Recurrent Aphthous Stomatitis

A study, with emphasis on host genetics, oral microbiota composition, and immunoregulatory networks

Maria Bankvall

Department of Oral Medicine and Pathology
Institute of Odontology
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2017

Cover illustration:
'The pillars of the thesis', by Lars and Maria Bankvall, including an adaption of *'The RAS model'*, originally designed by Maria Bankvall and Robert Carlsson.

Recurrent Aphthous Stomatitis

maria.bankvall@odontologi.gu.se

Paper II has been published in the *Journal of Oral Microbiology* and permission for reprinting has been obtained from Co-action Publishing. Paper IV has been published in *Immunology* and permission for reprinting has been provided by John Wiley and Sons Ltd.

ISBN 978-91-628-9994-3 (Print)
ISBN 978-91-628-9993-6 (PDF)
http://hdl.handle.net/2077/48668

Printed by Ineko AB, Gothenburg, Sweden 2017

'What we learn with pleasure we never forget'
Albert Mercier

'Recurrent aphthous stomatitis may be considered a condition that is the most tantalizing for the researcher, the most painful for the patient, and the most frustrating for the clinician'
Nihill Henry Somers, 1971

To my family

To those unfortunate enough to suffer from recurrent aphthous stomatitis

TABLE OF CONTENTS

ABSTRACT

Recurrent aphthous stomatitis (RAS) is one of the most common oral mucosal lesions. The aetiology is unknown and currently there is no consensus regarding suitable treatment regimens. RAS is recognised as a multi-factorial condition in which both endogenous and exogenous factors contribute to the recurrent oral ulcerations characteristic of this oral mucosal disease.

The overall aim of this thesis was to study the aetiological factors associated with RAS. Previously, it has been suggested that genetic factors, a microbiological component, and the abrogation of tolerance to specific food antigens are of importance in RAS. Hence, two clinical studies were conducted to explore the roles in RAS of host genetics and the composition of the oral microbiota. To reveal the actions of food components as exogenous triggering factors for RAS, it is necessary to understand the immunoregulatory networks involved in the induction of tolerance in the oral cavity. Extensive pre-clinical studies of these mechanisms are required before translating the acquired knowledge to the clinical setting. Therefore, two pre-clinical studies in mice were performed to explore the roles of the oral cavity and associated lymphoid tissues in comparison to those of the mesenteric lymph nodes (MLN), which are known to be of importance for oral tolerance induction.

The specific aims of the clinical studies were to: *(i)* identify patterns of association and segregation regarding genetic variants passed down to the offspring within families with RAS and to identify the genes and signalling pathways that determine the risk of developing this condition; and *(ii)* compare the oral microbiota profiles of patients with RAS and healthy control subjects, so as to define microbiotal changes in relation to disease activity. The specific aims of the pre-clinical studies were to: *(i)* identify differences between the murine APC and T-cell populations of the oral-associated lymphoid tissues [i.e., the nose-associated lymphoid tissues (NALT) and the cervical lymph nodes (CLN)] and the MLN; and *(ii)* determine whether the passage of an antigen through the oral cavity contributes to the overall immunological response and the degree of tolerance induced, as compared to gastric administration of the same antigen.

Buccal swabs were obtained from non-ulcerative areas of the mouths of patients with RAS (N=60) and healthy age- and gender-matched controls (N=60), with some of the patients (N=42) presenting with lesions upon sampling. Additional swabs from members of 16 families with RAS (N=91) were also included. The human DNA was analysed in a Genome-wide association study (GWAS), using a CoreExome array, and the bacterial DNA was analysed by Terminal-restriction fragment length polymorphism (T-RFLP). Flow cytometry and *in vitro* proliferation were used to analyse the APC and T-cell subsets at the different sites in the BALB/c mice. To compare oral and gastric administration of the antigen (ovalbumin, OVA), a DO11.10 TCR transfer model and an oral tolerance model, using BALB/c mice, were applied.

No pattern of association or segregation for genetic variants being passed down to the offspring within these families was detected. The most significant pathways implicated in RAS were the Ras signalling pathway, the PI3K-Akt signalling pathway, pathways in cancer, circadian entrainment, and the Rap 1 signalling pathway. The oral microbiota profiles differed between patients and controls, especially regarding the profiles of patients who presented with lesions during sampling, which clustered furthest from the profiles of the controls. The NALT contained a higher proportion of APCs and a lower proportion of T cells than the CLN and MLN. The APCs of the NALT displayed few signs of activation, instead showing high-level expression of markers associated with effector and tolerogenic functions. Furthermore, the T cells in the NALT more often showed a memory/effector phenotype, whereas those in the CLN and MLN had a naïve phenotype. In general, the cells of the NALT did not proliferate upon *in vitro* stimulation with concanavalin A, in contrast to the cells from the CLN and MLN. A similar activation pattern and degree of tolerance induction emerged when the two administration routes were compared.

In summary, understanding the genetic basis of RAS may allow the identification of individuals who are at risk of acquiring this condition. Changes to the oral microbiota may trigger the development of lesions or *vice versa*. The NALT displayed effector and tolerogenic functions as opposed to the other sites that demonstrated a strong capacity for primary immune activation. The contribution of the mucosal immune system, besides the intestine, for induction of oral tolerance remains to be further investigated. Suitable and efficient treatment strategies for RAS can be developed only when the aetiology of this condition is fully understood.

Keywords: Aphthous stomatitis, oral mucosa, oral medicine, genome-wide association study, genetic linkage, association, genetic polymorphism, microbiota, restriction fragment length polymorphism, antigen-presenting cell, T-lymphocyte, lymph node, flow cytometry

SAMMANFATTNING PÅ SVENSKA

Recidiverande aftös stomatit (RAS) anses vara en av de vanligast förekommande orala slemhinneförändringarna i världen idag. Trots att tillståndet är så utbrett saknas kunskap om orsakerna bakom och det finns idag inte heller någon formell konsensus kring de behandlingsalternativ som ska erbjudas de drabbade patienterna. RAS är förmodligen ett multifaktoriellt tillstånd där ett flertal både yttre miljöfaktorer och inre kroppsegna faktorer samverkar för att skapa de återkommande sårbildningar i den orala slemhinnan som så tydligt kännetecknar detta tillstånd.

Det huvudsakliga syftet med avhandlingen har varit att förstå mer kring orsakerna bakom RAS. Tidigare har det i litteraturen framhållits en möjlig ärftlighet bakom detta tillstånd, betydelsen av bakteriefloran i munhålan och att specifika födoämnen kan vara av vikt för att utlösa sår genom att det uppstår en bristande toleransutveckling mot vissa födoämnen. Avhandlingen består av två kliniska studier där betydelsen av ärftliga faktorer och bakteriefloran i munhålan undersökts. Dessutom har två prekliniska studier på möss genomförts med fokus på toleransutveckling och munhålans betydelse för denna mekanism. Tidigare har tarmens betydelse för toleransutveckling stått i centrum och munhålan har kommit i skymundan trots att den är en del av mag-tarmkanalen. Eftersom kunskaperna om munhålans och de tillhörande lymfkörtlarnas betydelse är bristande krävs fler prekliniska studier innan det är möjligt att vidare undersöka dessa mekanismer hos människa.

De huvudsakliga frågeställningarna för de kliniska studierna har varit att *(i)* ta reda på om det finns några skillnader i hur olika gener nedärvs från föräldrar till barn i familjer där RAS förekommer och att identifiera vilka gener och signalvägar som kan vara av betydelse för att utveckla det här tillståndet och *(ii)* ta reda på om bakterieprofilen i munhålan skiljer sig åt mellan patienter med RAS och friska kontrollindivider och även att undersöka om det uppstår skillnader hos patienter med RAS då de har sår jämfört med då de inte har sår, dvs. om vissa bakterier är kopplade till sjukdomsaktiviteten. De huvudsakliga frågeställningarna för de prekliniska studierna har varit att *(i)* undersöka möjliga skillnader i förekomsten och typen av immunceller i lymfvävnaderna i anslutning till munhålan (näs-associerad lymfoid vävnad, NALT och cervikala lymfnoder, CLN) jämfört med lymfvävnaden i anslutning till magen (mesenteriska lymfnoder, MLN) och *(ii)* ta reda på om det spelar någon roll för det immunologiska svaret och graden av toleransutveckling om ett födoämne tillförs mag-tarmkanalen genom att först passera munhålan och sedan magen eller om födoämnet tillförs magen direkt utan att först passera munhålan.

För de kliniska studierna samlades slemhinnestryk från kinden in, från patienter med RAS (N=60), och från kontrollindivider av samma ålder och kön (N=60). I patientgruppen uppvisade 42 individer sår vid provtagningstillfället men inte i anslutning till provtagningsområdet utan någon annanstans i munhålan. Slemhinnestryk från kinden togs också från 16 familjer där RAS förekom (N=91). Resultaten av de kliniska studierna visade att det inte verkar finnas några skillnader i hur gener nedärvs som är av betydelse för utvecklandet av det här tillståndet. RAS förefaller inte heller vara en monogen sjukdom dvs. orsakas av en enda gen utan är ett resultat av åtskilliga gener som samverkar. Ett flertal gener och signalvägar kunde också hittas som verkar vara av betydelse för att utveckla RAS. Gällande bakterieprofilen i munhålan så skiljde den sig åt mellan patienter med RAS och friska kontrollindivider. Skillnaderna var som störst då patienterna hade sår jämfört med när de inte hade sår.

För de prekliniska studierna samlades lymfoida vävnader in från de olika lokalerna från en experimentellt avlad stam av den vanliga husmusen (BALB/c mus) för att undersöka immuncellernas egenskaper och funktion. Vidare jämfördes det immunologiska svaret och graden av toleransutveckling genom att mata ett födoämnesprotein oralt jämfört med att föra ner det direkt i magen. För de här experimenten användes en genmodifierad musstam (DO11.10 TCR möss) samt BALB/c möss. Resultaten av de prekliniska studierna visade att det fanns skillnader i förekomst och funktion av immunceller och att skillnaderna var som störst för NALT jämfört med de andra lymfvävnaderna. Inga betydande skillnader kunde noteras i det immunologiska svaret eller i graden av toleransutveckling när de två olika sätten att tillföra ett födoämne jämfördes. Munhålans betydelse för utvecklandet av tolerans är därför fortfarande oklar.

Sammanfattningsvis är det av yttersta vikt att förstå mer kring orsakerna bakom RAS genom att studera de olika faktorerna var för sig men också att undersöka hur de samverkar för att kunna hitta lämpliga behandlingsstrategier och att kunna identifiera de patienter som riskerar att utveckla detta tillstånd.

PREFACE

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

I. **Bankvall M**, Östman S, Jontell M, Torinsson-Naluai Å. A genome-wide association study of recurrent aphthous stomatitis. *In manuscript*

II. **Bankvall M**, Sjöberg F, Gale G, Wold A, Jontell M, Östman S. The oral microbiota of patients with recurrent aphthous stomatitis. *J Oral Microbiol*. 2014 Oct 29;6:25739.

III. **Bankvall M**, Jontell M, Wold A, Östman S. Tissue-specific differences in immune cell subsets located in the oral-associated lymphoid tissues. *In manuscript*

IV. **Bankvall M**, Östberg AK, Jontell M, Wold A, Östman S. The engagement of oral-associated lymphoid tissues during oral versus gastric antigen administration. *Immunology*. 2016 Sep;149(1):98-110.

ABBREVIATIONS

Thesis Frame

IEL	Intraepithelial Lymphocyte
RAS	Recurrent Aphthous Stomatitis

Paper I

bp	Base pair
dFAM	Family-based association test
DNA	Deoxyribonucleic Acid
FDR	False Discovery Rate
GWAS	Genome-Wide Association Study
HLA	Human Leukocyte Antigen
IL	Interleukin
ORA	Over-Representation Analysis
RNA	Ribonucleic Acid
SNP	Single-Nucleotide Polymorphism
TDT	Transmission Disequilibrium Test
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor

Paper II

PCR	Polymerase Chain Reaction
PLS	Partial Least Squares Regression
PLS-DA	Partial Least Squares-Discriminant Analysis
rRNA	Ribosomal Ribonucleic Acid
T-RF	Terminal-Restriction Fragment
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
VIP	Variable Influence of Projection

Paper III

Ab	Antibody
APC	Antigen-Presenting Cell
α4β7	Alpha-4 Beta-7 Integrin – Lymphocyte Peyer´s Patch Adhesion Molecule (LPAM)
CCR	Chemokine Receptor
CD	Cluster of Differentiation
CLN	Cervical Lymph Node
Con A	Concanavalin A
CX3CR1	Chemokine (C-X3-Cmotif) Receptor 1
cDC	Conventional Dendritic Cell

DC	Dendritic Cell
FAE	Follicular-Associated Epithelium
FCS	Foetal Calf Serum
FoxP3	Forkhead Box P3
GALT	Gut-Associated Lymphoid Tissue
GARP	Glycoprotein A Repetitions Predominant
HEV	High Endothelial Venule
IFN-γ	Interferon Gamma
ILF	Isolated Lymphoid Follicle
IL-10	Interleukin-10
LAP	Latency Associated Peptide
LN	Lymph Node
LP	Lamina Propria
mAb	Monoclonal Antibody
MADCAM-1	Mucosal Addressin Cell Adhesion Molecule 1
MALT	Mucosal-Associated Lymphoid Tissue
M cell	Micro-Fold Cell
MLN	Mesenteric Lymph Node
NALT	Nose-Associated Lymphoid Tissue
PLN	Peripheral Lymph Node
PNAd	Peripheral Node Addressin
PP	Peyer´s Patch
PSGL-1	P-Selectin Glycoprotein Ligand 1
pTreg	Peripherally-Derived Regulatory T Cell
pDC	Plasmacytoid DC
SLIT	Sublingual Immunotherapy
TCR	T-Cell Receptor
Treg	Regulatory T Cell
tTreg	Thymus-Derived Regulatory T Cell

Paper IV

Alum	Aluminium Hydroxide
BAL	Broncho-Alveolar Lavage
Ig	Immunoglobulin
OVA	Ovalbumin
PBS	Phosphate-Buffered Saline
TMB	3,3´,5,5´-Tetramethylbenzidine Substrate

DEFINITIONS IN BRIEF

Thesis Frame

Aetiology — The origin of a disease involving the agents that trigger the disease.

Histopathology — Microscopic examination of a tissue to study the manifestations of a disease.

Immunopathogenesis — The development of a disease, emphasising the chain of events and focusing on the immunological mechanism/mechanisms that leads to the diseased state.

Major RAS (mRAS) — Lesions of RAS with a diameter >10 mm.

Minor RAS (miRAS) — Lesions of RAS with a diameter <10 mm.

Pathogenesis — The development of a disease, emphasising the chain of events and focusing on the biological mechanism/mechanisms that leads to the diseased state.

Paper I

Association analysis — This type of analysis involves comparing the frequency of a particular genetic variant between cases and controls, where the controls can be selected from a random population or from the same family as the case, to determine whether the variant is inherited more often in those who are affected in a population than in those who are not affected.

Call-rate — The proportion of genotypes of an SNP with non-missing data.

Candidate gene study — A hypothesis-based study design in which particular genes that are putatively involved in a particular disease are studied, either due to their location in a region of linkage or based on evidence that they might be involved in the particular disease being studied.

Dominant — Genes that influence the phenotype both in the homozygous and the heterozygous state.

Extended family — A family unit that extends beyond the nuclear family to include other relatives, such as aunts, uncles, and grandparents.

Genetic variation	Genetic differences both within and among populations caused by subtle differences in the human DNA sequence. These mutations lead to a permanent change in the chemical structure of a gene. Single nucleotide polymorphisms (SNP) are the most common types of genetic variation in humans. These variations are what make every human unique. Evolution relies on genetic changes being passed from one generation to the next.
GWAS	A study design that has no initial hypothesis regarding the genes that might be involved in the disease. Thousands of genetic variants are analysed simultaneously, and this approach has proved to be particularly successful in elucidating the genetic bases of common diseases and complex traits.
Linkage analysis	The analysis method traditionally used to identify genes of importance for disease, which has been used successfully for mapping genes that underlie monogenic Mendelian diseases. SNPs that lie close to the disease gene are identified, as they tend to be inherited together with the disease gene. The transmission and inheritance of this particular chromosomal fragment is then studied within a family.
Mendelian error	This describes an allele (a variant form of the same gene), in an individual, that is supposed to not have been received from either of the person's biological parents by Mendelian inheritance. This implies that the expected parent of an individual would then not actually be the parent indicated. This type of error is often due to a mistake in the genetic analysis, for example, a genotyping error or erroneous assignment of individuals as relatives when in fact they are not. Statistical genetics analysis is used to detect these errors.
Mendelian inheritance	The manner in which traits and diseases related to a single gene are passed down from parents to their children.
Minor Allele Frequency	This concept is used to determine how common or rare an allele is in the population, which is necessary to determine which SNPs in a GWAS that are of importance for the disease, being studied.
Monogenic	Monogenic inheritance occurs when a single gene influences the phenotype.
Nuclear family	A family group that consists only of the father, mother, and children.
Polygenic	Polygenic inheritance occurs when several genes act together to influence the phenotype.

Population stratification — Within a population, the prevalence of a disease may vary due to differences in ethnicity and heredity, which is unrelated to the disease but due to sampling from populations of different ancestries, giving rise to different subgroups with different genetical backgrounds, in which some individuals are more prone than others to a disease. In an association analysis, as opposed to a linkage analysis, this may lead to over-representation of the disease in a proportion of the disease cases where certain alleles in the population suffering from disease would appear to be associated with the disease although they may not have any disease-associated effects, i.e., false-positive results.

Recessive — Genes that influence the phenotype only in the homozygous state.

Segregation — The process whereby pairs of alleles are separated into different gametes (reproductive cells/sex cells, i.e., sperm and egg cells). This occurs during meiosis (a two-part cell division process that produces sex cells that have half the number of chromosomes as the parent cell).

SNP — A variation in a single nucleotide that occurs at a specific position in a genetic sequence, where each variation is present to some appreciable degree within a population.

Paper II

Bacterial taxon — In general, a taxon is used within the field of microbiology to systematically classify all living organisms into a common taxonomic system. There are different levels within this system, such as phylum, class, order, family, genus, and species, with species forming the basic unit.

Diversity — The range of different bacteria that co-exist within a specific bacterial community, described in terms of richness and evenness.

Evenness — The relative number (abundance) of the different species in a bacterial community. In T-RFLP, the area of the peak, i.e., the T-RF, reflects the number of each taxon, where a large area indicates a greater abundance than a small area.

Richness — The total number of species in a bacterial community. In T-RFLP, each peak/T-RF reflects a bacterial taxon.

Paper III

Gut tropism — The process of supplying the gut tissues with appropriate cells of the immune system, whereby the cells move from the inductive sites though a process called homing towards a specific stimulus, for example, an antigen found within the effector sites.

Homing — Cell-surface glycoproteins on lymphocytes and other leukocytes that mediate adhesion to specialised blood vessels, called high endothelial venules (HEVs). Several different classes of lymphocyte homing receptors have been identified, and they appear to target different surface molecules (addressins) on the HEVs in different tissues. This adhesion process plays a crucial role in the trafficking of lymphocytes.

Oral-associated lymphoid tissues — Lymphoid tissues that are located in the region of the oral cavity that encompass the nose-associated lymphoid tissue (NALT) and the cervical lymph nodes (CLN).

Paper IV

Adjuvant — Agents that increase the action of the principal drug or affect the absorption, mechanism of action, metabolism or excretion of the primary drug in such a way as to enhance its effects.

Effector sites — Regions of the mucosal immune system in which effector cells after extravasation, retention, and differentiation perform their actions.

