solvent) creates potent allergens. *Contact Dermatitis* 1992; **26**: 332-340.
3. Kroona L, Warfvinge G, Isaksson M et al. Quantification of l-carvone in toothpastes available on the Swedish market. *Contact Dermatitis* 2017; **77**: 224-230.
4. Paulsen E, Andersen KE, Carlsen L, Egsgaard H. Carvone: an overlooked contact allergen cross-reacting with sesquiterpene lactones? *Contact Dermatitis* 1993; **29**: 138-143.
5. Spiewak R, Samochocki Z, Grubska-Suchanek E et al. ESCD Abstracts: Posters, P075: Gallates, as well as hydroperoxides of limonene and linalool, are more frequent and relevant sensitizers than any cosmetic ingredient included in the European Baseline Series. *Contact Dermatitis* 2016; **75** (S1): 87.
6. Kroona L, Isaksson M, Ahlgren C et al. Carvone Contact Allergy in Southern Sweden: A 21-year Retrospective Study. *Acta Derm Venereol* 2018; .
7. Khamaysi Z, Bergman R, Weltfriend S. Positive patch test reactions to allergens of the dental series and the relation to the clinical presentations. *Contact Dermatitis* 2006; **55**: 216-218.
8. Clayton R, Orton D. Contact allergy to spearmint oil in a patient with oral lichen planus. *Contact Dermatitis* 2004; **51**: 314-315.
9. Isaac-Renton M, Li MK, Parsons LM. Cinnamon spice and everything not nice: many features of intraoral allergy to cinnamic aldehyde. *Dermatitis* 2015; **26**: 116-121.
10. Bakula A, Lugovic-Mihic L, Situm M et al. Contact allergy in the mouth: diversity of clinical presentations and diagnosis of common allergens relevant to dental practice. *Acta Clin Croat* 2011; **50**: 553-561.
11. Axell T, Rundquist L. Oral lichen planus--a demographic study. *Community Dent Oral Epidemiol* 1987; **15**: 52-56.
12. Kurago ZB. Etiology and pathogenesis of oral lichen planus: an overview. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2016; **122**: 72-80.
13. van der Waal I. Oral lichen planus and oral lichenoid lesions; a critical appraisal with emphasis on the diagnostic aspects. *Med Oral Patol Oral Cir Bucal* 2009; **14**: E310-4.
14. van der Meij EH, van der Waal I. Lack of clinicopathologic correlation in the diagnosis of oral lichen planus based on the presently available diagnostic criteria and suggestions for modifications. *J Oral Pathol Med* 2003; **32**: 507-512.
15. Andersen KE. Contact allergy to toothpaste flavors. *Contact Dermatitis* 1978; **4**: 195-198.
16. Bonamonte D, Mundo L, Daddabbo M, Foti C. Allergic contact dermatitis from *Mentha spicata* (spearmint). *Contact Dermatitis* 2001; **45**: 298.
17. Francalanci S, Sertoli A, Giorgini S et al. Multicentre study of allergic contact cheilitis from toothpastes. *Contact Dermatitis* 2000; **43**: 216-222.
18. Skrebova N, Brocks K, Karlsmark T. Allergic contact cheilitis from spearmint oil. *Contact Dermatitis* 1998; **39**: 35.
19. Hannuksela M, Salo H. The repeated open application test (ROAT). *Contact Dermatitis* 1986; **14**: 221-227.
20. Fregert S, Group ICDR, Group NACD, (eds.) Manual of Contact Dermatitis. 2nd edition, Copenhagen: Munksgaard: 1981. Fregert S., (ed.) Manual of Contact Dermatitis on Behalf of the International Contact Dermatitis Research Group and the North American Contact Dermatitis Group.
21. Escudier M, Ahmed N, Shirlaw P et al. A scoring system for mucosal disease severity with special reference to oral lichen planus. *Br J Dermatol* 2007; **157**: 765-770.
22. Williamson A, Hoggart B. Pain: a review of three commonly used pain rating scales. *J Clin Nurs* 2005; **14**: 798-804.
23. Slade GD, Spencer AJ. Development and evaluation of the Oral Health Impact Profile. *Community Dent Health* 1994; **11**: 3-11.