Gastric administration — The feeding of an antigen to an experimental animal using a sterile feeding needle inserted through the oesophagus into the stomach. This is the most common method for achieving oral tolerance in experimental animal models.

Inductive sites — Regions of the mucosal immune system where antigens that are selectively sampled from mucosal surfaces are transported and presented by DCs to naïve T and B cells, resulting in the induction of primary immune responses.

MALT — This is defined as the mucosa-associated lymphoid tissues and comprises the nose-associated lymphoid tissue (NALT), the gut-associated lymphoid tissue (GALT), and the bronchus-associated lymphoid tissue (BALT).

Oral administration	The feeding of an antigen to an experimental animal using a pipette inserted into the oral cavity, mimicking the natural route of an antigen.
Primary lymphatic organs	The sites at which lymphocytes develop (bone marrow and thymus).
Secondary lymphatic organs	The sites at which lymphocytes become activated and immune responses are induced (i.e., the lymph nodes, spleen and MALT).
Waldeyer´s ring	This lymphoid structure is found in humans and is believed to be equivalent to the NALT in mice. It consists of the unpaired nasopharyngeal tonsil (adenoid), the paired palatine tonsils, the unpaired lingual tonsil, and the paired tubule tonsils at the back of the pharynx.

INTRODUCTION

Recurrent aphthous stomatitis (RAS)

This is one of the most common and most challenging categories of oral mucosal lesions managed by clinicians, entailing great discomfort for patients causing problems with eating, drinking, swallowing, and speaking. In the U.S., RAS has even been reported as the most common oral mucosal lesion (Kleinman et al., 1994, Rees & Binnie, 1996). Despite this it is also one of the most poorly understood. RAS has been defined as an inflammatory condition of unknown aetiology that is characterised by painful, recurrent single or multiple ulcerations of the oral mucosa (Graykowski et al., 1966, Francis, 1970, Natah et al., 2004). The lesions may occur as a continuum that extends from occasional single ulcerations resulting in mild symptoms to more continuous episodes with persistent severe ulcerative lesions, causing more severe symptoms over an extended period of time.

Clinical and histopathological characteristics

RAS presents as well-demarcated single or multiple, round or ovoid, shallow, inflammatory ulcers in the oral mucosa, with the surrounding regions being clinically unaffected. These lesions consist of a necrotic centre covered with a yellowish or greyish-white pseudo-membrane surrounded by a raised, thin, erythematous halo (Figure 1). While the ulcers can be extremely painful, they do not bleed unless the pseudo-membrane is scraped off.

Lesions of this type are most commonly found in the non-keratinised oral mucosa, such as the labial and buccal surfaces. They may also present in the soft palate, ventral parts of the tongue, and floor of the mouth. They are rarely found on keratinised surfaces of the mouth, such as the gingiva, hard palate, or dorsum of the tongue. In addition, they are uncommonly found further down in the tonsils, uvula, and oropharynx, although when they are present in these areas they are highly debilitating for the patient and more difficult to treat. The shape of the aphthous lesion reflects its location. Those located on the lip or cheek mucosa are rounded or slightly elongated, whilst those in the vestibule or sulci or on the floor of the mouth can be elongated or linear.

This condition is generally categorised into three different types according to the size and number of lesions present. The most common category is 'minor RAS', which accounts for approximately 80% of all RAS cases with lesions of a diameter <10 mm, followed by the category of 'major RAS', with lesions of a diameter >10 mm (Truelove & Morris-Owen, 1958). The third category of RAS is 'herpetiform RAS', which is the most rare type with a prevalence of approximately 5% with up to a hundred millimetre large lesions covering the oral mucosa (Cooke & Armitage, 1960). The three types of RAS are all classified according to the International Classification of Diseases (ICD) created by the World Health Organization (WHO) with the ICD code K12.0 [which is concerned with recurrent oral aphthae with the sub-definitions of aphthous stomatitis (minor and major), Bednar aphthae, periadenitis mucosa necrotica recurrens, recurrent aphthous ulcers, and stomatitis herpetiformis], (http://apps.who.int/classifications/icd10/browse/2016/en). The ICD codes are used as a diagnostic tool to monitor the incidence and prevalence of diseases and other health problems, providing a picture of the general health situation in countries and populations worldwide.

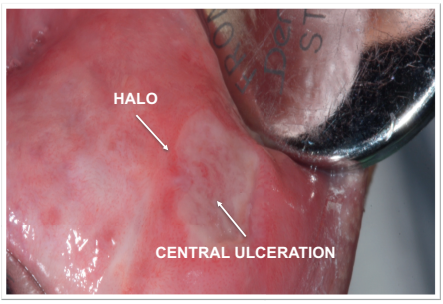

Figure 1. RAS lesion, located on the inside of the lower left lip and revealing the surrounding halo and the central ulceration coated by a pseudo-membrane.

The natural course of RAS can be divided into the following four stages:

- *Premonitory*
- *Pre-ulcerative*
- *Ulcerative*
- *Healing*

In general, the earliest tissue changes observed under the microscope are found within the epithelium. It is during the later stages that an inflammatory infiltrate in the connective tissue signals the development of an aphthous lesion.

Premonitory stage

During this stage, which can last up to 24 hours, many patients sense prodromal symptoms, such as a tingling or burning sensation with hyperaesthesia or pain (Stanley, 1972). The mucosa feels tense and sometimes it feels roughened or raw. No clinical changes are usually apparent. This is the area in which the lesion will subsequently develop.

Since biopsy specimens of true premonitory stages of RAS are difficult to obtain, not much is known about the histopathological characteristics and immunological processes during this stage. Changes are the most apparent in the epithelium where vacuolation of individual supra-basal cells occurs. (Stenman & Heyden, 1980) The underlying connective tissue shows no marked infiltration of inflammatory cells.

Pre-ulcerative stage

During this period, which lasts from 18 hours up to 3 days, the oral mucosa becomes slightly erythematous and a macule or slightly raised papule appears (Stanley, 1972). One nodule or several pin-headed nodules are seen, and with time an induration occurs. Gradually, a superficial membrane or coating emerges and the characteristic erythematous halo develops. At this stage the patient feels moderate pain.

The oral epithelium increases in thickness due to both a slight hyperplasia (an increase in the volume of a tissue due to an increased number of cells) and hypertrophy (an increase in the volume of a tissue due to enlargement of the cells), with extensive distribution of vacuolated keratinocytes throughout the spinous layer, which sometimes coalesce to form minor, intra-epithelial vesicles (Stenman & Heyden, 1980). In addition, slight degeneration of the supra-basal epithelial cells is observed. A minor mononuclear infiltrate of lymphocytes is seen within the epithelium. The connective tissue becomes

slightly oedematous with evidence of dilated capillaries and some accumulation of lymphocytes and plasma cells. At this stage, the number of mononuclear cells infiltrating the lamina propria is not high, and individual cells can easily be distinguished. The ducts and lobules of the minor salivary glands in the area of the developing aphthous lesion may also be surrounded by a mononuclear infiltrate (Stanley, 1972). The surrounding erythematous halo represents a localised vasculitis (Jurge et al., 2006).

Ulcerative stage

This period varies greatly between patients, in the range of 1–16 days. The central part of the superficial membrane undergoes necrosis and is sloughed off, leaving behind a shallow, well-defined ulcer (Stanley, 1972). The necrotic centre is then covered with a yellowish or greyish-white fibrinous pseudo-membrane and the surrounding thin erythematous halo becomes raised. The ulcer continues to enlarge over a couple of days until it reaches its maximum size. Several ulcers may coalesce to form larger lesions of up to 1 cm or more in diameter. During this period, the pain experienced by the patient is severe and greatly debilitating. Once the ulcer has reached its maximum size, it begins to shrink. The covering fibrinous pseudo-membrane now thickens and adheres to the underlying ulcer so as to create a protective barrier (Stanley, 1972). For the patient, this results in a gradual decrease in the level of pain to a feeling of slight discomfort.

This phase has from a histopathological perspective been nicely reviewed by Stanley (1972). An increase in the number of infiltrating immune cells is observed at this point throughout the tissue, especially in the epithelium. The oedema intensifies within the epithelium and the tissue starts to disintegrate upwards from the basal layer and is predominantly infiltrated by mononuclear cells and by some neutrophils. As the ulcer develops, the number of neutrophils increases and gradually they mix with the infiltrating mononuclear cells. There is a loss of the marginal epithelium with a fibrinous pseudo-membrane being formed on top. Subsequently, a purulent exudate accumulates at the junction of the marginal epithelium and the lamina propria, separating the epithelium even more from the underlying tissue. Mononuclear lymphocytes predominate in the connective tissue. Additional neutrophils are confined to the margins, i.e., the breach between the epithelium and the fibrinous pseudo-membrane and occasionally in adjacent tissues where the mononuclear lymphocytes are found. Neutrophils progressively become the dominant cell type, although plasma cells, eosinophils, and mast cells can also be detected. The infiltration of immune cells now becomes so intense that it is difficult to distinguish the architecture of the lamina propria. The infiltration of immune cells may extend deep into the connective tissue down to the underlying skeletal muscle. The number of neutrophils begins to decrease in the peripheral tissues that are distal to the ulceration. Plasma cells become a more prominent feature, and the superficial layers of the connective tissue contain many eosinophils. When the lesion stops expanding the inflammation decreases and numbers of inflammatory cells decline. Epithelial cell death is a key event in the development of ulcerations in RAS. In healthy epithelium, the most superficial cells, which are at the end of their life cycle, are simply desquamated off, as opposed to RAS lesions, which are characterised by top-to-bottom apoptosis such that dead cells slough off, leading to the formation of an ulcer (Al-Samadi et al., 2015).

The area adjacent to the ulcer is defined as the epithelium-covered area extending from the edge of the ulcer and sideways to the periphery of the biopsy. The infiltrate lateral to the ulcer is different from that at the centre of the actual ulceration (Schroeder et al., 1983). An infiltrate that consists predominantly of lymphocytes (outnumbering the neutrophils) is observed in this area (Mills et al., 1980, Hayrinen-Immonen et al., 1991). Monocytes/macrophages are also numerous in the tissues

adjacent and lateral to the ulcer. The number of mast cells is increased in the lamina propria (Lehner, 1969, Schroeder et al., 1984, Natah et al., 1998).

Healing stage
After 4–35 days, usually <21 days, the ulcer heals without clinically observable scar formation. The few lesions that are large and persist for longer periods of time heal with scarring. Granulated tissue is now found under the fibrinous pseudo-membrane (Stanley, 1972). This tissue contains numerous immature capillaries. In the epithelium, numerous proliferating epithelial cells are found. With time, the granulated tissue becomes collagenised, resulting in a decrease of vascularity and further the epithelium heals and becomes intact once again. Cells that mediate chronic inflammation, particularly plasma cells, may persist in the deeper zone of the lamina propria. The minor salivary glands in the area may exhibit minimal focal peri-ductal and peri-alveolar fibrosis, ductal ectasia, and a mild, focal infiltrate of cells that mediate chronic inflammation (Stanley, 1972). Unless the skeletal muscle bundles are damaged, scarring does not occur. Aphthous lesions heal more slowly than other mucosal wounds, presumably due to the massive infiltrate of lymphocytes which requires time to resolve the inflammation (Eversole, 1994).

Historical context

Hippocrates (460–370 BC) was the first known person to use the term 'aphtha', although he may have been referring to thrush rather than specifically aphthous stomatitis (Sircus et al., 1957). Later, in 'The Winter´s Tale' (Paulina, verse 2.2.32) and 'Romeo and Juliet' (Juliet, verse 3.2.90), William Shakespeare (1564–1666), described characters who declared that they would be cursed with blisters of the tongue for speaking the untruth. It could be RAS to which they were referring, since the tongue is a common site for this type of mucosal lesion (Rogers, 1977). The term 'aphthous' is derived from the Greek word 'aphtha', which was first introduced in the mid-17th century and means 'set on fire' (Oxford Dictionaries), which describes very well the burning sensation that patients experience during episodes of aphthous lesions.

In 1778, Jourdain-Berchillet discussed aphthae in considerable detail in his treatise on 'Diseases and Surgical Operations of the Mouth', although once again this may be a description of thrush. The first valid clinical description of RAS appeared in 1898 in a paper published in German by Kümmel and Mikulicz (1898). This was followed by the first description of RAS in the English language by Sibley in 1899. He described what is now recognised as minor RAS and attributed the cause to psychic stress, terming them 'ulcus neuroticum mucosae oris' or 'neurotic ulcers' (Sibley, 1899). In 1911, Sutton presented the first case of what is now known as major RAS and coined the phrase 'periadenitis mucosa necrotica recurrens' (Sutton, 1911). However, careful reading of this article reveals scarce mention of the glandular structures; instead, it states that the 'deeper glands' were hardly affected (Rogers, 1977). The third type of ulcer, the herpetiform type, was first described by Cooke and Armitage (1960), who pointed out their inability to discover a viral pathogen by cytological, serological, cultural or histopathological methods. Even so, they promoted the term 'herpetiform', since it accurately describes the clinical appearance of the lesions.

Epidemiology

In general, the epidemiological literature on oral mucosal lesions is fairly limited, as compared to the literature on dental caries and periodontal disease, the two major diseases of the oral cavity (Kleinman

et al., 1991). For RAS the prevalence varies widely where it has been estimated to be somewhere between 5% and 60% (Ship, 1972, Jurge et al., 2006), with a prevalence of approximately 20% in the general population (Akintoye & Greenberg, 2014). It is important to acknowledge that the different diagnostic criteria adapted at different oral medicine centres around the world may influence the estimated prevalence rates, as might the patient selection criteria (for example, geographic location, age- and gender distribution, genetic disposition, socio-economic status, and exposure to different environmental factors) (Axell & Henricsson, 1985, Shulman, 2004, Rivera-Hidalgo et al., 2004). Moreover, in calculating the prevalence, the time period considered, i.e., point prevalence (presence of lesions upon examination), period prevalence (history of lesions during a specified period of time) or life-time prevalence (presence of lesions during any point in life) (Kleinman et al., 1994, Shulman, 2004, Rivera-Hidalgo et al., 2004), is of importance, as is whether the population studied is selected at random or not (Shulman, 2004, Rivera-Hidalgo et al., 2004).

Axell *et al.* estimated the point prevalence of RAS in 1976 and 1985 as 2.0% in the general population of adults (≥15 years of age) in Sweden and the 2-year prevalence of RAS as 17.7% based on the same study population, including a total of 20,333 individuals (Axell, 1976, Axell & Henricsson, 1985). In 1990, the point prevalence was similarly estimated as 1.9% in another, smaller Swedish adult population (Salonen et al., 1990), whereas in 2013 the point prevalence was estimated as 0.5% (Robledo-Sierra et al., 2013). The results of this last study were, however, based on a non-referral adult Swedish population, who were registered by general dental practitioners, whereas the other studies were based on adults in the general population for whom the diagnosis was established by oral medicine specialists, which may explain in part the variability seen in the first three studies, as compared to the last study.

In the United States, two studies with large cohorts have been conducted in which the prevalences of RAS have been estimated. The first was the National Survey of Oral Health in U.S. school children (OHSC), which was conducted between 1986 and 1987 and involved children and adolescents in the age range of 5–17 years, including a total of 39,206 individuals (Kleinman et al., 1994). The second study was the Third National Health and Nutrition Examination Survey (NHANES III), which was conducted between 1988 and 1994 and focused on civilian, non-institutionalised individuals ≥2 months, and involved clinical examinations of 33,994 individuals. The point prevalence from the OHSC survey was 1.2% and the life-time prevalence was 36.5%. From the NHANES III survey, the point prevalence for RAS was 0.89% based on data from the adult population aged ≥17 years, including a total of 17,235 individuals (Rivera-Hidalgo et al., 2004, Shulman et al., 2004). In subsequent studies based on the data from these two studies, the point prevalences were estimated as 1.2% (OHSC) and 1.5% (NHANES III), the annual prevalence as 20% (NHANES III), and the lifetime prevalence as 40% (OHSC) in children and adolescents (Shulman, 2004), and based on the NHANES III study, the prevalences were estimated as 0.85% in adults and 1.5% in children (Chattopadhyay & Chatterjee, 2007).

The Swedish study carried out by Axell *et al.* (1985) and the American OHSC and NHANES III studies represent large probability samples from the general population. Another large study has also been conducted in which the life-time prevalence was estimated as 38.7% in men and as 49.7% in females (Embil et al., 1975). This study differed from the others in that it was based on populations from six different continents, including 21 countries, whereas the other three studies were based on populations within a single country (i.e., Sweden or the U.S.). Furthermore, the study population was more homogenous, including 635 armed-forces recruits and 9,897 health-profession students, as

opposed to the samples from the general population used in the other studies. In addition, the life-time prevalence was estimated rather than the point prevalence or period prevalence (in the other studies).

Several studies of RAS have reported a trend towards predominance of females (Embil et al., 1975, Miller & Ship, 1977, Axell & Henricsson, 1985), although studies reporting no gender differences (Miller et al., 1980, Kleinman et al., 1994) or a slight predominance of males (Rivera-Hidalgo et al., 2004, Chattopadhyay & Chatterjee, 2007) also exist. The most common period of life to suffer from this condition is before one reaches 40 years of age (Axell & Henricsson, 1985, Ship et al., 2000, Rivera-Hidalgo et al., 2004, Shulman et al., 2004, Chattopadhyay & Chatterjee, 2007), with increases in prevalence with age being observed for children and adolescents (Miller et al., 1980, Kleinman et al., 1994). Furthermore, several studies have shown significantly higher prevalences of RAS for whites than for blacks (Kleinman et al., 1994, Shulman, 2004, Rivera-Hidalgo et al., 2004, Chattopadhyay & Chatterjee, 2007), for non-smokers than for smokers (Rivera-Hidalgo et al., 2004, Chattopadhyay & Chatterjee, 2007), and for people with higher socio-economic status (Ship, 1972, Natah et al., 2004, Jurge et al., 2006, Akintoye & Greenberg, 2014). Interestingly, an association between herpes labialis and RAS has been demonstrated in some studies (Ship, 1972, Embil et al., 1975, Shulman, 2004, Rivera-Hidalgo et al., 2004) but not in others (Chattopadhyay & Chatterjee, 2007).

One problem with studying the prevalence of RAS is that it is greatly under-diagnosed, which renders epidemiological data unreliable. This is mainly due to the fact that there is, as of now, no curative treatment and a lack of consensus regarding the different treatment strategies available. Another limiting factor is that the criteria for the diagnosis of RAS are still relatively vague, which makes it difficult to compare selected study populations with regards to the diagnosis made and the inclusion and exclusion criteria. Furthermore, since RAS shows a recurring pattern, estimates of prevalence are more difficult to access, since over- or under-reporting is not uncommon. Thus, at the time of questioning/examination, the patient may: *(i)* have forgotten that they have had episodes of lesions during the time period in question; *(ii)* not be aware that they actually suffered from the specific condition; *(iii)* believe that they suffered from the condition when in fact they did not; or *(iv)* recall lesions at time-points other than those at which the lesions occurred (Kleinman et al., 1994, Rivera-Hidalgo et al., 2004). In addition, as the rates of allergies and autoimmune diseases have increased over the last decade (Okada et al., 2010), it is tempting to speculate that they may also influence the prevalence of RAS, even though there is currently no evidence to support this linkage.