24. Ni Riordain R, Shirlaw P, Alajbeg I et al. World Workshop on Oral Medicine VI: Patient-reported outcome measures and oral mucosal disease: current status and future direction. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2015; **120**: 152-60.e11.
25. Larsson P, List T, Lundstrom I et al. Reliability and validity of a Swedish version of the Oral Health Impact Profile (OHIP-S). *Acta Odontol Scand* 2004; **62**: 147-152.
26. John MT, Reissmann DR, Feuerstahler L et al. Exploratory factor analysis of the Oral Health Impact Profile. *J Oral Rehabil* 2014; **41**: 635-643.
27. John MT, Feuerstahler L, Waller N et al. Confirmatory factor analysis of the Oral Health Impact Profile. *J Oral Rehabil* 2014; **41**: 644-652.
28. Uter W, Geier J, Pfahlberg A, Effendy I. The spectrum of contact allergy in elderly patients with and without lower leg dermatitis. *Dermatology* 2002; **204**: 266-272.
29. Schäfer T, Böhler E, Ruhdorfer S et al. Epidemiology of contact allergy in adults. *Allergy* 2001; **56**: 1192-1196.
30. Prakash AV, Davis MD. Contact dermatitis in older adults: a review of the literature. *Am J Clin Dermatol* 2010; **11**: 373-381.
31. Wöhrl S, Hemmer W, Focke M et al. Patch testing in children, adults, and the elderly: influence of age and sex on sensitization patterns. *Pediatr Dermatol* 2003; **20**: 119-123.
32. Piaserico S, Larese F, Recchia GP et al. Allergic contact sensitivity in elderly patients. *Aging Clin Exp Res* 2004; **16**: 221-225.
33. International Fragrance Association. L-carvone: 6485-40-1, 2009: <http://www.ifraorg.org/en-us/standards>. Updated 2009. Accessed 2016 Aug 15.
34. McLelland J, Shuster S, Matthews JN. 'Irritants' increase the response to an allergen in allergic contact dermatitis. *Arch Dermatol* 1991; **127**: 1016-1019.
35. Johansen JD, Bruze M, Andersen KE et al. The repeated open application test: suggestions for a scale of evaluation. *Contact Dermatitis* 1998; **39**: 95-96.

IV

ABSTRACT

Background. The mucosal reaction in the presumably auto-immune disease oral lichen planus (OLP) is histologically very similar to oral allergic contact reactions. Contact allergy to the mint tasting flavour carvone (l-carvone) is common among patients with oral lesions such as OLP. In reverse, patients with contact allergy to carvone (CA) also often have OLP and it is therefore possible that contact allergy may be mistaken for OLP leading to inadequate treatment.

Objective. To investigate the immune reaction and immune cell profile in the oral mucosa in individuals with OLP compared to individuals with CA.

Methods. The inflammatory pattern and the immune expression were analysed in mucosal biopsies from subjects with OLP or CA. The expression of CD1a, CD3, CD4, CD8, CD11c, CD20, CD68, CD123, CD138 and MxA was immunohistochemically evaluated

Results. A lichenoid reaction with band-like lymphocytic infiltrates was seen in both OLP and CA but the infiltrates in CA were more cluster-like. The sub-epithelial expression of CD1a (Langerhans cells) was higher in OLP. All other markers were similar in OLP and CA.

Conclusion. Despite different aetiologies, the inflammatory patterns in lesions of OLP and CA are very similar differing only by means of an increased number of Langerhans cells in OLP.

INTRODUCTION

Contact allergy to the mint-tasting flavour carvone is more common in patients with oral lichen planus (OLP) or lichenoid lesions (OLL) than in other patch tested populations (1). Still, we do not know if the allergy is manifesting as lichenoid lesions or if the lesions of oral lichen planus facilitate sensitisation to carvone.

Dental hygienic products are often flavoured with different mint oils for a refreshing feel and to disguise the taste of active ingredients (2). The flavour carvone (l-carvone) is the main constituent of spearmint oil (3) and can be found in most toothpastes (4). Carvone is a mild skin sensitiser with a sensitisation rate between 1.6–3.5% in patch tested populations (5,6). We recently found that over 50 % of patients with a positive patch test to carvone had OLL or OLP (7).

OLP is a chronic inflammatory mucosal disease with unknown aetiology that affects 1–2% of the population. The disease may present at any age and in both sexes, but is most often seen in postmenopausal women (8,9). Clinically, OLP often has a bilateral, more or less symmetrical pattern. The lesions range from white plaques or reticular striae (classical appearance) to atrophic and erythematous lesions. Lesions of OLL are essentially identical to OLP but have either a known aetiology, such as contact allergy, or have an atypical distribution in the oral cavity e.g. unilateral lesions (10,11).

Histologically, both OLP and OLL present with a lichenoid reaction pattern where pathological components such as hyperkeratosis, keratinocyte liquefaction degeneration, basal cell apoptosis and a band-like infiltrate of lymphocytes can be seen (12). Attempts have been made to distinguish OLL from OLP by routine-stained sections, without much success (13,14).

Although several etiological factors have been discussed, there is evidence that OLP represents an autoimmune process where auto-reactive T-cells induce apoptosis of the basal cells of the oral epithelium (15).

The process is Th1-driven and the recruitment of cytotoxic T-cells is stimulated by Th1-cytokines (16,17). Accumulation of plasmacytoid dendritic cells (pDC) has been shown to play a role in several autoimmune skin diseases, including OLP, by upregulating a Th2-driven immune response through the production of different cytokines, mainly type I interferons (18,19). Although type I interferons are produced by virally infected cells, pDCs are the main producers (20). MxA protein is one of several proteins expressed in type I interferon-exposed cells. Besides expression in acute viral skin lesions, the protein is also found in inflammatory skin lesions such as lichen planus, lupus erythematosus and psoriasis but not in eczematous, urticarial or bullous dermatoses (21).

Manifestations of contact allergy in the oral mucosa have most commonly been described as lichenoid contact reactions to mercury in amalgam fillings (a variant of OLL) but

there are also several other non-lichenoid lesions reported (22-25). Similarly on skin, allergic contact dermatitis (ACD) may present with a range of clinical appearances. (26). In lesions of ACD, inflammatory infiltrates comprising lymphocytes, dendritic cells and macrophages, gather in the upper dermis around vessels. The composition of cells and to some extent the distribution of the infiltrates resembles what is seen in oral lichenoid reactions, especially in chronic ACD. The immune response in contact allergy is a delayed type hypersensitivity reaction and similar to OLP, the T-cell response is considered to be Th1- or Th17-driven (27). However, contact allergens are far from uniform and may trigger different types of immune responses. Dhingra et al (28) investigated the immune response to different allergens in human biopsies; IHC staining for different sub-types of T-lymphocytes and dendritic cells as well as gene-expression studies revealed differences in the immune response between the allergens nickel, fragrance and rubber. Fragrance had a more Th2-driven response compared to nickel which had a Th1/Th17 dominated response.

Despite many similarities between OLP and ACD the immune responses might differ, especially considering that carvone is a fragrance allergen. We have recently shown that individuals with contact allergy to carvone that were exposed to carvone in toothpaste reacted with more lichenoid lesions or perioral eczema compared to controls. With the aim to find means to distinguish oral contact allergy from non-allergic OLP this paper investigates the immune cell expression in the oral mucosa in individuals with OLP and in individuals with contact allergy to carvone.