Terminology

Currently, the terminology used for RAS is undergoing modification and there is still no widely accepted nomenclature for this condition. The categories RAS, aphthous-like ulcerations, and non-aphthous-like ulcerations have been proposed to differentiate between the different types of ulcerations (Scully, 2006, Scully, 2012). More recently, RAS has been suggested to have two presentation forms (simplex and complex aphthosis) and three morphological appearances (minor, major, and herpetiform) (Baccaglini et al., 2011). All three morphological types can be found within the two presentation forms. The differences between the presentation forms and morphological/clinical forms of RAS are illustrated in Table 1 and Figure 2. Complex aphthosis can be further subdivided into primary and secondary groups, where the underlying cause of the primary type remains idiopathic. The causes of secondary complex aphthosis include hematinic deficiencies, various systemic deficiencies and drug reactions (Baccaglini et al., 2011).

It remains a matter of debate as to whether the oral ulcerations associated with these hematinic deficiencies, various systemic deficiencies, and drug reactions, are truly RAS or instead represent oral ulcers that closely resemble RAS. The truth may lie somewhere in the middle, in that some systemic conditions are associated with RAS whilst others are not. Rather than being a single entity, RAS may instead present with different phenotypes, whereby the more common forms display discrete ulcers that typically develop during childhood or adolescence, abate to some degree with age, are not associated with any of yet defined systemic disorder, and may be associated with a positive family history of recurrent oral ulcerations (Scully, 2012). In the absence of a standard nomenclature, it is difficult to define the aetiology of RAS. It is imperative that research groups around the world agree on the terminology to be used for all studies conducted on RAS, to uncover the true aetiology of this condition. It is also important to mention that this issue is complicated by the fact that some patients present with more than one morphological/clinical appearance at any given phase of the disease, and that changes in the presentation form and morphological/clinical appearance may occur over time. For example, a patient who predominantly presents with aphthous lesions of the major type may also suffer from lesions of the minor type, whereas the opposite pattern is rarely observed, i.e., a patient who mainly suffers from lesions of the minor type and who suffers simultaneously from lesions of the major type. Furthermore, a patient who mainly suffers from lesions of the minor type may proceed to suffer from lesions that are predominantly of the major type for a period of time and *vice versa*.

Table 1. Classification of recurrent aphthous stomatitis (RAS).

	Presentation forms of RAS lesions	
	Simplex	*Complex*
Incidence	Common	Uncommon
Frequency	Episodic	Episodic or continuous
Duration	Short-lived lesions	Persistent lesions
Number	Few	Few to many
Recurrence rate	Three to six lesions/year	Frequent or continuous ulcerations
Rate of healing	Rapid	Slow
Grade of pain	Low to moderate	Moderate to severe
Grade of disability	Low to moderate	Moderate to severe
Location	Limited to the oral cavity	May present with genital lesions

	Morphological/Clinical appearances of RAS lesions		
	Minor	*Major*	*Herpetiform*
Size *(mm)*	< 10[a]	>10	1-2[a]
Shape	Round to oval[b]	Round to oval[b]	Round to oval
Number	1–5	1–10	10–100
Location	Non-keratinised mucosa	Non-keratinised mucosa	Any intra-oral site
Duration *(days)*	4–14	>30	<30
Recurrence rates *(months)*	1–4	<1	<1
Evidence of permanent scarring	Uncommon	Common	Uncommon

[a]Larger if coalesced. [b]May appear as crater-forming.

Adapted from: Porter *et al*. Recurrent Aphthous Stomatitis. Crit Rev Oral Biol Med. 1998; 9(3):306-321., Rogers RS III. Complex aphthosis. Adv Exp Med Biol. 2003; 528: 311–316, Baccaglini *et al*. Urban legends: recurrent aphthous stomatitis. Oral Dis. 2011 Nov;17(8):755-70, and Akintoye *et al*. Recurrent Aphthous Stomatitis. Dent Clin North Am. 2014 April; 58(2):281-297.

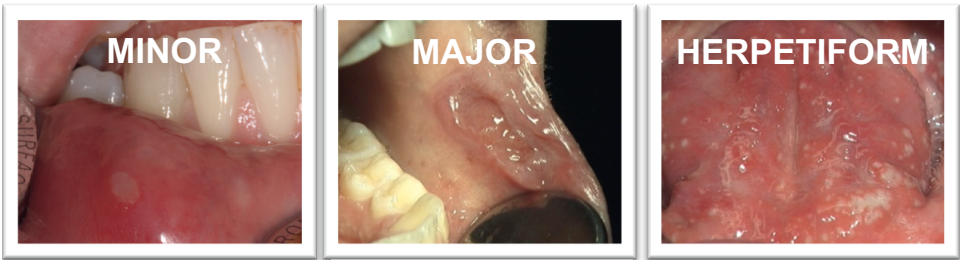

Figure 2. Clinical images illustrating the three morphological/clinical appearances of RAS lesions (minor, major and herpetiform).

Diagnosis

For the diagnosis of RAS, the clinical picture and the history of the patient are of importance. There are currently no clinical tests designed specifically for RAS, and from the histopathological perspective this condition presents as non-specific ulcerations of the oral mucosa, which does not help the clinician or pathologist to establish unambiguously the diagnosis. However, the analysis of a biopsy may help to rule out other potential diagnoses, and there are diagnostic tests available that can be used to exclude other diseases.

There are several differential diagnoses associated with RAS, with the most common one being infection with the herpes simplex virus (HSV). The lesions of these two conditions are normally localised to different areas, with RAS lesions located in the non-keratinised mucosa and herpes lesions being found on keratinised surfaces. Herpes lesions may also in some cases occur only unilaterally, which is not the case for aphthous ulcers. In addition, there is a risk of transmission of the infection for HSV, since there is a viral aetiology. The symptoms of the two conditions are similar once the lesion has been established, with pain and soreness resulting from the ulceration. However, for HSV infection, it is not uncommon for the patient to experience heightened pain 24 hours before the debut of an ulcer, as well as post-herpetic pain. For aphthous lesions, prodromal symptoms can be present, although the severity is not as extensive as is sometimes observed for HSV infection. The simplest way to verify a herpes simplex diagnosis and exclude a diagnosis of RAS is to perform a virus detection test. Moreover, since the aetiologies of the two conditions differ, the strategies for their treatment differ. Interestingly, as previously mentioned in the section *'Epidemiology'*, several reports have indicated an association between RAS and HSV, whereby patients with RAS more commonly present with herpes labialis than do control subjects.
Other recommended differential diagnoses are (Natah et al., 2004, Jurge et al., 2006, Baccaglini et al., 2011, Chavan et al., 2012):

- Behçets syndrome
- Inflammatory bowel diseases (IBD; Crohn´s disease and ulcerative colitis)
- Gluten-sensitive enteropathy
- Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA)
- Hematinic deficiencies
- Cyclic neutropenia and benign familial neutropenia
- Primary and secondary immunodeficiencies, including infection with human immunodeficiency virus (HIV)
- Mouth and genital ulcers with inflamed cartilage (MAGIC syndrome)

- Sweet´s syndrome
- Food hypersensitivity
- Drug reactions

Pathogenesis

Currently, it is unclear if RAS qualifies as a disease *per se* or if it is part of a broad spectrum of underlying systemic disorders (Baccaglini et al., 2011). Clearly, RAS has a multi-factorial aetiology, in that multiple endogenous and exogenous factors contribute to establishing an inflammatory process in the oral mucosa. The particular locations of the ulcerations have baffled researchers for decades. Why do specific lesions occur at particular locations as opposed to a more generalised inflammation of the oral mucosa? The oral cavity provides a range of environments in which ideal milieus occur randomly that favour the development of aphthous lesions in particular (Miller et al., 1977). However, the compositions of these niches are currently unknown.

Aetiology

Despite decades of research, the origin of RAS remains to be uncovered. Traditionally, research on RAS has had a mono-causal approach, which has in part been detrimental, since the aetiologies of many disease entities are known to be multi-factorial. In this aspect, RAS is probably similar. Individuals who are prone to RAS may share certain endogenous features (factors in the oral milieu, in combination with genetic susceptibility and systemic factors) that create immunological dysregulation, which triggers lesion development when the individual is exposed to certain exogenous components (foodstuffs, medications, local trauma, pathogens and/or psychological factors). This hypothesis is presented in greater detail in terms of *'The RAS model'* in the *'Hypothesis'* section of this thesis. Whilst the roles of the oral microbiota composition and host genetics are presented in detail in the papers appended to this thesis, this particular section focuses on other factors of importance such as the mucosa, saliva, immune system and food components, also believed to contribute to the aetiology of RAS. The remaining endogenous factors (systemic factors) and exogenous factors (medications, local trauma, pathogens and psychological components) have been reviewed extensively elsewhere.

Mucosa

The oral mucosa itself most probably plays a role in RAS. This tissue consists of a stratified squamous epithelium and does not, unlike the gut mucosa, have a natural adsorptive capacity. Instead, it shows regional variations in permeability that are in direct correlation with the thickness of the epithelial barrier (Squier, 1991). Aphthous lesions tend to occur in non-keratinised mucosa. It has been proposed that this is due to the fact that antigenic stimulation is more likely in the lining mucosa than in the masticatory mucosa, since the former has a lower degree of keratinisation and possibly higher permeability than the latter (Subramanyam, 2011). This could also explain why RAS is less common in smokers, since it is believed that tobacco increases the degree of keratinisation. Paradoxically, patients with RAS have been reported to be significantly less likely to be smokers than control subjects, and patients with RAS who do smoke have been found to smoke fewer cigarettes than the controls (Atkin et al., 2002, Natah et al., 2004). Furthermore, local traumas are known to result in aphthous lesions in patients who suffer from RAS (Wray et al., 1981, Natah et al., 2004, Jurge et al., 2006). The oral cavity is easily traumatised because it has high functional activity with large masticatory forces and close contact between soft and hard tissues, i.e., the oral mucosa and teeth. Thereby, the non-keratinised mucosa is also more easily traumatised, since it is not as thick as the

keratinised mucosa. Other pre-disposing factors for RAS, such as psychological stress, nutritional deficiencies, and hormonal imbalances, may alter the epithelial integrity of the mucosal lining, resulting in aphthous lesions (Subramanyam, 2011). In addition, patients with RAS who lead stressful lives seem to be more prone to para-functional habits that cause trauma to the oral mucosa, e.g., chewing of the lips resulting in an aphthous lesion (Albanidou-Farmaki et al., 2008). It has also been demonstrated that the degree of keratinisation of the oral mucosal tissue in patients with RAS is significantly lower than that in a group of controls (Banoczy & Sallay, 1969). Macroscopic hyperkeratosis of the oral mucosa and the presence of leukoplakia have not been observed in patients with RAS (Sallay & Bánóczy, 1968). Similarly, patients with leukoplakia rarely suffer from RAS (Sallay & Bánóczy, 1968). Currently, there are no studies that have conclusively examined the oral mucosal properties of patients with RAS.

Saliva

In general, the salivary flow rate does not seem to be impaired in patients with RAS, either during the acute or the remission phase (Wu-Wang et al., 1995, Brozovic et al., 2002, Mohammad et al., 2013). Recently, increased salivary levels of IL-2 have been demonstrated in patients with RAS, who presented with active ulcers at the time of sampling, as compared to healthy controls. Interestingly, the highest increases in the level of this pro-inflammatory cytokine were observed in the age group of 16–30 years and in females (Kalpana et al., 2014), which are groups suggested to hold an increased risk of RAS. Elevated levels of TNF-α have also been detected in patients during the period when the lesions are present (Boras et al., 2006, Eguia-del Valle et al., 2011), as well as during the remission phases of the disease (Boras et al., 2006). In contrast, for IL-6, there were no differences in the levels detected during either the active or passive phase between the patients with RAS and the controls (Boras et al., 2006). In addition, reduced salivary levels of epidermal growth factor (EGF) during both the active and passive stages of the condition have been reported (Adisen et al., 2008). This change in EGF levels may disturb the mucosal integrity and hinder mucosal healing. In addition, lower levels of vascular endothelial growth factor (VEGF), which is important for angiogenesis and wound healing, have been found in patients suffering from major RAS (but not in those with minor RAS) during the active phases of the disease but not during the passive phases, as compared to control subjects (Brozovic et al., 2002). Salivary and peripheral blood neutrophils of patients with RAS exhibit significantly lower phagocytic activities than those of controls, although no significant differences in phagocytic activity were observed when the neutrophils of the two different locations of the same patients were compared (Kumar et al., 2010). In a pilot study involving patients with RAS, differential MUC7 glycosylation was observed, which might alter the function of the salivary mucins in terms of their protective properties against mucosal pathogens (Zad et al., 2015). Although there have been several reports on the constitution of saliva and its possible effects on the development of RAS, there is no conclusive evidence to support the role of saliva in this condition.

Immune system

While the immunopathogenic mechanisms underlying RAS remain unclear, they most likely involve local and/or systemic stimulation that results in an atypical cytokine cascade, which in turn leads to enhanced innate and cell-mediated immune responses directed towards focal areas of the oral mucosa. Here, a T cell-mediated immune response is likely to be a central feature due to the heavy infiltration of T cells into the epithelium, possibly as the consequence of the entry of an endogenous or exogenous antigen, resulting in damage to the epithelium. Furthermore, the ulcers seen in RAS have features suggestive of vasculitis.

Support for the role of T cells is strengthened by the fact that $CD3^+$ T cells are central inflammatory cells found in tissue specimens from RAS lesions, where $CD4^+$ T cells comprise approximately 50% and $CD8^+$ T cells approximately 20% of the T-cell population (Hayrinen-Immonen et al., 1991). During the pre-ulcerative stage, $CD4^+$ T cells predominate over $CD8^+$ T cells, with a $CD4^+/CD8^+$ ratio of 2:1, which changes during the ulcerative stage, where the $CD8^+$ T cells predominate, with a $CD4^+/CD8^+$ ratio of 1:10, while in the healing phase, the $CD4^+$ T cell numbers increase again, yielding a $CD4^+/CD8^+$ ratio of 10:1 (Savage et al., 1985, Porter & Scully, 1991). Patients who are infected with HIV and suffer from AIDS, who show a progressive decline of the $CD4^+$ T-cell populations in their peripheral blood and a reversal of the $CD4^+CD8^+$ ratio, commonly manifest with aphthous lesions. The prevalence of RAS in these patients is not increased but the severity of the condition is, which is consistent with the systemic changes in the T-cells ratios for this condition and the local changes in T-cell ratios typical of the ulcerative lesions. Therefore, the systemic defect is believed to amplify the local defect in immune regulation (MacPhail & Greenspan, 1997). Furthermore, the proportions of $CD4^+CD25^{high}$ regulatory T cells in peripheral blood samples have been reported to be decreased in patients with RAS during both the acute and remission phases (Lewkowicz et al., 2005, Lewkowicz et al., 2008). The Tregs of patients with RAS are also reported to have impaired function, since they are less efficient at suppressing the cytokine production of $CD4^+$ effector T cells than are the Tregs of healthy individuals (Lewkowicz et al., 2008).

Support for the role of vasculitis comes from the fact that RAS is a major feature of Behçets disease, which is a systemic vasculitis disorder. Early reports suggested an immune complex–mediated vasculitis in patients with RAS (Schroeder et al., 1984, Dagalis et al., 1987) whereby antibody-antigen immune complexes form and are deposited into the blood vessel walls, causing inflammation. Increased levels of anti-endothelial cell antibody (AECA), an autoantibody that is associated with autoimmune vascular damage, has been reported in blood samples from patients with RAS, whereas the absence of anti-neutrophil cytoplasmic antibody (ANCA) in these samples suggests that neutrophil-mediated vasculitis is not involved (Healy et al., 1996). Increased levels of AECA were found during both the active and passive phases of RAS (Healy et al., 1996).

During the ulcerative stage, neutrophils become the dominant cell type in the actual ulceration, although they are also occasionally observed intravascularly and in areas characterised by mononuclear cell infiltrates, i.e., in the areas peripheral to the ulcer (Hayrinen-Immonen et al., 1991). The migration and chemotactic function of neutrophils (Abdulla & Lehner, 1979, Dagalis et al., 1987) as well as their phagocytic function (Ueta et al., 1993) have been reported to be normal in patients with RAS. However, a more recent report has described significant reductions in phagocytic activity for salivary and peripheral blood neutrophils in patients with RAS compared to control subjects (Kumar et al., 2010). Moreover, the production of oxygen radicals, which are required for the process of phagocytosis, has been found to be similar in the neutrophils of patients with RAS and control subjects (Wray & Charon, 1991). Interestingly, aphthous lesions are a prominent feature of cyclic neutropenia, which is a rare haematological disorder that is characterised by recurrent episodes of neutropenia (abnormally low concentrations of neutrophils in the blood). In addition, patients infected with HIV who present with major RAS lesions have been observed to show signs of neutropenia (MacPhail et al., 1991). At present, the precise role of neutrophils in the development and/or healing of the lesions of RAS is unclear, though it is suspected that they play an active role given that they are so dominant throughout the tissue in the ulcerative stage.

During the ulcerative stage, numerous macrophages loaded with phagolysosomes that contain the debris of neutrophils are observed, indicating that the main function of these cells is to clear the

remnants of neutrophils from these tissues (Schroeder et al., 1983). The proportion of $CD11b^+$ macrophages has been reported as approximately 14%, particularly in the periphery of the lymphoid cell infiltrate, whereas monocytes are found in low numbers (Hayrinen-Immonen et al., 1991).

Mast cells, which represent approximately 2–5% of the inflammatory cells in RAS lesions, have been found mainly in the richly vascularised connective tissue that lies beneath the basement membrane (Hayrinen-Immonen et al., 1991), i.e., the sub-epithelial lamina propria, as opposed to the epithelium, in which only a few mast cells have been detected (Natah et al., 1998). Mast cells are present in several-fold higher numbers in aphthous lesions than in normal mucosa (Schroeder et al., 1983). A significant increase in the number of mast cells is apparent when one compares the RAS lesions to oral traumatic ulcers or the healthy tissues of control subjects, revealing clear signs of activation/degranulation in the RAS lesions, which indicates an active role for these cells in the immunopathogenesis in RAS (Natah et al., 1998).

Approximately 5–12% of the cells found in RAS lesions comprise B cells that stain positively for CD19, which is a prominent B-cell marker (Hayrinen-Immonen et al., 1991). The B cells are found both in the epithelium and in the lamina propria (Griffin, 1981), particularly in the periphery of the lymphoid cell infiltrate (Hayrinen-Immonen et al., 1991). Activated B cells are rarely found in RAS lesions, which suggests that T cells are the predominant lymphocytes in this condition (Hayrinen-Immonen et al., 1991). However, a B cell-mediated mechanism may also be implicated that involves antibody-dependent, cell-mediated cytotoxicity (ADCC), and possibly also immune complexes (Jurge et al., 2006).