MATERIAL AND METHOD

Tissue samples

The experimental design of this study was approved by The Ethics Committee of the Swedish Southern Health Care Region. The study samples came from a clinical use test study on carvone contact allergy, OLP and OLL. Participants with or without contact allergy to carvone were instructed to use toothpaste containing 1% carvone during a month's time and were subsequently evaluated and biopsied. The allergic participants receiving carvone toothpaste reacted with more lichenoid lesions or perioral eczema compared to controls (Kroona L, *et.al. Use test with l-Carvone in toothpaste on sensitised individuals – manuscript*). Twenty-eight participants were selected for this study. Eleven participants were clinically classified as having OLP and had no contact allergy to carvone or to any other clinically relevant allergen. These participants used toothpaste containing 1% carvone and are referred here to as OLP-C. Fourteen participants had contact allergy to carvone and all but one had some form of clinical oral lichen (OL), either OLP or OLL. Nine of these used toothpaste with 1% carvone (referred to as CAOL-C) and five used toothpaste with no flavour (referred to as CAOL-NF) of which one had no OL. Three participants were controls with no contact allergy and no OL. They had normal mucosa and used toothpaste with carvone (referred to as NM-C). Incisional biopsies were taken at the end of the use test or when the use test was terminated ahead of time. Punch biopsies, 6 mm Ø, were taken in the buccal mucosa in an area with clinically evident inflammation or lichenoid lesion if possible.

Immunohistochemistry

Immunohistochemistry was performed on 3-µm-thick serial sections of formalin-fixed paraffin-embedded tissues. Antibodies and their characteristics are listed in Table 1. For analysis of anti- CD1a, CD3, CD4, CD8, CD20, CD68 and CD138, the tissue sections were deparaffinised and rehydrated prior to heat-induced antigen retrieval (HIAR) in TRIS-EGTA buffer, pH 9.0 at 95°C for 40 min in a Decloaking Chamber™ (NxGen, Biocare Medical, Concord, CA, USA). After cooling and washing the sections in TRIS-buffered saline with 0.1% Tween 20, pH 7.6 (TBS-T), the endogenous peroxidase was blocked for 5 min with peroxidase blocking reagent (HPBK; S2023; DAKO, Glostrup, Denmark) followed by block of the nonspecific background staining with background punisher (M4U534L; Biocare Medical, Concord, CA, USA) for 10 min at room temperature. The sections were then incubated for 30 min with primary antibodies

Antibody	Clone	Target cells	Source	IHC dilution
CD1a M3571	Monoclonal Mouse anti-Human, clone 010	Langerhans cells	DAKO, Glostrup, Denmark	1:400
CD3 A0452	Polyclonal Rabbit anti-Human	Peripheral T-cells	DAKO, Glostrup, Denmark	1:500
CD4 ab133616	Monoclonal Rabbit anti-Human	Helper T-cells	Abcam, Cambridge, UK	1:2000
CD8 M7103	Monoclonal Mouse anti-Human, clone C8/144B	Cytotoxic T-cells	DAKO, Glostrup, Denmark	1:400
CD11c 111M	Monoclonal Mouse anti-Human, clone 5D11	Myeloid dendritic cells Macrophages	Sigma-Aldrich Sweden AB	1:200
CD20cy M0755	Monoclonal Mouse anti-Human, clone L26	B-cells	DAKO, Glostrup, Denmark	1:2000
CD68 M0814	Monoclonal Mouse anti-Human, clone KP1	Macrophages	DAKO, Glostrup, Denmark	1:8000
CD123 554527	Monoclonal Mouse anti-Human, clone 7G3	Plasmacytoid dendritic cells	BD Biosciences, San Jose, CA, USA	1:400
CD138 M7228	Monoclonal Mouse anti-Human, clone MI15	Plasma cells (B-cells)	DAKO, Glostrup, Denmark	1:800
MxA MABF938	Monoclonal Mouse anti-Human, clone CL143	Plasmacytoid dendritic cells	Merck Millipore, Darmstadt, Germany	1:4000

Table 1. Antibodies used for immunohistochemical staining

diluted in antibody diluent (S2022; DAKO, Glostrup, Denmark) at optimal dilutions obtained by serial dilution experiments (Table 1). Next, the sections were incubated with secondary antibody (goat anti-rabbit/horseradish peroxidase (HRP); MACH4; M4U534L; Biocare Medical, Concord, CA, USA) for 30 min at room temperature. For mouse antibodies (CD1a, CD8, CD20, CD68 and CD138) the sections were incubated for 15 min with Mouse Probe (M4U534L; Biocare Medical, Concord, CA, USA) prior to incubation with secondary antibody. The immunoreaction was then visualized with diaminobenzidine (DAB; MACH4; M4U534L; Biocare Medical, Concord, CA, USA) for 5 min. The tissue sections were counterstained with haematoxylin, dehydrated, and mounted. After each step, slides were washed with TBS-T.

For analysis of anti-CD11c, CD123, and MxA, HIAR was performed in 10 mM citrate buffer, pH 6.0 at 95°C for 40 min. After cooling and washing with TBS-T, sections were incubated for 10 min with HPBK followed by background punisher for 10 min at room temperature. At optimal dilutions (Table 1), sections were incubated with primary antibody over night at 4°C followed by Mouse Probe for 15 min prior to incubation with secondary antibody for 30 minutes.

The immunoreaction was then visualized with DAB for 5 min and counterstained with haematoxylin, dehydrated, and mounted. N-Universal negative control mouse (N1698;

DAKO, Glostrup, Denmark), and N-Universal negative control rabbit (N1699; DAKO, Glostrup, Denmark) were the negative control.

Evaluation of immunohistochemistry

Two of the authors (Z.P. and L.K.) evaluated the staining patterns quantitatively and semi-quantitatively. Sections were scanned with Nano Zoomer histology scanner (Hamamatsu Photonics K. K., Japan) using a 40x objective. For each sample, an area of interest, approximately 2 mm across the surface, was chosen according to the presence inflammatory infiltrates. Three areas in 40x magnification were subsequently chosen in the epithelial-connective tissue junction, one at each border and one centrally in the area of interest (figure 1A). The selected areas were used throughout the serial tissue sections for quantification of positive immunostaining. Immunostaining of CD3, CD4, CD8 and CD68 was automatically evaluated by IHC profiler (National Institutes of Health, Bethesda, MD, USA)(29) while evaluation of CD11c, CD123, CD20 and CD138 was performed by manual counting of immunostained cells in both epithelium and connective tissue. CD1a-positive immunostained cells were evaluated only in the connective tissue while MxA was evaluated both by IHC profiler and by counting immunostained cells in connective tissue. Cut-off values for IHC profiler were adjusted to define immunostaining as negative = 0, weak positive = 1, positive = 2 and strong positive = 3. Cut-off values for the quantitatively assessed antibodies (CD1a, CD11c, CD20, CD123, CD138 and MxA) were set as positive if >5 cells showed immunostaining. Mean values of positively immunostained cells were retrieved from the three areas investigated by the two observers.

Statistical analysis

The immunohistochemical data was statistically analysed with SPSS Statistics 24.0 software (IBM, Armonk, NY, USA). The differences in expression of CD1a, CD3, CD4, CD8, CD11c, CD20, CD68, CD123, CD138 and MxA in the studied groups were evaluated by Mann-Whitney test. The OLP-C group was compared to carvone allergic participants receiving carvone (OLP-C vs. CAOL-C). Carvone allergic participants receiving carvone toothpaste were compared to carvone allergic participants receiving non-flavoured toothpaste (CAOL-C vs. CAOL-NF).

The findings from the NM-C group were not used in the statistical analysis.