TNF-α, which is a major pro-inflammatory cytokine, is secreted by activated monocytes, macrophages, B cells, T cells, and mast cells. It has an important role in the recruitment of immune cells by inducing the up-regulation of adhesion molecules on endothelial cells, thereby stimulating neutrophil chemotaxis and T-cell proliferation (Natah et al., 2000). The expression of TNF-α has been found to be higher in patients with RAS than in control subjects, suggesting that this condition is characterised by high-level expression of this cytokine (Taylor et al., 1992, Buno et al., 1998, Natah et al., 2000). Furthermore, increased levels of TNF-α have been found in samples of saliva collected from patients with RAS (Eguia-del Valle et al., 2011). In addition, treatment strategies that involve the use of Thalidomide and Pentoxifylline, which prevent the synthesis of endogenous TNF-α, have been shown to be successful in treating RAS (Jurge et al., 2006). Besides TNF-α, increased levels of IL-6, an interleukin that has both pro-inflammatory and anti-inflammatory effects, seem to play important roles in the disease process of RAS (Yamamoto et al., 1994, Lewkowicz et al., 2005), as well as IFN-γ (Buno et al., 1998, Lewkowicz et al., 2005). IL-10 is an anti-inflammatory cytokine that is expressed at lower levels in patients with RAS than in controls (Buno et al., 1998, Lewkowicz et al., 2005). This suggests that suppression of the inflammatory reactions is defective in patients with RAS. IL-2 is another important pro-inflammatory cytokine, the binding of which to the IL-2 receptor on T cells results in the induction of proliferation and increased cytokine secretion, as well as stimulation of the secretion of pro-inflammatory cytokines IL-1, TNFα, and TNF-β. Increased plasma levels of IL-2 and increased expression of the IL-2 receptor by the activated peripheral lymphocytes have been reported during the active phases of RAS lesions (Sun et al., 2000). Polymorhisms in the genes encoding cytokines thought to be of importance in RAS development have been reported. In one study, no specific polymorphisms in the gene for TNF-α was found (Bazrafshani et al., 2002b), whereas in other studies such polymorphisms have been reported (Guimaraes et al., 2007, Sun et al., 2013). Polymorphisms of the gene that encodes IL-6 have been observed (Bazrafshani et al., 2002a, Karakus et al., 2014). While variations in certain IL-10 SNPs have been noted (Najafi et al., 2014) no

differences in the inheritance patterns of specific IL-10 polymorphisms have been reported (Bazrafshani et al., 2003).

Food components

The involvement of food components and preservatives in the aetiology of RAS has been discussed in the literature for many decades. However, no studies have specified the foods that are relevant for RAS development or the exact mechanisms by which these foods trigger lesion development. An association between coeliac disease and aphthous ulceration has been reported in approximately 5% of cases (Tursi et al., 2001, Field & Allan, 2003). It has also been discussed whether gluten sensitivity in patients with RAS can arise in the absence of coeliac disease (Wray, 1981), since gastro-intestinal symptoms may not always be present and other features of coeliac disease are seen in these patients (Field & Allan, 2003). However, there are also studies that indicate no association between coeliac disease and RAS (Yasar et al., 2012), since a gluten-free diet may not always alleviate the symptoms (Hunter et al., 1993). The association of RAS with coeliac disease has not been completely resolved (Field & Allan, 2003). Cow´s milk protein allergy has also been suggested as being linked to RAS (Calderon et al., 2008, Besu et al., 2009, Besu et al., 2013a, Besu et al., 2013b). Again, an unequivocal association has not been established.

Studies within this field have mainly been based on anamnestic data, elimination/provocation diets, allergy testing or combinations of the three aspects. Studies based on anamnestic findings suggest that patients with RAS consume a diet that differs from that of controls, whereby the patients with RAS report a higher intake of acidic foods (Gonul et al., 2007), lower daily consumption of calcium, iron, vitamin B_1, and vitamin C, and higher consumption of preservatives, such as benzoic acid and cinnamaldehyde (Ogura et al., 2001). In a non-referral, adult Swedish population, the incidence of self-reported allergies was significantly higher for patients with RAS than for the controls, as registered by general dental practitioners (Robledo-Sierra et al., 2013). In addition, patients with atopy have been reported to associate ingestion of certain foods with the development of ulcers (Wilson, 1980). Self-reporting of food allergies is, however, known to over-estimate the prevalence of allergies (Prescott et al., 2013). Nevertheless, the incidence of food allergies is increasing globally (Yu et al., 2016).

Elimination and/or provocation diet interventions have in part been successful in treating RAS, in that a proportion of the patients in each study have experienced a reduction of symptoms concomitant with the elimination of certain foods (Hay & Reade, 1984, Wright et al., 1986, Nolan et al., 1991, Wray et al., 2000). However, there are also studies that show that this type of intervention has no effect on lesion development (Eversole et al., 1982). Apart from an immediate type I hypersensitivity reaction a delayed-type IV hypersensitivity reaction could also be involved in RAS, since it has been shown that there is often a time delay between when the particular food component is ingested and when the clinical symptoms appear. This makes it difficult to define which food component represents the triggering factor, as a considerable number of different foods are consumed each day. Furthermore, it should be stressed that this type of diet requires a high level of persistence on the part of the patients. Patch-testing and contact urticaria testing are well-known methods for identifying Type I and Type IV allergic reactions of the skin. Patch-testing of patients with RAS led to the conclusion that this diagnostic tool may indeed be a useful adjunct for identifying food allergies in these patients (Nolan et al., 1991, Wray et al., 2000). Ultimately, despite extensive research efforts within this field to date the roles of food components in the aetiology of RAS are still unknown.

Treatment strategies

At present, there is no cure for RAS. Instead, all the available treatment strategies are aimed primarily at relieving symptoms, such as pain, and secondarily focus on promoting healing and reducing the duration and frequency of lesions. While various topical and systemic therapies have been used to treat RAS, there are few publications demonstrating the efficacies of the different substances applied. Currently there is no consensus available regarding appropriate treatment regimens.

A systematic Cochrane review from 2012 of systemic treatments involving tablets and injections revealed that the studies of treatments for this condition are not sufficiently comprehensive (Brocklehurst et al., 2012). Out of 25 randomised control trials (RCTs) in which 21 different interventions were assessed, not one single treatment was found to be effective. This was mostly due to the poor methodology used and the fact that not all drugs used for the treatment of RAS were included. It should also be appreciated that different drugs are more or less effective in different patients, and that systemic interventions are most often applied to patients with a more severe and persistent form of RAS for whom topical treatments strategies have failed. The immunomodulatory/anti-inflammatory interventions included in this systematic review were Beta-glucan, Clofazimine, Colchicine, Levamisole, Montelukast (leukotriene receptor antagonist), Pentoxifylline, Prednisone and Sulodexide. Other interventions studied were camel thorn, homeopathy, LongoVital (herbal plus vitamin), Longovital (herbal alone), multi-vitamin, propolis, sub-antimicrobial doxycycline, tetracycline, and vitamin B_{12}.

A protocol for a systematic review of topical treatment strategies has also been registered in the Cochrane database but it has yet to be completed (Taylor J. Topical interventions for recurrent aphthous stomatitis (mouth ulcers). Cochrane Database of systemic reviews 2013). This type of treatment involves, for example, the use of antiseptics, anti-inflammatory agents, and analgesics (chlorhexidine mouthwash or gel, triclosan gel, topical diclofenac, amlexanox), antibiotics (doxycycline gel), topical corticosteroids (triamcinolone acetonide, fluocinolone acetonide, Clobetasol proprionate), hyaluronic acid (gel), topical anaesthetics (lidocaine spray or gel), and other modalities (laser therapy and natural substances) (Belenguer-Guallar et al., 2014).

In Sweden, the Swedish Agency for Healthy Technology Assessment and Assessment of Social Services (SBU) proposed to compare the effects of rinsing with Clobetasol proprionate 0.025% or Aureomycin (tetracycline), which are drugs commonly used in Sweden for the treatment of RAS, in a systematic review (http://www.sbu.se/globalassets/publikationer/upplysningstjanst/pdf_er/skoljning-med-klobetasolgel-eller-aureomycin-tetracyklin-vid-aftosa-munsar-afte.pdf). Unfortunately, no comparative studies of these two drugs have been performed, so there was no possibility to perform the planned comparison.

Mucosal immunity

The mucosal immune system

The immune system, which comprises all the mucosal surfaces of the body (commonly referred to as the mucosal immune system) plays a dual role: on the one hand it is required to protect the body from harmful antigens (pathogens) by inducing protective immunity, while on the other hand it needs to

induce tolerance towards harmless antigens (food proteins and the commensal microbiota). These two important roles are complete counterparts, and ideally, when the body is exposed to harmful pathogens an inflammatory process is initiated to eliminate these pathogens whilst tolerance to food proteins and the commensal microbiota is maintained. However, on occasion, the immune system has difficulties deciding between when to induce protective immunity and when to induce tolerance, resulting in immunoregulatory diseases, such as autoimmune diseases and allergies.

This part of the immune system is strategically located at the entrance points of the body, and it is constantly exposed to numerous antigens from the external environment. The mucosal immune system is found in the ocular cavity, the respiratory tract (nose, sinus, trachea and lungs), the gastro-intestinal tract (oral cavity, oesophagus, stomach, and small and large intestines), and the female urogenital tract (uterus, bladder, and vagina) (Lamichhane et al., 2014). The associated secretory glands of these compartments (the lachrymal, salivary and mammary glands) are also included. The epithelium acts in part as a physical barrier with tight junctions between the cells, as well as the mucus layer overlying the epithelium (Kim & Surh, 2015). Fluids secreted from the associated glands contain antimicrobial factors that create a biochemical barrier (Kiyono & Azegami, 2015). However, antigens may penetrate this barrier, necessitating the assistance of innate and adaptive immune responses (Kim & Surh, 2015).

The mucosal immune system is composed of inductive sites, which are areas in which antigens, selectively sampled from mucosal surfaces, are transported and presented by dendritic cells (DCs) to naïve T and B cells, inducing a primary immune responses, and effector sites, which are areas in which effector cells, after extravasation, retention, and differentiation, perform their actions. The respiratory and gastro-intestinal compartments of the mucosal immune systems in humans and mice are shown in Table 2. The mucosa-associated lymphoid tissues (MALTs), which are considered as inductive sites of the mucosal immune system, consist mainly of the nose-associated lymphoid tissues (NALTs) and the gut-associated lymphoid tissues (GALTs). The bronchus-associated lymphoid tissues (BALTs) are also classified as MALT structures but since they are not found in mice and only occasionally found in humans, they will not be discussed further here. All MALT structures consist of a follicle-associated epithelium (FAE) that contains micro-fold (M) cells. The FAE overlies the follicular zones of B cells and naïve- and memory T cells with surrounding para-follicular zones that contain T cells and DCs, and where high endothelial venules (HEVs) are also found (Pabst, 2015) (Figure 3). At the bottom of the follicular zone lies a germinal centre. These tissues also have efferent lymphatics. MALT structures therefore resemble lymph nodes but they lack afferent lymphatics and are not encapsulated (Brandtzaeg, 2010). In general, the antigen is taken up by the M cells in the FAE and presented to DCs, which process the antigen into peptides and load them onto their MHC class I or class II molecules and transport them to the para-follicular T-cell zones. The antigen is then presented to the naïve T cells, causing them to proliferate and differentiate into antigen-specific T cells. These cells support the induction of IgA-producing B cells and their differentiation into plasma cells in the follicular zones. Thereafter the B and T cells migrate to the regional lymph nodes through lymph vessels, and then through the circulation to the effector sites (Lamichhane et al., 2014).

The major immunoglobulin within the mucosal immune system is secretory immunoglobulin A (SIgA), as it predominates in most secreted bodily fluids. IgA is produced by plasma cells locally at mucosal surfaces. IgA attaches to the polymeric immunoglobulin receptor (pIgR) found on mucosal epithelial cells, which allows for transcytosis into the cell and then secretion into the mucus. During transcytosis a fragment of the pIgR is cleaved off, while the remainder remains attached to the IgA molecule. This complex forms the secretory component (SC). The SC is not found in the systemically circulating IgA but only in the mucosal secretory IgA. At the mucosal surface and in the lumen, the

function of SIgA is to bind and agglutinate bacteria and other antigens so as to facilitate their clearance, to neutralise bacterial toxins, and to prevent penetration of the mucosal membrane by these molecules. In addition, the SIgA-antigen complex can be taken up by M cells in the FAE, thereby inducing an adaptive immune response (Shakya et al., 2016).

Lymph nodes play an important role in protecting the body against foreign antigens, since they are the sites of the primary immune responses. Antigen-presenting cells (APCs) display antigens to naïve lymphocytes, which become reactive and undergo clonal expansion to produce new lymphocytes and plasma cells (Willard-Mack, 2006). Antibodies are produced by the plasma cells and are secreted into the lymph, while activated lymphocytes are transported by the circulation to effector sites where harmful antigens are eliminated (von Andrian & Mackay, 2000). Under normal conditions, the lymphocytes then leave the effector site and home to the lymph nodes located in the area from which they originated from (Miyasaka & Tanaka, 2004).

Table 2. The respiratory and gastro-intestinal compartments of the mucosal immune systems in humans and mice.

	Human	Mouse
Inductive sites		
Upper respiratory tract	Waldeyer´s ring and ILFs	NALT and ILFs
Gastro-intestinal tract	GALT (PPs, ILFs and appendix)	
Local/regional lymph nodes	MLNs, CLNs	
Effector sites		
Upper respiratory tract	Lamina propria of the nasal mucosa	
Gastro-intestinal tract	Lamina propria of the oral cavity and intestinal mucosa	
Secretory glandular tissues	Salivary glands	
Surface epithelium	FAE and IEL compartments	

Abbreviations: ILF, isolated lymphoid follicle; NALT, nose-associated lymphoid tissue; GALT, gut-associated lymphoid tissue; PP, Peyer´s patch; FAE, follicle-associated epithelium; IEL, intraepithelial lymphocyte.

Adapted from: Brandtzaeg *et al*. Terminology: nomenclature of mucosa-associated lymphoid tissue. Mucosal Immunol. 2008 Jan;1(1):31-7, and Brandtzaeg. Function of Mucosa-Associated Lymphoid Tissue in Antibody Formation. Immunol Invest. 2010;39(4-5):303-55.

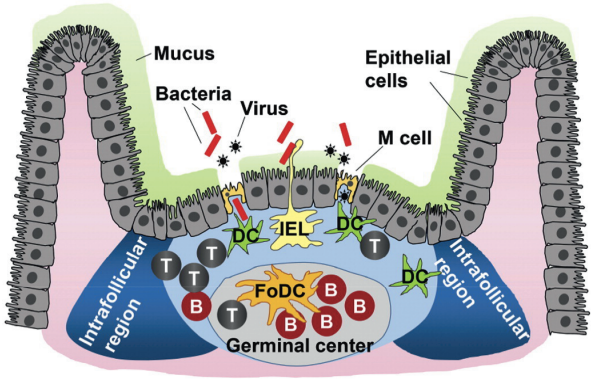

Figure 3. Schematic of a Peyer´s patch, which is a mucosa-associated lymphoid tissue (MALT) structure found in the intestine. *All MALT structures consist of a follicle-associated epithelium (FAE), that contains micro-fold (M) cells. The FAE overlies the follicular zones of B cells and naïve- and memory T cells, with surrounding intra-follicular/para-follicular zones that contain T cells and DCs. At the bottom of the follicular zone lies a germinal centre. T, T cell; B, B cell; DC, dendritic cell; IEL, intraepithelial lymphocyte; FoDC, follicular dendritic cell. Adapted from Shakya et al. 2016. Mucosal vaccine delivery: Current state and a pediatric perspective. J Control Release. 240:394-413.*

The nasopharyngeal immune site

The nose forms the point of entry to the upper respiratory tract and in mice it is divided into two distinct passages separated by the nasal septum. Each nasal passage extends from the nostril to the nasopharynx and consists of a main chamber that is composed of a lateral wall, a septal wall, a roof, and a floor (Harkema et al., 2006). The lumen of the main chamber is lined with a richly vascularised and innervated mucosa, consisting of the surface epithelium and the underlying lamina propria.

The NALT in rodents comprise bilateral lymphoid cell aggregates that are present on both sides of the nasopharyngeal duct located dorsally to the cartilaginous soft palate, and are believed to be analogous to the Waldeyer´s ring in humans (Kuper et al., 1990, Brandtzaeg et al., 2008). This tissue is recognised as the major MALT structure in the upper respiratory tract, and is believed to be an inductive site for mucosal immune responses, similar to the Peyer´s patches (PP), which are the GALT equivalent in the intestine (Kiyono & Fukuyama, 2004). The NALT is covered by a FAE that contains M cells similar to those found in the gut epithelium and contains a follicular zone, as well as a para-follicular zone (Kiyono, 2013). The NALT together with the isolated lymphoid follicles (ILFs) and draining CLNs make up the inductive sites of the nasal immune system. Equivalently, the nasal mucosa, FAE, and associated IELs make up the effector sites.

Antigens and vaccines are inhaled through the nose and transported *via* the nasal mucosa through the M cells of the FAE, where the DCs in the lamina propria are loaded with the antigen and migrate to the NALT where they present the antigens to naïve T cells and B cells. Activated and memory B and T cells then migrate from the NALT *via* the HEVs to the CLN (Kuper et al., 1992) where the immune response is amplified (Zuercher, 2003). These cells then pass into the circulation and home to the effector sites, such as the lamina propria of the nasal passages, and to exocrine glands, such as the submandibular glands (Kiyono, 2013).

In normal adult, female BALB/c mice, the NALT contain approximately 1×10^6 cells (Heritage et al., 1997). Approximately 30% $CD4^+$ T cells and 10% $CD8^+$ T cells have been reported in the NALT (Wu et al., 1996, Heritage et al., 1997, Asanuma et al., 1997). However, in another study, the percentage of $CD4^+$ T cells (but not $CD8^+$ T cells) was different (Rodriguez-Monroy et al., 2007). The ratios of $CD4^+$ T cells to $CD8^+$ T cells in the NALT and MLN have been reported to be similar (3.9 and 3.8 respectively), and somewhat higher than in the CLN (2.9) (Asanuma et al., 1997). The majority of the $CD3^+$ T cells in the NALT co-express CD45RB, which suggests T cells with a naïve phenotype (Wu et al., 1996). Furthermore, the percentage of $CD11c^+$ DCs in the NALT has previously been reported as approximately 3% (Nacer et al., 2014). In addition, the percentage of $CD11b^+Ia^+$ (MHC class-II) macrophages has been reported as 4.5% (Heritage et al., 1997), indicating that there could be a greater proportion of $CD11b^+$ than $CD11c^+$ APCs in this compartment. The B cell to T cell ratio has been estimated at approximately 50% B cells and 50% T cells (Wu et al., 1996, Heritage et al., 1997, Asanuma et al., 1997).

Although the NALT is recognised as the central structure responsible for antigen-specific immune responses of the upper respiratory tract, it has also been proposed that there are other, additional inductive sites that are of importance, i.e., the NALT-independent pathways. This arose from the discovery of respiratory M cells, which possess the functional characteristics of classical M cells but reside in epithelium separated from the NALT (Kim et al., 2011). After the antigens have been sampled by the respiratory M cells, they are loaded onto DCs in the lamina propria, and then transported onwards in a fashion similar to that of the NALT-dependent pathway.

The oral immune site

From the mouth, exogenous antigens are taken up by the oral mucosa or swallowed down into the gut. The oral cavity is lined with a stratified squamous epithelium, which does not have a natural adsorptive capacity, unlike the gut and the respiratory epithelium. The latter two consist of a single layer of epithelium (apart from the most anterior parts of the nose, which are lined with a stratified squamous epithelium), whereas the oral mucosa shares features with the skin and exhibits regional variations in permeability that are in direct correlation to the thickness of the epithelial barrier (Squier, 1991). The areas that are exposed to the highest levels of wear, i.e., the attached gingiva, hard palate, and dorsum of the tongue are covered by a keratinised epithelium, which hinders passage through the epithelium. In contrast, the surfaces that are not as exposed, i.e., the labial and buccal surfaces of the mouth, as well as the soft palate, ventral parts of the tongue, and floor of the mouth, are covered by a non-keratinised mucosa, which is more permeable.

Unlike the nose and the intestine, the oral cavity of mice lacks a clear inductive MALT structure of its own, like the NALT and GALT, respectively. Instead, this region displays effector sites, such as the epithelium and lamina propria lining the oral cavity, as well as the salivary glands. In humans, the Waldeyer´s ring is located in the transition zone between the oral cavity and the nasopharynx, thereby establishing an inductive site in close proximity to the oral cavity and being exposed to both airborne and alimentary antigens. In addition, in mice, the NALT lie in close proximity to the oral cavity. Therefore, there are a variety of lymphoid tissues within the naso-oropharyngeal cavities, i.e., the NALT in mice and Waldeyer´s ring in humans, as well as the ILFs scattered throughout these mucosal passages. However, the interplay between the nasal and oral compartments is not fully understood.