RESULTS

Histological findings

Hematoxylin-eosin staining of tissue sections in the OLP-C group showed lichenoid reaction in all cases with distinct subepithelial band-like lymphocytic infiltrates. A majority (7/9) of the tissue sections from CAOL-C group showed lichenoid reaction however the lymphocytic infiltrates were less band-like but rather arranged as rounded lymphocytic infiltrates. A weak lichenoid reaction or a weak unspecific inflammation was observed in 3 out of 5 tissue sections from CAOL-NF group (Figure 1).

Figure 1 – Hematoxylin-eosin-stained sections of study groups. A) Overview of a tissue section with the areas of interest indicated by squares. B-D) examples of lichenoid reaction in B) OLP-C, C) CAOL-C and D) CAOL-NF. Scale bar indicate 500 μ m.

Characterisation of infiltrating cells

In the OLP-C group, the band-like infiltrates predominantly stained for T-cell markers CD3, CD4 and CD8, Type I interferon marker MxA, macrophage markers CD68 and CD11c and B-cell marker CD20. Less present were Langerhans cell marker CD1a, plasmacytoid DC marker CD123 and plasma cell marker CD138. A similar pattern was observed in study group CAOL-C. However, a difference was seen between the groups in expression of Langerhans cell marker CD1a and plasma cell marker CD138 (Figure 2 and 3). There was a statistically significant increase in the expression of CD1a in OLP-C compared to CAOL-C ($p = 0.038$). Less than five CD138 positive cells were observed in the tissue sections of the CAOL-C group while 45% of the OLP-C group had more than

five cells present per tissue sample, however the difference was not statistically significant ($p = 0.067$). The main difference was observed between CAOL-NF compared to CAOL-C (Figure 2 and 3). There was a statistically significant decrease in the expression of all markers except for CD1a and CD138 in CAOL-NF compared to CAOL-C.

Figure 3 – Representative sections from the different study groups. IHC staining for CD1a (A-D), CD11c (E-H), CD68 (I-L), CD123 (M-P) and MxA (Q-T). Objective magnification 40x.

DISCUSSION

We here describe for the first time the immunohistopathological features of mucosal contact allergy to carvone and compare them with biopsies from participants with OLP as well as carvone allergic participants not exposed to carvone.

In the clinical use test study preceding the biopsies taken for this study, carvone contact allergic patients who received carvone had more symptoms, had a higher mucosal index i.e. more active lichenoid lesions and had a reduced quality of life-score compared to patients with only OL and to allergic participants receiving non-flavoured toothpaste. Still, no major differences in the different immunostainings were seen between OLP-C and CAOL-C. All allergic participants who were triggered by carvone had OL and it is possible that the superimposed lichenoid reaction conceals any contact allergic reaction. CD1a positive cells in the connective tissue were more prevalent in the OLP-C group than in CAOL-C. Similar findings were reported by Souto et al (30) where OLP had higher quantities of CD1a+ dendritic cells in the sub-epithelial connective tissue compared to specimens of amalgam lichenoid reactions. Seemingly contradictory findings were reported by Gueiros et al (31) where no differences were seen between lesions of OLP and OLL, however, these OLL-lesions were not defined as contact allergic reactions and the biopsies were taken at two different mucosal sites, either the buccal or lingual mucosa. The features of the sub-epithelial inflammatory infiltrate differed slightly between OLP-C and CAOL-C. While the OLP-C infiltrates were uniformly band-like the CAOL-C infiltrates had in some cases a more lobular character, often arranged around vessels, indicating an ongoing migration of cells such as seen during the elicitation process of ACD (32).

The expression of several IHC markers differed significantly between CAOL-C and CAOL-NF. CAOL-NF displayed fewer inflammatory cells and the lichenoid component was very discrete. This can be seen as evidence that the CAOL-C group truly reacted to the carvone in the toothpaste. However, the sampling may be biased as the CAOL-NF group was small and one of the participants had no lichenoid lesions. Also, before being exposed to carvone the clinical appearance of the lichenoid reactions in the CAOL-NF group were less intense compared to the CAOL-C group.