A unique feature of the oral cavity is that it is protected by both the mucosal immune system, manifested through the oral mucosa, saliva, and salivary glands, and the systemic (peripheral) immune

system, through the gingival crevicular fluid (Walker, 2004, Kiyono, 2013). The principal cell type of the oral epithelium is the keratinocyte, although there is also a large proportion of DCs among which the Langerhans cells predominate.

It has been proposed that antigens are sampled from the lumen of the oral cavity and loaded onto DCs that migrate to regional lymph nodes, i.e., the CLNs, where they present the antigens to naïve T cells (Eriksson et al., 1996). While, the exact mechanisms of antigen up-take are currently not known, the most prominent subsets in the mouse oral mucosa are $CD11c^{neg}CD11b^{+}$ APCs and $CD11c^{+}CD11b^{+}$ DCs (Mascarell et al., 2008). The antigen-specific T cells and B cells in the CLN then migrate to the lamina propria of the oral cavity and salivary glands, where they exert their effector functions.

The submandibular glands display a predominance of IgA-producing cells but they also contain high numbers of T cells. Approximately 50% of the lymphocytes are $CD3^{+}$ T cells, whereas both $CD4^{+}$ T cells and $CD8^{+}$ T cells are found in equal numbers. $CD4^{neg}CD8^{neg}$ cells are also found. However, $CD4^{+}CD8^{+}$ T cells are essentially lacking (Kiyono, 2013). At least 95% of the IgA found in the saliva is produced by plasma cells located in the salivary glands (Brandtzaeg, 2013). In the CLN, there are approximately 80% $CD3^{+}$ T cells and 20% B cells. Among the T cells, approximately 50% are $CD4^{+}$ and 20% are $CD8^{+}$ (Asanuma et al., 1997).

The gastro-intestinal immune site

The gastro-intestinal tract extends from the oral cavity to the anus, encompassing the largest mucosal surface, and it is continuously exposed to various antigens. However, since the upper gastro-intestinal tract (oral cavity, pharynx, oesophagus, and stomach) contains few lymphoid tissues compared to the lower gastro-intestinal tract (small intestine and large intestine), the majority of immune cells are found in the latter (Mowat & Agace, 2014). This is also one of the reasons why the lower gastro-intestinal tract is the most extensively studied compartment of the mucosal immune system. The induction of oral tolerance within the intestine occurs mainly in the small intestine, whereby only this compartment will be reviewed in the current section.

Villi are finger-like projections, characteristic of the small intestine, that extend into the lumen to increase the surface area of the epithelium and allow for greater uptake of intestinal antigens. The epithelium is dominated by enterocytes, which form a simple, columnar layer and have a strong absorptive capacity, since they consist of microvilli, which increase the surface area even more. Underneath the epithelium lies the lamina propria, and it is within these two segments that most of the immune cells reside. Interspersed within the intestinal epithelium lie the GALT structures, comprising the PPs and ILFs. Together with the MLNs they form the inductive sites of the lower gastro-intestinal tract. Equivalently, the effector sites are the intestinal mucosa, the FAE, and the IELs. As with other MALT structures, the PPs consist of follicular B-cell areas and para-follicular T-cell areas that are covered by a FAE that contains M cells. The PPs number approximately 8–10 in mice (Kunisawa et al., 2008). In contrast to the PPs, the ILFs consist of B-cell zones but no clearly evident T-cell zones (Mowat & Agace, 2014).

Antigens are sampled across the intestinal epithelium *via* a number of different routes, either to the GALT or to the lamina propria. In the GALT, this is accomplished through one of two routes (Pabst & Mowat, 2012): *(i)* through the M cells in the FAE of the PPs and ILFs, where the antigens are then loaded onto DCs; or *(ii)* the antigens are sampled directly from the lumen by DCs in the underlying tissue extending their processes through the FAE. In the remainder of the small intestine, antigens are

sampled across the epithelium to the lamina propria through the following routes (Pabst & Mowat, 2012, Kim & Surh, 2015): *(a)* small antigens may pass directly across the epithelium by para-cellular diffusion through pores in the tight junctions that connect the epithelial cells; *(b)* larger antigens can be taken across enterocytes by transcellular routes, i.e., passing through the epithelial cells; *(c)* by exosomes; *(d)* by CX3CRX1-expressing macrophages that extend their processes through the epithelial barrier; *(e)* through apoptotic bodies derived from intestinal epithelial cells; and *(f)* by capturing antigens passed on *via* goblet cells.

It has been proposed that the microbiota is mainly sampled through the GALT, and that food antigens are taken up by DCs in the lamina propria rather than through the GALT (Pabst & Mowat, 2012). Ultimately, the PPs may not play as important a role in the induction of oral tolerance to food proteins as previously described, since oral tolerance has been shown to be established in the absence of PPs (Spahn et al., 2001, Spahn et al., 2002, Kraus et al., 2005). Irrespective of the route, all drainage of immune cells occurs to the MLN before they migrate onwards to the effector sites.

Tolerance

Induction of tolerance mainly occurs within the intestinal immune site, since (as mentioned above) the gastro-intestinal tract comprises the largest mucosal surface and is continuously exposed to various antigens, such that the majority of immune cells are found here (Mowat & Agace, 2014). This is also one of the reasons why it is the most extensively studied compartment of the mucosal immune system. Induction of tolerance to food antigens in the small intestine is commonly referred to as 'oral tolerance', which represents the normal immune response to the harmless protein antigens to which humans and animals are exposed *via* the mucosal route. The intestine has the capacity to distinguish harmless antigens from potential pathogens and to develop active antigen-specific tolerance towards the former. This is vital for maintaining a healthy immune balance and avoiding pathological immune reactions. The abrogation of oral tolerance is thought to underlie the development of allergies and inflammatory bowel disease (Pabst & Mowat, 2012), both of which are growing concerns globally (Okada et al., 2010).

Terminology

'Oral tolerance' is defined as the body´s ability to prevent reaction to a harmless, orally administered, soluble antigen, for example, a food antigen, thereby avoiding an aggressive immunological response specifically in the small intestine. This term should be distinguished from 'mucosally induced tolerance', since oral tolerance affects both the local and systemic immune responses, whereas mucosally induced tolerance involves gut bacteria and only induces a local response in the other parts of the intestine, for example, the colon (Pabst & Mowat, 2012). Induction of oral tolerance is therefore confined to the gastro-intestinal tract, more specifically the small intestine. However, mechanisms for tolerance induction must exist along the entire respiratory, gastro-intestinal, and urogenital tracts, since they are all mucosal surfaces, parts of the mucosal immune system, and are exposed to large amounts of antigens. Currently, there is no fully established terminology for tolerance induction that refers to all parts of the mucosal immune system. 'Mucosal tolerance' has been suggested as a more appropriate term (Kim & Surh, 2015) for tolerance that is induced specifically towards dietary antigens. However, this phrasing is confusingly similar to the term 'mucosally induced tolerance', which is proposed to describe tolerance induction to gut bacteria in the intestine. The induction of

tolerance in the oral cavity has been referred to as 'oral mucosal tolerance' (Novak et al., 2008). Ultimately, there is a need for a more stringent and concise terminology within this area.

Historical context

The concept of oral tolerance was first introduced in the 19th century by Dakin (1829). He observed that the development of contact dermatitis in South American Indians to poisonous ivy could be prevented by chewing the leaves of this plant. In 1911, Wells and Osborne demonstrated experimentally that feeding egg protein to guinea pigs could inhibit systemic anaphylaxis to this food component (Wells & Osborne, 1911). Later, in 1946, Chase showed that by pre-treating animals orally with an allergenic compound to which they had not earlier been exposed, for example, 2:4 dinitrochlorobenzene (a known allergen used for experimental sensitisation in humans), a degree of protection was obtained that was higher than if the compound had been given intravenously, cutaneously or parenterally prior to a challenging sensitisation (Chase, 1946). The mechanism by which the immune system acts non-responsively to antigens was not well understood at the time.

The importance of the small intestinal epithelium for oral tolerance induction was demonstrated in the 1980s. Initially, in 1983, Strobel *et al.* showed that it was not the antigen single-handedly that induced tolerance but that it had to be confined within serum, since mice became tolerant after serum transfer from an OVA-fed mouse to a naïve donor mouse. Therefore, this tolerogenic moiety was named 'the serum factor' (Strobel et al., 1983). Subsequently, Bruce and Ferguson demonstrated, in 1986, that parenteral administration of an antigen was not sufficient to induce tolerance but that gut processing seemed to be required (Bruce & Ferguson, 1986). In 1990, Peng *et al.* showed that the tolerogenic activity in serum was not correlated to the serum levels of antigen absorbed by the intestine but that it was time-dependent, since serum collected 5 minutes after the feeding of OVA was unable to transfer tolerance despite the fact that significant levels of immunoreactive OVA were present (Peng et al., 1990). Instead, it required approximately 45–60 minutes in BALB/c mice. In addition, in 1994, Furrie *et al.* showed that severe combined immunodeficiency (SCID) mice, which lack functioning mature B and T cells, failed to generate the oral tolerance-inducing serum factor (Furrie et al., 1994). Therefore, a fully functioning intestinal immune system is a requirement for oral tolerance induction. An attempt was made to characterise the features of the serum factor (Furrie et al., 1995), but this issue remained unsolved until the beginning of the 21st century when it was shown to consist of exosomes (Karlsson et al., 2001). Exosomes are small membrane-bound vesicles that are secreted by the fusion of intracellular multi-vesicular late endosomes/lysosomes with the plasma membranes from the small intestinal epithelial cells after processing of the sampled antigen, and the exosomes are then transported to the circulation and disseminated to different locations within the body. Exosomes have been detected throughout the body in physiological fluids, for example, broncho-alveloar lavage (BAL) fluids (Admyre et al., 2003), serum (Caby et al., 2005), urine (Pisitkun et al., 2004), breast milk (Admyre et al., 2007), and saliva (Ogawa et al., 2008), and they are believed to mediate communication between cells. Furthermore, they express MHC class II molecules that are loaded with antigens (Ostman et al., 2005) and can possibly initiate antigen presentation either by themselves or through exosome uptake by other APCs resulting in the activation of T cells.

Induction of oral tolerance in the small intestine

Several factors influence the induction of oral tolerance, such as the age, genetic background, and immunological status of the animal, as well as the nature of the antigen and the dose and frequency of administration. Low doses of antigen favour active suppression, whereby an inhibitory environment is

created by the induction of a multitude of different Treg subsets, among which the FoxP3$^+$ Tregs are the most important and well-characterised. High doses of antigen initiate T-cell activation, resulting in clonal anergy or the deletion of antigen-specific T cells (Gregerson et al., 1993, Friedman & Weiner, 1994, Chen et al., 1995). Multiple feedings of antigen are more effective at inducing oral tolerance than a single feeding, while continuous feeding of an antigen in the drinking water enhances suppression to both Th1 and Th2-type responses, as compared with a single feed or feeding the antigen once per day over several days (Faria et al., 2003).

Tregs are immunosuppressive cells that are essential for maintaining peripheral tolerance, preventing the development of autoimmunity and limiting chronic inflammatory diseases. They are broadly classified into two subsets: the thymus-derived regulatory T cells (tTregs), which acquire their regulatory capacity in the thymus; and the peripherally-derived regulatory T cells (pTregs), which acquire their regulatory function in the periphery through the differentiation of conventional CD4$^+$ T cells (Abbas et al., 2013, Li et al., 2015). FoxP3$^+$ Tregs employ an array of mechanisms to maintain oral tolerance through either the secretion of suppressive molecules or cell-cell interactions. Tregs secrete inhibitory cytokines (e.g., TGF-β and IL-10), express granzymes (which results in the cytolysis of effector T cells), diminish local IL-2-inhibiting effector T-cell proliferation through metabolic disruption, and/or modulate the maturation or function of DCs (Vignali et al., 2008). The type of Treg activity is dictated by the environmental context, as well as on the location of the inflammation (Chaudhry & Rudensky, 2013).

A multi-step process for the induction of oral tolerance in the small intestine has been proposed according to the following (Hadis et al., 2011, Pabst & Mowat, 2012, Kim & Surh, 2015) (Figure 4):

1) **Sampling of the antigen and migration of DCs to the draining lymph node**
   CD11c$^+$CD103$^+$ DCs, which represent the most abundant subset of APCs in the lamina propria of the small intestine, sample antigens from the gut lumen and migrate to the MLN. CX3CR1$^+$ macrophages play an important role in sampling the antigens and loading them onto the CD11c$^+$CD103$^+$ DCs. Migration to the MLN is assisted by the up-regulation of CD62L and CCR7, markers that are expressed on the lymphocytes, which allows them to enter the secondary lymphoid tissues *via* HEVs. CCL19 and CCL21 are the ligands for CCR7 expressed by the lymphatic endothelium and in the MLNs that help guide the cells.

   The MLN are a pre-requisite for the establishment of oral tolerance, as surgical removal of the MLN (Worbs et al., 2006), as well as the lack of this lymph node due to genetic mutation (Spahn et al., 2001, Spahn et al., 2002) have been shown to prevent the induction of oral tolerance. Furthermore, in CCR7-deficient mice, the ability of cells to migrate into the MLN is impaired (Worbs et al., 2006, Jang et al., 2006). There also seems to be a distinct difference in function between CD103$^+$ DCs and CX3CR1$^+$ macrophages, in that CD103$^+$ DCs are less efficient at sampling antigens themselves from the intestinal lumen (Farache et al., 2013, Chang et al., 2013) and require the assistance of CX3CR1$^+$ macrophages for this activity (Niess et al., 2005, Hadis et al., 2011, Mazzini et al., 2014).

2) **Presentation of the antigen to naïve lymphocytes in the lymph node**
In the MLN, the $CD11c^+CD103^+$ DCs present the antigens to naïve B and T cells, such that they become activated.

3) **Induction of gut tropism and differentiation of $FoxP3^+$ Tregs**
The $CD11c^+CD103^+$DCs produce retinoic acid (RA) from dietary retinoids (vitamin A) through the activity of the enzyme retinaldehyde dehydrogenase 2 (RALDH2). The local stromal cells in the lymph node also perform this task. This stimulates gut tropism by inducing the expression of the gut-homing receptors $\alpha_4\beta_7$ and CCR9 on activated antigen-specific T cells, which favours homing to the small intestinal mucosa. It also stimulates the differentiation of $FoxP3^+$ Tregs assisted by TGF-β. The induction of $FoxP3^+$ Tregs is putatively more important for the induction of oral tolerance than anergy and the deletion of T cells. In the absence of $FoxP3^+$ T cells, oral tolerance does not develop.

Adding TGF-β to a culture that contains $CD11c^+CD103^+$ DCs induces the differentiation of $FoxP3^+$ Tregs; this phenomenon does not occur when TGF-β is added to $CD11c^+CD103^{neg}$ DCs. The latter cells cannot expand the Treg population *in vitro* (Coombes et al., 2007). Furthermore, $CD11c^+CD103^{neg}$ DCs require the addition of exogenous TGF-β in order to metabolise vitamin A (retinol) from the diet into RA through the action of RALDH2, which is not necessary for $CD11c^+CD103^+$ DCs (Coombes et al., 2007, Benson et al., 2007, Sun et al., 2007). Mice that are fed a vitamin A-deficient diet, have severely impaired capacity for gut homing of activated $CD4^+$ T cells, resulting in reduced numbers of these cells in the lamina propria of the small intestine (Iwata et al., 2004, Cha et al., 2010).

4) **Homing of antigen-specific T cells and $FoxP3^+$ Tregs to the lamina propria and secondary expansion of the $FoxP3^+$ Tregs**
The activated antigen-specific T cells and the $FoxP3^+$ Tregs home back to the intestinal lamina propria. Then the $FoxP3^+$ Tregs undergo secondary expansion under the influence of IL-10 produced by the resident $CX3CR1^+$ macrophages. These latter cells cannot migrate within the lymph vessels and are not very good at priming naïve T cells. Their functions are instead expressed within the local mucosa, where they sample antigens from the intestinal lumen and load them onto the DCs, while also facilitating the local differentiation of Tregs.

Besides promoting the differentiation of $FoxP3^+$ Tregs, $CD103^+$ DCs are unable to produce the pro-inflammatory cytokines IL-6 and TNF-α and instead produce the anti-inflammatory cytokines IL-10 and TGF-β (Kim & Surh, 2015). Interestingly, most $CD4^+$ T cells in the lamina propria display an activated or memory phenotype, even in the absence of pathogens (Kim & Surh, 2015).

5) **Dissemination of the $FoxP3^+$ Tregs putatively promotes systemic tolerance**
Some of the Tregs are then believed to exit the mucosa *via* the lymph or blood and spread throughout the immune system, promoting systemic tolerance.

6) **Involvement of the liver in the induction of systemic tolerance**
   Apart from the gut processing of the antigen, it has been demonstrated that the liver assists in establishing systemic tolerance from intestinally derived antigens, whereby a certain fraction of the antigens is transported from the GALT and/or lamina propria of the intestine to the MLN and then on to the PLNs and through the thoracic duct to the circulation, whereas another fraction is transported through the portal vein to the liver and then onwards through the circulation.

   The portal vein drains blood from the intestine to the liver. Injecting an antigen directly into the portal vein without first passing it through the intestine is known to induce oral tolerance (Thomson & Knolle, 2010). Moreover, hindering the blood flow away from the liver by inserting a porto-caval shunt prevents the induction of oral tolerance (Callery et al., 1989, Yang et al., 1994).

It is currently not clear as to whether a proportion of the effector T cells and $FoxP3^+$ Tregs disseminate through the circulation from the local lamina propria to other mucosal sites of the body during the induction of oral tolerance. This dissemination pattern has however been established for B cells, which migrate from the inductive sites to effector sites through the circulation assisted by adhesion molecules and chemokine-chemokine receptors (Brandtzaeg, 2013). Experiments conducted in mice and humans show that activated B cells migrate from the GALT to the salivary glands (Brandtzaeg, 2013). However, the NALT may be more important for the production of memory/effector B cells that are destined for the salivary glands rather than the GALT (Brandtzaeg, 2013). Therefore, regional antigen stimulation may be more effective initiating appropriate immune responses than immune induction at more distal sites. This could be due to significant differences in the compositions and functions of the immune systems at the various sites, for example, regarding homing properties. The effects of oral tolerance are impaired T-cell proliferation by clonal deletion, clonal anergy or bystander suppression, decreased expression of pro-inflammatory cytokines, and a reduced systemic delayed-type hypersensitivity (DTH) reaction.

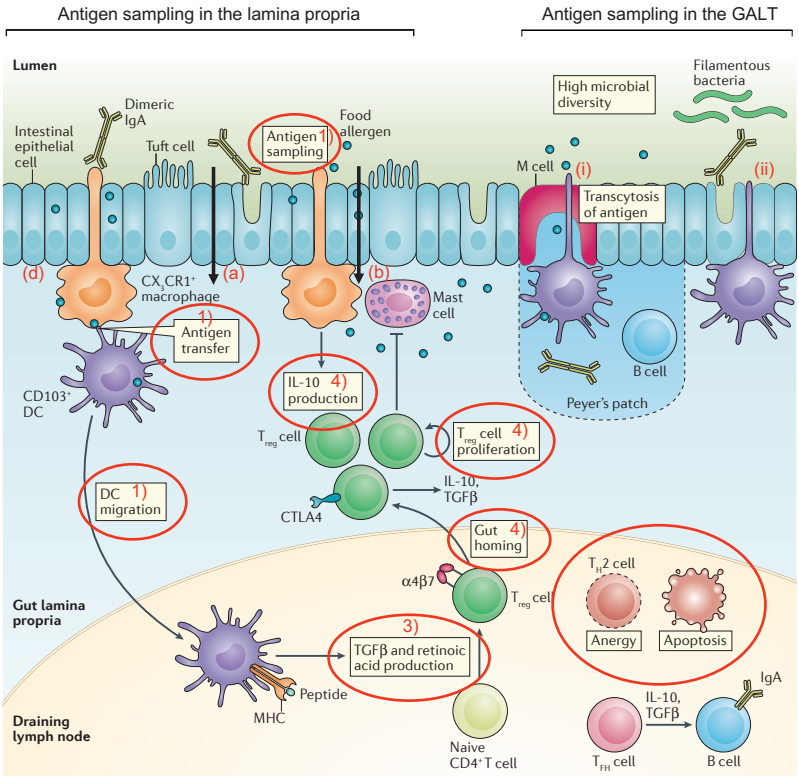

Figure 4. Induction of oral tolerance in the small intestine. *A multi-step process for the induction of oral tolerance has been proposed as follows, resulting in clonal anergy, clonal deletion, as well as the induction and differentiation of Tregs: 1) sampling of the antigen and migration of DCs to the draining lymph node; 2) presentation of the antigen to naïve lymphocytes in the lymph node (not shown in the illustration); 3) induction of gut tropism and differentiation of FoxP3$^+$ Tregs; and 4) homing of antigen-specific T cells and FoxP3$^+$ Tregs to the lamina propria and secondary expansion of the FoxP3$^+$ Tregs. Antigen sampling through the intestinal epithelium occurs in the GALT: (i) through M cells in the FAE of the PPs and ILFs, where the antigens are then loaded onto DCs; or (ii) antigens are sampled directly from the lumen by DCs in the underlying tissue extending their processes through the FAE. Antigens are sampled across the intestinal epithelium to the lamina propria through the following routes: small antigens may pass directly across the epithelium (a) by para-cellular diffusion through pores in the tight junctions that connect the epithelial cells; (b) larger antigens can be taken across enterocytes by transcellular routes, i.e., passing through the epithelial cells; (c) by exosomes; (d) by CX3CRX1-expressing macrophages that extend their processes through the epithelial barrier; (e) through apoptotic bodies derived from intestinal epithelial cells; and (f) by capturing antigens passed on through goblet cells. Routes c), e), and f) are not shown in the illustration. Figure adapted from Yu W et al. 2016. Food allergy: Immune mechanisms, diagnosis and immunotherapy. Nat Rev Immunol. doi:10.1038/nri.2016.111. Oral tolerance model adapted from Pabst O and Mowat AM. 2012. Oral tolerance to food protein. Mucosal Immunol. 5(3):232-239 and Kim KS and Surh CD. 2015. Induction of immune tolerance to dietary antigens. Adv Exp Med Biol. 850:93-118. DC, dendritic cell; FAE, follicular-associated epithelium; GALT, gut-associated lymphoid tissue; ILF, isolated lymphoid follicle, M cell, microfold cell; PP, Peyer's patch, Treg, regulatory T cell.*

Induction of tolerance in the nasal and oral cavities

There must also be mechanisms for establishing tolerance within the nasal and oral cavities since inflammation due to nasal and oral microbiota, food antigens and other environmental factors are rarely seen in these regions. In addition, post-operative infections are seldom observed within the oral

cavity, despite the high bacterial load in saliva, which continuously enters the surgical wound.

It has previously been demonstrated that DCs from the oral cavity have the capacity to induce FoxP3$^+$ Tregs *in vitro*, as has been shown in the intestine (Yamazaki et al., 2012). However, the mechanism of induction seems to be different in the oral cavity, and is not dependent upon CD103$^+$ DCs, RA, and RALDH, as is the case in the intestine (Yamazaki et al., 2012). Other reports have indicated that it may not be the CD11c$^+$CD11b$^+$ DCs that transport the antigens sampled from the lumen of the oral cavity to the CLN, as has been observed in the intestine, where CD103$^+$ DCs transport antigens to the MLN, but instead the antigens in the oral cavity are transported by CD11c^{neg}CD11b$^+$CX3CR1$^+$ cells (Mascarell et al., 2011). Furthermore, the expression of RALDH2 is higher in the lingual mucosa than in the intestinal mucosa (Mascarell et al., 2011). Moreover, it has been demonstrated that RA-producing skin-derived DCs are capable of triggering the generation of Tregs, although these cells are CD103^{neg} and not CD103$^+$ as in the intestine (Guilliams et al., 2010). These results indicate that the induction of FoxP3$^+$ Tregs outside of the intestine is dependent upon the production of RA by RALDH, however CD103$^+$ DCs may not necessarily be involved. Instead, the process may be dependent upon CD11c^{neg}CD11b$^+$CX3CR1$^+$ cells, at least with regards to the oral-associated lymphoid tissues. Both CD103$^+$ and CD103^{neg} DCs have been identified in the NALT (Lee et al., 2015). Previously, no evidence was found to support the notion that mucosal airway DCs can capture pathogens from the lumen of the nasal passage by extending their dendrites (Kim et al., 2011). Recently, CD11c$^+$ DCs have however been shown to sample the luminal content through dendrites in the nasal passage (Qin et al., 2015). Whether or not CD11c^{neg}CD11b$^+$CX3CR1$^+$ cells are present in the NALT is currently unknown.

Previous studies comparing the homing patterns of the NALT and PPs have demonstrated that T cells, generated in the NALT, preferentially home back to the same site, as well as to the CLN and MLN. Reciprocally, T cells from these nodes home successfully to the NALT. However, it has been shown that cells from the PPs do not home as successfully to these sites and that cells from these sites do not home as effectively to the PPs (Kuper et al., 1992). In studies of the homing preferences mediated by the lymphocyte integrins and adhesion molecules on HEVs, it has been observed that homing to the PPs is heavily dependent upon interactions between the integrin α4β7 and the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) (Nakache et al., 1989, Berlin et al., 1993) Conversely, the homing of conventional T cells of the NALT is mediated by homing receptor L-selectin and Peripheral node addressin (PNAd) interactions, whereas the homing of CD4$^+$CD25$^+$ Tregs are more dependent on interactions between P-selectin glycoprotein ligand 1 (PSGL-1) and CD44-HA interactions (Ohmichi et al., 2011). The HEVs in the NALT strongly express PNAd, in contrast to other lymphoid tissues (Berlin et al., 1993). In addition, the interactions that occur between α4β7 integrin on lymphocytes and mucosal addressin cell adhesion molecule 1 (MADCAM-1) on HEVs is of greater importance for lymphocyte homing to the PPs than for lymphocyte homing to the NALT (Ohmichi et al., 2011, Kawashima & Fukuda, 2012). The PNAd and L-selectin interactions, although of greater importance in the NALT, are also of importance for recirculation and homing to all the PLN and MLN, including the CLN (Lyszkiewicz et al., 2011). Here, homing is also mediated by interactions between α4β7 and MADCAM-1 (Lyszkiewicz et al., 2011). Although the NALT is a MALT tissue that is similar to the PPs, its expression pattern of vascular addressins, which are the counter-receptors for the lymphocyte homing receptors, is more similar to that of the PLN (Kawashima & Fukuda, 2012). The ability to home to the oral cavity has not been extensively studied. However, SLIT immunisation in mice results in the dissemination of immunity according to homing patterns similar to those observed for the NALT (Brandtzaeg, 2013). Collectively, the roles of the nasal and oral cavities in the induction of tolerance have not been as well elucidated as those of the intestinal compartment.

Lymph nodes and tolerance

Studies have shown that the removal of specific CLN that drain the nasal mucosa abrogates oral tolerance, and that this can be restored by transplantation of a lymph node from the same site, but not by replacing it with a PLN or MLN (Wolvers et al., 1999, Kraal et al., 2006). In these studies, the NALT remained intact but could not establish oral tolerance on its own. Surgical removal of the MLN also prevents the induction of oral tolerance (Worbs et al., 2006). These results imply that only certain lymph nodes are crucial for the induction of oral tolerance, and that specific lymph nodes are able to compensate for the absent functions of other lymph nodes, if required.

RAS as a consequence of tolerance abrogation

That food components might be factors of importance for the aetiology of RAS remains a highly debated issue within this field of research. For a specific subgroup of patients with this condition, dysregulation of the immune responses to particular food constituents may occur, resulting in an untoward hypersensitivity or allergic reaction in which RAS emerges as part of the clinical picture. However, there is currently a lack of knowledge as regards to the tolerance mechanisms operating in the oral cavity, as the majority of knowledge of tolerance stems from studies of the intestine. Before clinical investigations can be carried out on how tolerance may be abrogated in patients with RAS, more extensive pre-clinical studies of tolerance mechanisms must be conducted.

HYPOTHESIS

During the course of this thesis, I have constructed and implemented a theoretical frame called *'The RAS Model'*, to study the aetiology of RAS (Figure 5). This model has allowed me to examine the roles of both endogenous and exogenous factors in the development of this condition. However, the focus of this thesis has been on the following endogenous factors: host genetics; the oral microbiota composition; and immune regulatory networks.

The overall hypothesis, in accordance with on-going research and results from our department, is that individuals who suffer from RAS acquire a genetic susceptibility for this condition where there in certain individuals exists a hereditary component. Furthermore, these individuals, despite forming a largely heterogeneous group, may share certain factors/features locally in the oral cavity, which are also influenced by the genetic make-up of the individual. These factors/features modulate a specific type of immune response, when exogenous antigens penetrate the oral mucosa, resulting in oral ulcerations.

The local factors in the mouth that influence susceptibility to RAS comprise alterations to: the oral microbiota composition; the structure and permeability of the oral mucosa; the composition of the saliva; and the immune response to antigens. These local factors together with the genetic susceptibility of the host and various systemic factors, such as systemic disease, hematinic deficiencies and/or a hormonal imbalance, enable certain exogenous stimuli, such as different food components, medications, local trauma, pathogens, and/or psychological factors, to trigger lesion development. An altered oral microbiota might promote degradation of the mucin-layer that coats the oral mucosa, thereby promoting increased permeability, which in turn would facilitate exposure of the underlying tissues to antigens, thereby causing ulcerative lesions. Changes to the composition of the saliva might also facilitate this process by reducing the clearance of oral bacteria, allowing for the selection of bacteria in the oral cavity, which would increase degradation of the salivary components. Furthermore, structural changes to the oral mucosa could promote increased permeability. In addition, an altered immune response would facilitate the development of the ulceration.

To define precisely the aetiology of RAS, it is essential to consider it as a multi-factorial condition and to focus on both endogenous and exogenous factors that could potentially trigger the disease and drive the formation of lesions. Effective treatment strategies can be developed only when the aetiology of RAS has been detailed and fully understood.

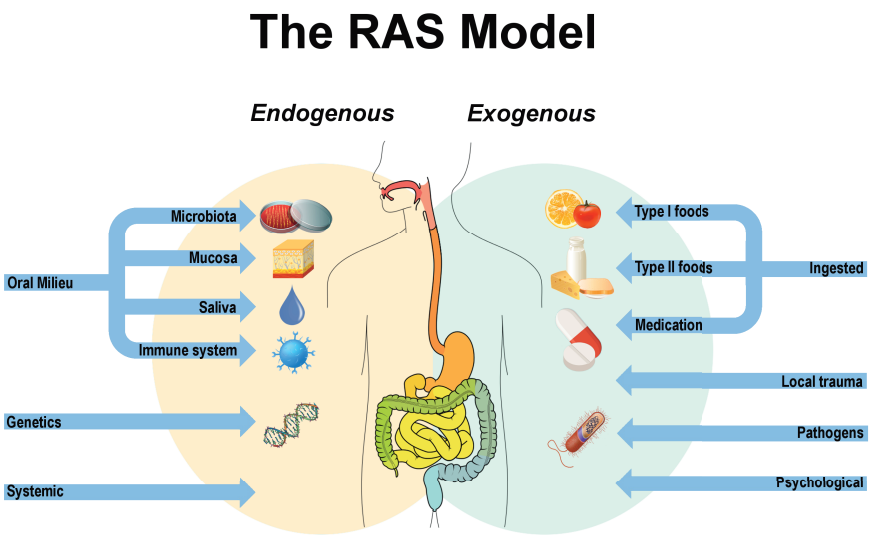

Figure 5. The RAS model. *Both endogenous and exogenous factors are suggested to contribute to the development of an aphthous lesion. The endogenous factors (on the left) consist of local factors in the oral milieu, such as the oral microbiota, the oral mucosa, the saliva, and the local immune system, together with genetics and systemic factors, such as systemic disease, hematinic deficiencies and/or a hormonal imbalance. The exogenous factors (on the right), which trigger lesion development in individuals who are pre-disposed to RAS by some or all of the suggested endogenous factors, consist of ingested substances, such as food components and medications, local trauma, pathogens (e.g., bacteria and viruses), and psychological factors (e.g., stress). Furthermore, the food components are proposed to be divided into type I and type II foods. Type I foods represent acidic foods (e.g., citrus and tomatoes), which in this model are hypothesised to generate an etch wound of the oral mucosa, as opposed to the type II foods (e.g., cow´s milk protein and gluten), which are suggested to provoke a hypersensitivity or allergic reaction.*

OBJECTIVES

Studies I and **II** are clinical studies that involved patients with RAS and that were designed to explore the underlying mechanisms of this condition by investigating the roles of host genetics and the oral microbiota composition in the development of RAS. Furthermore, since food components are considered potential exogenous triggering factors, we also explored the immunoregulatory networks involved in the induction of tolerance within the oral cavity based on existing knowledge of the tolerance mechanisms in the intestine. Therefore, the second part of this thesis, including **Studies III** and **IV** described the results of pre-clinical studies that were conducted in mice to explore the roles of the oral-associated lymphoid tissues, encompassing the NALT and CLN.

Scientific questions

The studies that make-up this thesis were designed to answer the following scientific questions:

STUDY I:

- Are there specific patterns of association and segregation of genetic variants that are passed down to the offspring within families with RAS?
- Which signalling pathways and genes are linked to the genetic basis of RAS?

STUDY II:

- Do the oral microbiota profiles of patients with RAS and healthy control subjects differ?
- Does the oral microbiota profile change in relation to disease activity in patients with RAS?

STUDY III:

- Are there any differences in the phenotypes of the APC and T-cell populations of the oral-associated lymphoid tissues, as compared to the intestinal lymphoid compartment, the MLN, and the peripheral lymphoid compartment, the PLN?
- Are there any differences in the cellular proliferative responses *in vitro* between the NALT and CLN, as compared to the MLN and PLN?

STUDY IV:

- Does the passage of an antigen through the oral cavity contribute to the overall immunological response and the degree of tolerance induced, as compared to the situation in which this passage is bypassed and the antigen is instilled by gavage directly into the stomach?
- Are the activation patterns of ovalbumin (OVA) -specific $CD4^+$ T cells and of Treg differentiation different when the OVA antigen is administered *via* the oral route rather than the gastric route?

MATERIALS AND METHODS

Detailed descriptions of the patients recruited and the materials and methods used in the different studies are found in the respective papers. Described below are the most important details of the materials, the study designs and experimental designs, and short descriptions of the various methods used and the statistical analyses employed.

Materials

Patients

Studies I and **II** include patients with RAS and control subjects, from whom the guidelines of the World Medical Association Declaration of Helsinki were followed. Written informed consent was obtained from all the patients and control subjects after they were fully informed about the aims of the studies and the methods to be used. In addition, for all the enrolled children, written parental consent was also obtained. Both studies were approved by the Regional Ethics Review Board in Gothenburg.

All patients were selected from the referral population at the Clinic of Oral Medicine, Public Dental Health, Gothenburg, Region Västra Götaland, Sweden, in the period 2010–2013 or from our, *'Afte Studie'* Facebook page (www.facebook.com). The use of social media has in our experience been useful for coming into contact with patients who are suffering from RAS and who have an interest in contributing to scientific research. Many patients with RAS have learned through previous encounters with dental and health-care units that there is a lack of consensus regarding the available treatment regimens. Therefore, these patients do not always seek medical help and are infrequently referred to a specialist clinic like our own, unless they are suffering from a severe form of RAS. Thus, social media have provided an excellent forum for recruiting a representative cohort of individuals who are suffering from this condition. Furthermore, enrolling individuals for research purposes can entail logistical challenges. In this aspect, social media provide an efficient mode of rapid communication. The control subjects for **Study I** consisted of family members and for **Study II** consisted of individuals recruited from local Public Dental Health clinics across the region. Clinical data concerning the study subjects were recorded as single data entries in the web-based Medview programme used for research purposes (Jontell et al., 2005).

For both **Study I** and **Study II** the following inclusion and exclusion criteria were applied to patients with RAS, as some individuals participated in both studies, which were run to a certain extent in parallel:

Inclusion criteria

- Episodes of aphthous ulcers at least once a month during the past year

Exclusion criteria

- Diagnosis of other oral mucosal lesions
- Severe caries or periodontal disease
- Smoking

- Excessive consumption of alcohol (alcohol intake more than three times weekly)
- Use of antibiotics or anti-bacterial mouth rinses during the previous month
- Use of drugs for the treatment of mouth ulcers in the previous six months

In **Study I** 16 families with RAS were enrolled, comprising a total of 91 individuals. Overall, 66 individuals were considered to be affected by the disease because they had suffered from RAS at some point in their life (in the past or the present), and 25 individuals were considered to be healthy because they had never suffered from RAS. The healthy individuals served as controls for their affected family members. Therefore, no additional control subjects were enrolled. The rationale used here was that family members are more similar genetically than are individuals randomly selected from a population. In general, family members without a disease differ from members with a disease with regards to aspects of importance for the disease, which is of interest in genetic studies of the type conducted here. Thus, the probability of finding differences related to disease between healthy and diseased individuals within a family are greater than when comparing randomly chosen individuals. Moreover, family-based studies avoid the problem of population stratification bias.

In **Study II** 60 patients with RAS were included, with 42 of the patients presenting with lesions upon sampling. For six of the patients, it was not clear from their oral medical journals whether or not they presented with an ulcer upon sampling. These individuals were therefore excluded from certain parts of the analysis. However, sampling was not carried out in the ulceration itself but instead in the healthy buccal mucosa, distant from the ulcer(s). Equal numbers of healthy age- and gender-matched control subjects were included, and a prerequisite was that the patients and controls were not related to each other or shared a common household, as this could influence their oral microbiota profiles.

Mice

Studies III and **IV** are pre-clinical studies involving experiments on mice that were bred and housed under standard conditions in the animal facility of the University of Gothenburg according to institutional guidelines. Both studies were approved by the Regional Ethics Review Board in Gothenburg. For **Study III** BALB/c mice were used, and for **study IV** BALB/c mice and DO11.10 TCR transgenic mice were used. For **Study III,** all the mice were fed a conventional diet. For **Study IV,** the experimental groups were administered ovalbumin (OVA grade V) as well as conventional chow, and the control groups were fed phosphate-buffered saline (PBS) in addition to the normal diet.

BALB/c – This is a laboratory-bred strain of the common house mouse, albino in colour (white coat with pink eyes), and one of the most widely used inbred strains for animal experiments. The strain was developed by H.J. Baggs in 1913 from a stock of mice obtained from a pet dealer and named the 'Bagg albino', and later was inbred by E.C. MacDowell in 1923 (Potter, 1985). The name BALB/c originates from the strain name ('BALB' from 'Bagg albino') and 'c' from the genotype of the colour locus 'c/c'. Some common sub-strains have been derived over the years. These mice are particularly well suited to experiments in cancer research and immunology. In addition, since it is a Th2-dominant mouse strain (Watanabe et al., 2004), it is particularly appropriate for studies on allergy.