Despite the fact that carvone allergic individuals displayed a higher clinical activity of OL in our previous study we could not identify any increased immune expression in those with carvone contact allergy and OL when comparing them to participants with OLP only. Instead, there was an increased expression of CD1a positive cells in participants with OLP. The overall histological and immunohistochemical characteristics for both groups were very similar and the same dilemma can be seen on skin where it is difficult to separate several inflammatory diagnoses solely based on histological features. Instead, clinical signs and patch testing are additional tools necessary for a correct diagnosis (33).

However, the clinical expression of oral carvone contact allergy does not seem to fit in the OLP/OLL dichotomy as a majority of the CAOL-C group had bilateral lichenoid lesions mimicking clinical OLP. Hence, it is possible that some OLP patients may have a non-diagnosed contact allergy although there may not be any clinical or histological suspicions. Clinicians should be made aware of soluble allergens such as carvone and that they may play a role in giving increased symptoms in OLP patients.

REFERENCES

- Ahlgren C, Axell T, Möller H et al. Contact allergies to potential allergens in patients with oral lichen lesions. *Clin Oral Investig* 2013; **18**: 227-237.
- Lippert F. An introduction to toothpaste - its purpose, history and ingredients. *Monogr Oral Sci* 2013; **23**: 1-14.
- Surburg H, Panten J. Ch 3. natural raw materials in the flavor and fragrance industry. In: Common Fragrance and Flavor Materials, 6th edition, Surburg H, Panten J, editors. (eds.): Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA, 2016: 193-264.
- Kroona L, Warfvinge G, Isaksson M et al. Quantification of l-carvone in toothpastes available on the Swedish market. *Contact Dermatitis* 2017; **77**: 224-230.
- Paulsen E, Andersen KE, Carlsen L, Egsgaard H. Carvone: an overlooked contact allergen cross-reacting with sesquiterpene lactones? *Contact Dermatitis* 1993; **29**: 138-143.
- Spiewak R, Samochocki Z, Grubska-Suchanek E et al. ESCD Abstracts: Posters, P075: Gallates, as well as hydroperoxides of limonene and linalool, are more frequent and relevant sensitizers than any cosmetic ingredient included in the European Baseline Series. *Contact Dermatitis* 2016; **75** (S1): 87.
- Kroona L, Isaksson M, Ahlgren C et al. Carvone Contact Allergy in Southern Sweden: A 21-year Retrospective Study. *Acta Derm Venereol* 2018; .
- Axell T, Rundquist L. Oral lichen planus--a demographic study. *Community Dent Oral Epidemiol* 1987; **15**: 52-56.
- Salonen L, Axell T, Hellden L. Occurrence of oral mucosal lesions, the influence of tobacco habits and an estimate of treatment time in an adult Swedish population. *J Oral Pathol Med* 1990; **19**: 170-176.
- van der Waal I. Oral lichen planus and oral lichenoid lesions; a critical appraisal with emphasis on the diagnostic aspects. *Med Oral Patol Oral Cir Bucal* 2009; **14**: E310-4.
- van der Meij EH, van der Waal I. Lack of clinicopathologic correlation in the diagnosis of oral lichen planus based on the presently available diagnostic criteria and suggestions for modifications. *J Oral Pathol Med* 2003; **32**: 507-512.
- Andreasen JO. Oral lichen planus. 1. A clinical evaluation of 115 cases. *Oral Surg Oral Med Oral Pathol* 1968; **25**: 31-42.
- Thornhill MH, Sankar V, Xu XJ et al. The role of histopathological characteristics in distinguishing amalgam-associated oral lichenoid reactions and oral lichen planus. *J Oral Pathol Med* 2006; **35**: 233-240.
- Larsson A, Warfvinge G. The histopathology of oral mucosal lesions associated with amalgam or porcelain-fused-to-metal restorations. *Oral Dis* 1995; **1**: 152-158.
- Kurago ZB. Etiology and pathogenesis of oral lichen planus: an overview. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2016; **122**: 72-80.
- Khan A, Farah CS, Savage NW et al. Th1 cytokines in oral lichen planus. *J Oral Pathol Med* 2003; **32**: 77-83.
- Iijima W, Ohtani H, Nakayama T et al. Infiltrating CD8+ T cells in oral lichen planus predominantly express CCR5 and CXCR3 and carry respective chemokine ligands RANTES/CCL5 and IP-10/CXCL10 in their cytolytic granules: a potential self-recruiting mechanism. *Am J Pathol* 2003; **163**: 261-268.
- Saaddeh D, Kurban M, Abbas O. Update on the role of plasmacytoid dendritic cells in inflammatory/autoimmune skin diseases. *Exp Dermatol* 2016; **25**: 415-421.
- Santoro A, Majorana A, Bardellini E et al. Cytotoxic molecule expression and epithelial cell apoptosis in oral and cutaneous lichen planus. *Am J Clin Pathol* 2004; **121**: 758-764.
- Siegal FP, Kadowaki N, Shodell M et al. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 1999; **284**: 1835-1837.
- Fäh J, Pavlovic J, Burg G. Expression of MxA protein in inflammatory dermatoses. *J Histochem Cytochem* 1995; **43**: 47-52.
- Bakula A, Lugovic-Mihic L, Situm M et al. Contact allergy in the mouth: diversity of clinical presentations and diagnosis of common allergens relevant to dental practice. *Acta Clin Croat* 2011; **50**: 553-561.