DO11.10 TCR transgenic mice – This is a transgenic mouse strain on a BALB/c background with T-cell receptors that are MHC class II-restricted, i.e., the $CD4^+$ T cells present with a T-cell receptor (TCR) that recognizes exclusively the immunodominant epitope $OVA_{323–339}$ (Robertson et al., 2000).

This strain was originally developed by researchers at Washington University in St Louis, St Louis, MO, USA (Murphy et al., 1990) and has proven particularly useful for studies of tolerance induction.

Samples

Studies I and **II –** Sampling of the buccal mucosa was carried out with a sterile swab (Isohelix/Cell Projects Ltd., Harrietsham, Kent, UK) at a location distant from the ulcer(s). The buccal mucosa is a representative localisation for aphthous ulcers and represents an extensive area of the oral mucosa. All participants were requested not to brush their teeth and not to consume any food or beverage within 1 hour prior to sampling, as a number of these participants were enrolled in both studies, which were in part running in parallel. For **Study I**, one dentist collected all the samples using a standardised technique, according to the protocol of the manufacturer (Isohelix/Cell Projects), except for the family members who lived far from Gothenburg, who sampled themselves at home under careful instruction from the clinician and returned the samples by post. In **Study II**, two dentists participated in the collection of samples using the same standardised technique.

Study III – the following lymphoid tissues were dissected from BALB/c mice: the NALT; CLN (*Ln. mandibularis*, *Ln. mandibularis accessorius,* and *Ln. parotideus superficialis*, all of which are superficial lymph nodes); MLN (*Lnn. jejunales*); and PLN (*Ln. subiliacus*, *Ln. axillaris proprius* and *Ln. axillaris accessorius*). A detailed description of the locations of the various lymphoid tissues is provided in **Paper IV**.

Study IV – The equivalent lymphoid tissues as were used in **Study III** were dissected from DO11.10 TCR transgenic mice that were undergoing the DO11.10 TCR transfer model**.** In addition, the following samples were collected from BALB/c mice that were undergoing the oral tolerance model: broncho-alveolar lavage (BAL) fluid; blood; and spleens.

Study designs/experimental designs

Studies I and **II** were case-control studies, whereby **Study I** was based on patients and their family members, i.e., a family-based case-control study, and **Study II** was based on patients and age-and gender-matched control subjects.

Study III consisted of two parts. The first part was based on a flow cytometric analysis of cells from the BALB/c mice, and the second part involved *in vitro* stimulation of the murine cells with a mitogen.

Study IV involved two separate experimental designs. The first one involved the DO11.10 TCR transfer model, and the second one involved an oral tolerance model.

Methods

Genome-wide association studies (GWAS) (Study I)

GWAS allows examination of the entire human genome at high resolution, across all the chromosomes, without the need for an initial hypothesis as to which genes might be involved in a particular disease (Hirschhorn & Daly, 2005). Previously, genetic analysis of a disease involved carefully selecting potential candidate genes to be analysed in so-called 'candidate gene studies'. With GWAS, thousands of SNPs can be analysed simultaneously in different SNP arrays. The results of a GWAS are used to identify the most common genetic variations that are likely to influence the risk of developing a certain disease (i.e., SNPs that occur more frequently in people with a particular disease than in people without the disease) or to identify heritable quantitative traits (biological traits that show continuous variation, for example, body mass index, blood pressure, and blood lipid levels) that are potential risk factors for disease development. These types of studies are particularly valuable for studying common, complex diseases that are polygenic in nature, as opposed to diseases that entail Mendelian inheritance (Ott et al., 2011). However, GWAS cannot specify the actual causal genes, only the genetic variants that are associated with a disease. Different GWAS approaches can be adopted, and in this study, linkage analysis and association analysis were combined, where the linkage was dependent upon association. This allowed for the investigation of genetic variants separately within each family (linkage analysis) and the segregation of a genetic variant within the population as a whole (association analysis).

Terminal-restriction fragment length polymorphism (T-RFLP) (Study II)

This method, which was developed by Liu *et al.* (1997), has been widely used to study bacterial communities in complex environments, such as the oral cavity, using variations in the 16S rRNA gene, which is present in most bacteria. The 16S rRNA gene contains 1500 bp and is built up of various sequences, some of which are highly conserved among bacteria, while others are from variable regions (V-regions) that differ in sequence between bacterial species. Nine V-regions (V1-V9), ranging in length from 50 bp to 100 bp (Wang et al., 2007), have been identified. All the V-regions are included using this method, in contrast to other methods, for example, next-generation sequencing (NGS) where only particular V-regions are analysed. In T-RFLP, the 16S rRNA gene is amplified and the terminal fragment is fluorescently labelled at the 5´-end during the polymerase chain reaction (PCR), thereby facilitating its detection. The PCR products are then digested with a specific restriction enzyme, and the labelled fragments are separated and detected using capillary electrophoresis. Only the fluorescently labelled fragments are analysed. The rest are ignored. Each fragment generates an individual peak, a terminal-restriction fragment (T-RF), the size of peak being dependent upon the length of the fragment, which represents a specific bacterial taxon, while the pattern of the peaks reveals the bacterial profile of an individual. The principles of T-RFLP are illustrated in Figure 6. In general, T-RFLP permits differentiation to the genus level, and occasionally even to the species level. Only the dominant genera are identified with this method, as it is a semi-quantitative method that allows determination of the presence or absence of a bacterium but is not able to specify the abundance of this bacterium. Moreover, this technique does not differentiate between dead and live bacteria.

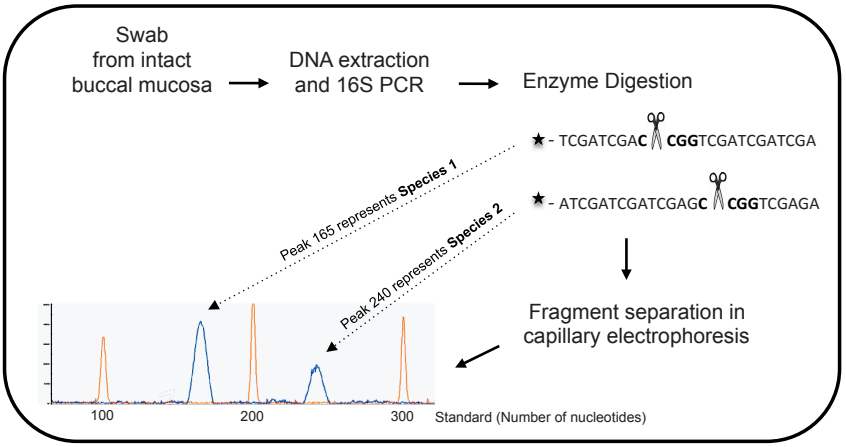

Figure 6. The principles of Terminal-restriction fragment length polymorphism (T-RFLP). *This method is based on variations in 16S rRNA genes within bacterial populations. In **Study II**, bacterial DNA samples were prepared from swabs taken from the intact buccal mucosa of the study subjects and used as a template for PCR amplification of the 16S rRNA genes using universal primers. The PCR products were digested with a restriction enzyme that yielded a fragment fingerprint representative of a specific bacterial species, and the cleaved PCR products were detected with a fragment analyser. The size of each fragment/height of each peak is representative of an individual bacterial genus/species.*

Flow cytometry (Studies III and IV)

This method is widely used to measure the optical and fluorescence properties of individual particles (e.g., cells) based on light scattering (light deflected around the edges of a cell after being struck by light from the excitation source) or fluorescence emission (the light that is emitted (sent out) by an atom or molecule when it passes from a higher energy state back to the original lower energy state after being struck by light from the excitation source). Light scattering gives information about the structural and morphological properties, i.e., size and granularity, while the fluorescence emission gives information about the fluorescent properties of the cells. These features are detected by staining the particles with dyes or monoclonal antibodies using intra- and/or extra-cellular staining. There are various applications for flow cytometry, for example, cell counting, cell sorting, and the detection of biomarkers. The main components of flow cytometers are the fluidics, optics (excitation and collection), electronic network (detectors), and a computer (Adan et al., 2016) (Figure 7).

- **Fluidics:** This involves the ordering of the particles into a single stream through hydrodynamic focusing. The sample in solution is injected into the flow cytometer through a central channel/core, which is enclosed by an outer sheath that contains a faster-flowing fluid (e.g., PBS). As the fluid in the outer sheath moves, the particles in the central channel/core are dragged downwards into a single stream of particles. In the absence of hydrodynamic focusing, the exit of the central channel/core would be blocked due to all the cells wanting to exit at the same time.

- **Optics:** After hydrodynamic focusing, each particle in the stream passes through one or more beams of light. The source of the light is most commonly a laser beam. The optic step is divided into two parts: excitation and collection. Excitation

involves focusing the light source on the particles, whereby lenses are used to shape and focus the laser beam so that the light will hit the particles at the right angle. Collection involves transmitting the light scatter or fluorescent light of the particle to an electronic network. Light emitted from the particle-laser beam interaction is collected by a detector after passing through a filter and is converted to voltage, which allows detection of the signal. Light scattered in the forward direction is collected by a detector known as the forward scatter channel (FSC). The FSC is roughly equivalent to the particle´s size and is useful to distinguish between living cells and dead cells or cellular debris. Light scattered in a sideways direction is collected by the side scatter channel (SSC) and provides information regarding the granularity, i.e., the granular content of a particle, which corresponds to the complexity of the cell. In addition, various optical mirrors and filters are used to separate and direct specific wavelengths of the collected light to the appropriate optical detectors.

- **Electronic network:** This involves detecting the signal and converting it into digital data. The obtained signal is analogue in form and must be converted to a digital signal for computer processing.

- **Computer:** This is used to analyse the data. Different software packages are available for the analysis of flow cytometry data, i.e., the fluorescence properties of the particles. The most common gating strategy used is FSC and SSC plotting.

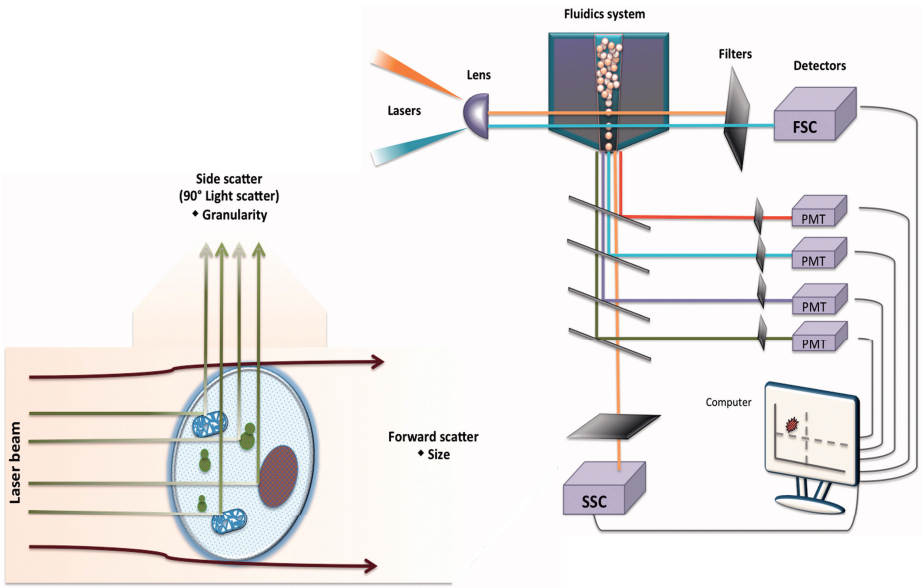

Figure 7. The principles of flow cytometry. *The panel to the left shows light scattered in the forward direction (corresponding to the particle´s size), as well as light scattered in the sideways direction (providing information on the granularity, i.e., the granular content of a particle). The panel to the right shows the main components of flow cytometry. Adapted from Adan et al. 2016. Flow cytometry: Basic principles and applications. Crit Rev Biotechnol.1-14.*

Cell proliferation assays (Studies III and IV)

This method is used under *in vitro* conditions to measure the rate of proliferation of cells at a certain time-point when the cells are exposed to a specific proliferation-inducing agent, for example, an antigen or a mitogen. As the entire course of proliferation is not analysed it is necessary to select a time-point at which the process of proliferation has begun but before all the cells have proliferated or the medium has been completely depleted. Mitogens are molecules that can activate T cells and/or B cells regardless of the antigen-specificity of the cells. Some mitogens are lectins that bind to carbohydrates found in the cell membrane of the lymphocytes. However, there are also mitogens that are not lectins, and thus induce proliferation through other mechanisms. A mitogen can activate a large proportion of lymphocytes, such as 5–10%. Conversely, the proportion of cells that are activated after exposure to a specific antigen is fractions of a per mille of lymphocytes. Commonly used mitogens are phytohaemagluttinin (PHA; mitogenic for human cells) and concanavalin A (Con A; mitogenic for murine cells). A pre-defined number of cells are seeded into the wells of a 96-well plate, after which the proliferation-affecting agent is added to a certain number of the wells. The remainder of the wells, which contain cells but not the proliferating agent, functions as control wells. The plate is then incubated at 37°C in an incubator. Thymine is one of the nucleotides in DNA and thymidine is a nucleoside (a nucleotide that lacks the phosphate group). Since thymidine closely resembles thymine, it is easily incorporated into the DNA strand. Thus, the cell proliferation test plate is removed from the incubator, ^{3}H-thymidine is added, and the plate is re-incubated for an additional period of time. Proliferating cells incorporate the radioactive thymidine into their DNA during each round of cell division. After the incubation step, the cells are harvested by washing the cells from the plate. The cell materials, and especially the radioactive DNA, adheres to a filter and the number of cells in the well or the number of cell divisions during incubation is measured using a scintillation counter (Kruisbeek et al., 2004).

The DO11.10 TCR transfer model (Study IV)

In general, an adoptive cell transfer implies the transfer of cells from a donor to a recipient, for example, from one strain of mouse to another, for the purpose of studying a specific cell line of interest. Regarding T cells, a successful adoptive T-cell transfer depends on the ability to select cells from a donor or to engineer genetically cells with targeted antigen-specificities, and thereafter induce the cells to proliferate while preserving their effector functions and homing abilities in a recipient (June, 2007). The labelled cells are either endogenously fluorescent, i.e., isolated from a transgenic mouse that expresses the fluorescent protein of interest, or the cells are labelled before transfer (Matheu et al., 2011). In the current study, DO11.10 TCR cells were transferred from DO11.10 TCR-transgenic mice into BALB/c mice, according to a modified version of the protocol devised by Kearny *et al.* (1994). DO11.10 mice have an abundance of OVA-specific T cells, which does not represent the natural state. However, DO11.10^{+}CD4^{+} T cells allow for the detection of OVA-specific CD4^{+} T cells. The KJ1.26 monoclonal antibody is used to detect these cells (of the clone KJ1.26) by binding to the T-cell receptor (TCR), that they express (Murphy et al., 1990). The use of DO11.10 and KJ1.26 cells has proven valuable for uncovering many of the intricate details of T cell-mediated immunity.

The oral tolerance model (Study IV)

The most common way of inducing tolerance in an experimental animal model involves three separate phases:

- **Initial exposure:** The mouse, preferably a young naïve mouse, is fed a specific antigen (e.g., OVA) through the mucosal route in the diet (food or water). The naïve mouse has not previously been exposed to this antigen so the animal develops tolerance.

- **Sensibilisation:** In this phase, the mouse is immunised with the same antigen (in this case OVA) in combination with an adjuvant [e.g., Freund´s complete adjuvant (FCA), to initiate a Th1-type response)] or Alum (aluminium hydroxide, to initiate a Th2-type response). The animal is challenged with the antigen through a non-mucosal route, for example, with an injection into the skin or blood-stream. The adjuvant enhances the immune response if tolerance has not previously been developed. However, if tolerance has already been established the adjuvant does not contribute to an increased immune response but rather induces a decreased response or no response. Responses to other antigens are not affected.

- **Challenge:** The mouse is exposed to a high dose of OVA to evoke an immunological reaction. The route of administration dictates the animal´s physiological response: if nasally, the outcome is airway allergy; administration of the antigen by gavage gives diarrhoea; and administration through the skin results in a delayed-type hypersensitivity reaction (DTH).

In this particular study a modified version of the acute model of allergic airway disease was adopted (Meyer-Martin et al., 2014).

Enzyme-linked immunosorbent assay (ELISA) (Study IV)

This is a plate-based assay technique designed for the detection and quantification of peptides/proteins, antibodies or hormones in a sample using antigen-antibody reactions and a colour change based on an enzyme-linked conjugate and an enzyme substrate (Aydin, 2015). There are three different types of ELISA: direct assay; indirect assay; and capture assay (sandwich).

- **Direct assay:** This is the simplest form of ELISA. An antigen is fixed to a solid surface by adsorption to the assay plate. Then, a labelled primary antibody is added to bind to the antigen. The primary antibody is labelled with an enzyme [horse-radish peroxidase (HRP) and alkaline phosphatase (AP) are the most commonly used]. This results in the formation of an antigen-antibody-enzyme complex. Any excess of the complex is washed away and the enzyme activity is detected by adding an enzymatic substrate to produce a visible signal colour, which can be measured spectrophotometrically and compared to a standard curve to calculate the quantity of antigen in the sample.

- **Indirect assay:** The antigen is fixed to a solid surface of the plate in the same way as for the direct assay. Then, an unlabelled primary antibody is added, followed by a labelled secondary antibody. This results in the formation of an antigen-antibody-enzyme complex. Any excess of the complex is washed away and the enzyme activity is detected by adding substrate.

- **Capture assay (sandwich):** A capture antibody is attached to the plate. The antigen binds to the capture antibody, after which a primary antibody is added, followed by a labelled secondary antibody, which allows for detection of the antigen-antibody-enzyme complex.

To increase the specificity of the method, i.e., to ensure a stable antigen-antibody-enzyme complex that is not mistakenly washed away, biotinylation may be applied. The antibody to be linked to the enzyme (the primary antibody or the secondary antibody, depending on whether the assay used is direct or indirect) is labelled with biotin and the enzyme is labelled with streptavidin. Biotin and streptavidin have a high affinity for each other. Lastly, the antigen-antibody-enzyme complex is detected in a spectrophotometer, a fluorometer or a luminometer. Determination of the antigen concentration in a sample requires the production of a standard curve using antigens of a known concentration. The concentration of antigen in a sample can then be calculated using the optical density (OD) values.

This method was used to detect the total IgE levels in sera of the mice, as well as the OVA-specific IgE and IgG1 and IgG2a. The latter three factors were detected using specific kits, as described in **Paper IV**. The production of OVA-specific IgE and eosinophil recruitment to the lungs are both common features of allergic airway inflammation in OVA-immunized mice. These parameters are however, easily affected, which implies that the production of OVA-specific IgE and the recruitment of eosinophils may decrease despite the fact that the selected OVA dosage may not be sufficient to induce oral tolerance. A previous study has shown that there is a hierarchical pattern of suppression during the induction of oral tolerance, whereby IgG1 (produced during a Th2-type response) is more resistant to suppression. Therefore, IgG1 may be a better parameter to measure if one wants to determine whether or not oral tolerance has truly been induced (Keller et al., 2006).