23. Clayton R, Orton D. Contact allergy to spearmint oil in a patient with oral lichen planus. *Contact Dermatitis* 2004; **51**: 314-315.
24. Isaac-Renton M, Li MK, Parsons LM. Cinnamon spice and everything not nice: many features of intraoral allergy to cinnamic aldehyde. *Dermatitis* 2015; **26**: 116-121.
25. Khamaysi Z, Bergman R, Weltfreund S. Positive patch test reactions to allergens of the dental series and the relation to the clinical presentations. *Contact Dermatitis* 2006; **55**: 216-218.
26. So JK, Hamstra A, Calame A et al. Another Great Imitator: Allergic Contact Dermatitis Differential Diagnosis, Clues to Diagnosis, Histopathology, and Treatment. *Current Treatment Options in Allergy* 2015; **2**: 333-348.
27. Nosbaum A, Vocanson M, Rozieres A et al. Allergic and irritant contact dermatitis. *Eur J Dermatol* 2009; **19**: 325-332.
28. Dhingra N, Shemer A, Correa da Rosa J et al. Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response. *J Allergy Clin Immunol* 2014; **134**: 362-372.
29. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One* 2014; **9**: e96801.
30. Souto GR, Nunes LF, Tanure BB et al. CD1a+ dendritic cells in oral lichen planus and amalgam lichenoid reaction. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2016; **121**: 651-656.
31. Gueiros LA, Gondak R, Jorge Jr. J et al. Increased number of Langerhans cells in oral lichen planus and oral lichenoid lesions. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology* 2012; **113**: 661-666.
32. Gawkrödger DJ, McVittie E, Carr MM et al. Phenotypic characterization of the early cellular responses in allergic and irritant contact dermatitis. *Clin Exp Immunol* 1986; **66**: 590-598.
33. Frings VG, Boer-Auer A, Breuer K. Histomorphology and Immunophenotype of Eczematous Skin Lesions Revisited-Skin Biopsies Are Not Reliable in Differentiating Allergic Contact Dermatitis, Irritant Contact Dermatitis, and Atopic Dermatitis. *Am J Dermatopathol* 2018; **40**: 7-16.

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