Cytospin (Study IV)

This is a rapid cytological method that has been specifically designed to collect and concentrate hypocellular fluids. After the actual cytospin is completed, other methods, such as immunocytochemistry, can be performed to identify the cells. This method can also be used to identify cells that have previously been processed for flow cytometry. Initially, the cells are washed in a protein-containing medium, for example, a culture medium or a serum- and/or albumin-based PBS solution. Thereafter, a maximum of 0.5×10^6 cells/ml are re-suspended in up to 500 µl of the solution (in the current study, 100,000 BAL cells in 500 µl were used), and each sample is pipetted into a plastic chamber, placed in a cytocentrifuge, and forced by centrifugation onto a glass slide (Shanholtzer et al., 1982). The cells can be spun at various speeds and times depending on the cell type (in the current study, we used 800 rpm for 3 minutes). The volume-to-cell ratio must be sufficiently dilute to ensure the formation of a monolayer of cells. The slides are then dried, fixed, and stained (for **Study IV** staining with May–Grünwald/Giemsa stain was performed). For the current study, 200 cells were counted for each sample and classified as eosinophils, neutrophils or other nucleated cells (Figure 8). The percentage of each cell type was calculated as the percentage fraction of the total number of cells. Eosinophils are typically present in samples from patients with allergies and asthma. Neutrophils are the most abundant type of white blood cells and are the first cells to arrive at the site of an infection.

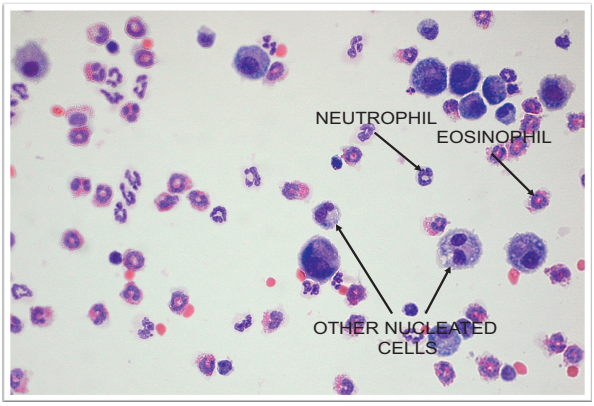

Figure 8. Light microscopy image of a cytospin preparation of murine bronch-oalveolar lavage (BAL) fluid stained with May–Grünwald/Giemsa. *The arrows indicate an eosinophil, a neutrophil, and other types of nucleated cells, which were the cells that were counted in* ***Study IV*** *using the IMAGEJ software (http://imagej.nih.gov/ij/). The percentage of each cell type was calculated as the percentage fraction of the total number of cells.*

Statistical analysis

Study I – The data were analysed using the free, open-source, whole genome association analysis software PLINK ver. 1.07, (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007) and the licensed software SNP & Variation Suite ver. 8, (http://goldenhelix.com/SNP_Variation/) (Golden Helix Inc., Bozeman, MT, USA). These software packages are used for complex analyses and the visualisation of genomic and phenotypic data, which allows for the management of data, the performance of statistical checks for quality control, and the carrying out of basic association testing. Data management was performed with the PLINK software, and quality controls were carried out using the SNP & Variation Suite software. For testing linkages in the presence of association, the GWAS approach chosen for this particular study, the dFAM analysis (family-based association for disease trait), was run in PLINK. The dFAM test allows for comparisons to be made between family members (linkage analysis), as well as between different families (association analysis). dFAM incorporates the transmission disequilibrium test (TDT), the sibling TDT, and case-control data into a single Cochran-Mantel-Haenszel test for each marker. Since they are all incorporated into the same test, it is not possible to distinguish the results of one test from another. A genome-wide significant threshold of 5×10^{-8} was applied. This threshold has been proposed for declaring a significant association in a GWAS, since it corresponds to a *p*-value of 0.05 after a Bonferroni (Dunn´s) correction for 1 million independent tests. In a GWAS, thousands of genetic variants are analysed, which is both costly and labour-intensive. Therefore, there is a limit to the sample size. However, since variants that contribute to complex diseases are likely to have modest effects, the use of large sample sizes is important. Since multiple testing is performed, the *p*-values must be corrected. Therefore, this threshold has been proposed (Risch & Merikangas, 1996). Thereafter, set-level statistics, in the GeneTrail2 software, were used for pathway analysis of the data. GeneTrail2 is a web-interface that provides access to different tools for the statistical analysis of molecular signatures (https://genetrail2.bioinf.uni-sb.de/) (Stockel et al., 2016). This approach allows one to ascertain if a certain biological pathway is significantly enriched or depleted among the identified genes to an extent greater than would be expected by chance. Eventually, a pathway is declared to be significantly

enriched if the upper-tailed *p*-value (H_1: $\mu>\mu_0$) of a test is significant, and is judged to be depleted if the lower-tailed *p*-value (H_1: $\mu<\mu_0$) is significant. The specific test chosen for this particular step was over-representation analysis (ORA), which relies on a reference set for comparison. The test examines whether the entries that belong to a certain pathway are more or less present in the data-set, as compared to the reference set. In an enrichment analysis, multiple pathways are tested simultaneously, which requires *p*-value adjustments. False discovery rate (FDR) adjustments were made with the Benjamini-Yekutieli procedure. FDR corrections identify the rate of type I errors (false-positive predictions) in null hypothesis testing when conducting multiple comparisons. The Benjamini-Yekutieli procedure controls the FDR. The significance level was set at $p<0.05$. The minimum size of the pathway was 2 genes and the maximum size was 700 genes.

Study II - Multivariate analysis was used to project associations for the microbial patterns between the patients with RAS and the controls, as well as within the group of patients with RAS. This was achieved using a Partial Least Squares-discriminant analysis (PLS-DA) in the SIMCA P+ ver. 13 software (Umetrics AB, Umeå, Sweden). Thereafter, univariate data analysis was performed, according to the SIMCA analysis, on relevant T-RFs (corresponding to different bacterial taxons) using the Fisher's exact test. Adjustments for mass-significances were not performed, since the univariate analysis was based on SIMCA results, where only the most important peaks were analysed further. The Fisher´s exact test is used to compare small data-sets with two independent unpaired groups with binomial data (only two possible outcomes; the T-RFs do not assume numerical values). Univariate data analysis regarding richness was performed using the Mann-Whitney U-test, as the data were not normally distributed according to The D´Agostino-Pearson omnibus normality test. If the data had passed this test an unpaired *t*-test would have been preferred for the richness analysis. The Mann-Whitney U-test is a non-parametric test that is used for the comparison of two independent groups (equivalent to the Students *t*-test used for a normally distributed data-set). All the univariate analyses were performed in GraphPad Prism, with *p*-values <0.05 being considered statistically significant. Furthermore, Shannon's Index ($H = -\sum_{i=1}^{s} piInpi$) was used to estimate the diversity (richness and evenness). A high H-value represents a diverse and equally distributed bacterial community, whereas a low H-value characterises a bacterial community with lower levels of diversity. A H-value of 0 represents a community with just one taxon. Evenness within a group was calculated with Shannon's Evenness Index ($E=H/lnS$), which assumes a value between 0 and 1, where 1 represents complete evenness. The statistical methods used in this study are explained in detail in **Paper II**.

Study III - Statistical analysis was conducted using the Kruskal-Wallis test followed by Dunn´s correction for comparisons of the percentages of cells in the various lymphoid compartments. Here *p*-values <0.05 were considered statistically significant. The reason for using this statistical test was that there were a fair number of outliers in the data-set, suggesting that the data were not normally distributed. However, the number of mice was too low to explore this notion. Therefore, a non-parametric test was applied, in this case the Kruskal-Wallis test, which is the equivalent of a one-way ANOVA (used with a normally distributed set of unpaired, numerical data when comparing three or more groups that are independent). For this reason, the Kruskal-Wallis test is not as powerful as the one-way ANOVA. Non-parametric tests are based on median values and therefore, they handle outliers more adequately. To use the median value the data are sorted in ascending order and the middle value represents the median. This means that 50% of the values lie above the median and 50% lie below the median value. This is why the median is shown with percentages. Within these tests a number of tests are performed step-wise to compare the groups. Therefore a *post hoc* test is required (Tukey´s test for the one-way ANOVA and Dunn´s correction for the Kruskal-Wallis test).

Corrections must be made when many tests have been carried out, in order to adjust the level of significance for the number of tests that have been performed.

Study IV - Statistical analysis was conducted using the Kruskal–Wallis test followed by Dunn´s correction for comparisons of the percentages of cells in the various lymphoid compartments. For comparisons of the OVA-fed mice and the PBS-fed control mice, the Mann-Whitney U-test was performed since, as this is a non-parametric test for the comparison of two independent groups (equivalent to the Students *t*-test used for a normally distributed data-set). Here *p*-values <0.05 were considered statistically significant. A correlation analysis was conducted using the Spearman´s rank correlation, as the data were assumed not to be normally distributed; if instead the data had been normally distributed a Pearson´s correlation coefficient would have been used. The correlation coefficient is a measure of linear association between two variables. A coefficient of 1 implies a strong positive correlation, whereas a coefficient of -1 implies a strong negative correlation. A coefficient of 0 corresponds to a weak correlation. For the correlation analysis, the data from all of the different sites and for both routes of administration was processed in the same analysis.

RESULTS

Detailed descriptions of the results are found in the respective papers. Therefore only the most important findings are presented below.

STUDY I

This study was conducted using a genome-wide association approach designed as a family-based case-control study in which a total of 16 families with RAS (N=91) were included and each individual provided a buccal swab sample. The family members that had never suffered from RAS (N=25) served as controls for the individuals that suffered from this condition at the present time or that had suffered from RAS at some point in the past (N=66).

Are there specific patterns of association and segregation of genetic variants that are passed down to the offspring within families with RAS?

In total 547,644 SNPs derived from across all the chromosomes, were analysed in the CoreExome array, with a certain proportion of the SNPs being removed due to high rates of missing calls or Mendelian inconsistencies or low minor allele frequencies, leaving a total of 288,452 SNPs for the final analysis. The specific aim was to test for linkage in the presence of association in the selected families, thereby allowing comparisons within the family using linkage analysis, and on a population level, comparing between the families, using association analysis. This step was performed with a dFAM test.

The dFAM analysis revealed that none of the included SNPs reached the genome-wide significant threshold of 5×10^{-8}. Therefore, no pattern of association or segregation of genetic variants that were passed down to the offspring within these families was detected. Moreover, no specific gene with a Mendelian pattern of segregation for the disease as a whole was detected, which means that no one specific gene was found to be more significant than any other gene with respect to causing RAS. This confirms previous findings that RAS is not a monogenic disease.

Which signalling pathways and genes are linked to the genetic basis of RAS?

The top 1000 associated SNPs with the best *p*-values from the dFAM analysis were selected for the subsequent pathway analysis. The KEGG and Reactome pathway databases were applied to identify pathways that influence the risk of developing RAS. From the KEGG database, the Ras signalling pathway, the PI3K-Akt signalling pathway, pathways in cancer, circadian entrainment, and the Rap 1 signalling pathway were identified. In the Reactome database, the G alpha (i) signalling events pathway and the peptide ligand-binding receptors were recognised as being of importance. The SNP rs4705140, which corresponds to the gene PPP2R2B, was found to be significantly overrepresented in the above analysis. The rs4705140 SNP was linked to the PI3K-Akt signalling pathway, the dopaminergic synapse pathway, and the mRNA surveillance pathway. Overall, these results give clues as to the signalling pathways and genes that influence the risk of developing RAS.

STUDY II

For this case-control study, 60 patients suffering from RAS and 60 age- and gender-matched control subjects were selected. Buccal swabs were obtained at a single time-point, with 42 of the patients presenting with a lesion upon sampling. The bacterial DNA was extracted and the 16S rRNA gene was analysed using the T-RFLP methodology. Multivariate analyses were performed to identify differences in the oral microbiota profiles between the patients and controls, as well as between the patients. Furthermore, univariate testing was conducted to analyse the T-RFs that were identified as being of importance in the multivariate analyses, so as to separate the groups. In addition, diversity measures were carried out.

Do the oral microbiota profiles of patients with RAS and healthy control subjects differ?

A wider range of T-RFs (each T-RF corresponding to a specific bacterial taxon) was identified in the controls than in the patients (156 versus 118, making up a total of 192 different T-RFs in the overall study population). Despite this, the richness was equal between the two groups as well as the diversity measured with Shannon´s diversity index. However, the Shannon´s evenness index showed a slight tendency towards a higher value in the patient group, as compared to the control group, indicating that the numbers of bacteria within the different taxa may be lower in the RAS group than in the control group.

Comparing the oral microbiota profiles between the two groups, differences were observed, in that the patients clustered together as one group and the controls clustered together as another group, with both groups being separate from one another, indicating a difference in their microbiota profiles. Furthermore, the univariate analysis revealed a number of T-RFs that were associated with the patients, and others that were associated with the controls. Overall, these results indicate a difference in the oral microbiota profiles between patients with RAS and control subjects. With this methodology, it is however not possible to determine the specific differences in the profile, regarding the represented bacterial taxons. However, in an *in silico* analysis, the T-RFs present in the patients with RAS did not correspond to the bacterial species *Streptococcus sanguinis*, *Streptococcus oralis* or *Helicobacter pylori*, which have previously been associated with RAS.

Does the oral microbiota profile change in relation to disease activity in patients with RAS?

A more comprehensive analysis of the oral microbiota profiles within the group of patients with RAS was performed, whereby the patients were sub-divided into the following groups: 1) *Presence of a lesion* (reflecting whether or not the patient presented with a lesion at the time of sampling); 2) *Lesion size* [reflecting whether the patient suffered from minor RAS (Ø<10 mm) or major RAS (Ø>10 mm) at the time of sampling]; and 3) *Regular medication* (reflecting whether or not the patient was on medication at the point of sampling). These parameters were chosen because they could potentially influence the composition of the microbiota. The study group comprised 60 patients in total, with 30 individuals who suffered from minor RAS and 30 individuals who suffered from major RAS at the time of sampling. Of the total group of 60 individuals, 42 presented with a lesion upon sampling (25 with minor RAS and 17 with major RAS) and 24 individuals used some form of regular medication (excluding the use of antibiotics).

Multivariate analysis was performed to take into account the presence or absence of a lesion and if the patient suffered from minor or major RAS upon sampling. The use of medication was excluded, since the groups, based on the sub-division according to the type of medication, would have become too small and skewed to allow performance of a reliable analysis. Again, a difference in the oral microbiota was observed between the patients with RAS and the controls, whereby the patients that presented with a lesion upon sampling clustered furthest away from the controls, especially when the patients presented with a lesion of the major type upon sampling (Figure 9). In addition, for the peaks that were identified as being of importance in the patients with RAS as a whole compared to the control group, univariate analyses was performed now with the different subgroups of patients.

Overall, the results showed that the presence of a lesion altered the oral bacterial composition and, to a certain extent, the size of the lesion also contributed to this change. The skewness of the groups must, however, be taken into consideration here, and it should be emphasised that the presence of a lesion appeared to have a greater impact on the composition of the microbiota than did the actual size of the aphthous lesion. Univariate analysis revealed that the use of medication did not seem to influence the composition of the oral microbiota. In conclusion, it was not possible to ascertain whether the presence of a lesion alters the microbiota of the oral cavity or if a changed microbiota triggers the development of lesions.

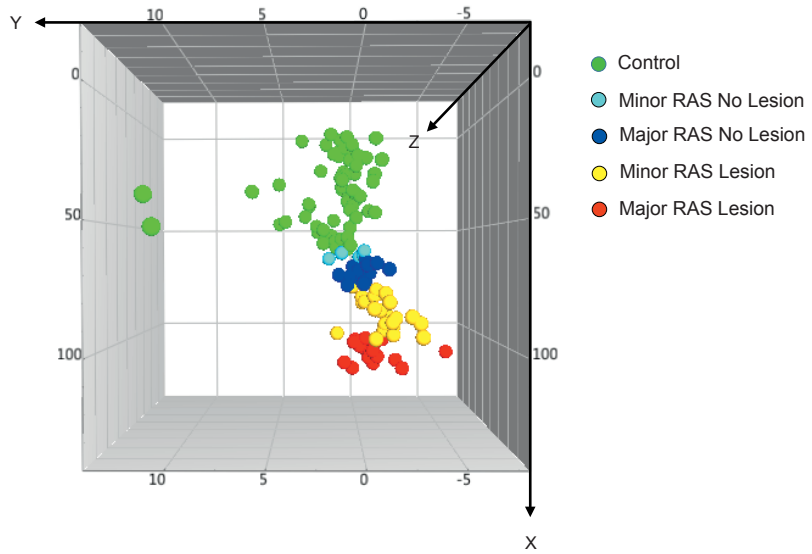

Figure 9. Multivariate analysis reveals that the presence of lesions during sampling contributes to the observed differences in oral microbiota profiles between the patients with recurrent aphthous stomatitis (RAS) and control subjects. *The patients with RAS were assigned to subgroups based on: 1) the size of the lesion (minor RAS Ø<10 mm and major RAS Ø>10mm); and 2) presenting with or not presenting with a lesion at the time of sampling (RAS Lesion/RAS No Lesion). A Partial Least Squares-discriminant analysis (PLS-DA) 3D-score scatter plot depicts the relationship between the X-variables, i.e., the buccal microbiota (identified as T-RFLP peaks) and the Y-variables, i.e., the study population (identified as minor RAS patients with and without lesions, major RAS patients with and without lesions, and control subjects). Each symbol represents the peak pattern of one individual. Individuals with similar buccal microbiotas cluster together, whereas individuals that have different microbiotal profiles are separated. The six major RAS patients for whom it was not known if they presented with a lesion upon sampling were excluded from this part of the analysis. R2Y indicates how well the variation of Y is explained, while Q2 indicates how well Y can be predicted. For this model: R2Y = 0.35 and Q2 = 0.03.*

STUDY III

For the identification and comparison of the different murine APC and T-cell subsets, BALB/c mice were sacrificed and the NALT, CLN, MLN, and PLN were dissected and prepared as single-cell suspensions before staining for flow cytometry. In addition, an *in vitro* proliferation assay involving treatment with the mitogen Con A was performed. Proliferation was estimated by radioactive ^{3}H-thymidine incorporation.

Are there any differences in the phenotypes of the APC and T-cell populations of the oral-associated lymphoid tissues, as compared to the intestinal lymphoid compartment, the MLN, and the peripheral lymphoid compartment, the PLN?

Overall, the oral-associated lymphoid tissues displayed certain variations in their APC and T-cell subsets compared to their intestinal and peripheral counterparts, the MLN and PLN, respectively. The largest differences were observed between the NALT and the other sites, i.e., the CLN, MLN, and PLN, although the CLN also displayed variances compared to the MLN and PLN, while showing features characteristic of both these tissues.

The NALT had a greater abundance of APCs (approximately 4.2% of the live gated cells) as compared to the CLN, MLN, and PLN (approximately 1.5% for each). Differences in the activation patterns of the APC subsets, categorised based on the markers CD11c and CD11b, were also observed between the different sites. The $CD11c^{+}CD11b^{+}$ DCs, which were abundant in the NALT, displayed a low degree of activation with limited expression of CD40, CD86, and IAd (MHC class II), as compared to the CLN, MLN, and PLN (Figure 10). The CLN and PLN showed the highest degrees of activation of this subset, with similar expression levels of CD40 and CD86, which were higher than those of the MLN, whereas the expression levels of IAd were similar in all three sites (Figure 10). Furthermore, CX3CR1 and CD206, which are markers associated with a tolerogenic APC function, were more readily expressed in the $CD11c^{neg}CD11b^{+}$ APC subset, which was also found abundantly in the NALT, as compared to the CLN, MLN, and PLN, where the expression levels of these markers were similar (Figure 10). Overall, the NALT presented with the highest percentages of APCs among the lymphoid compartments; while the degree of activation of APCs was lower in the NALT, the tolerance-inducing capacity was greater than at the other sites.

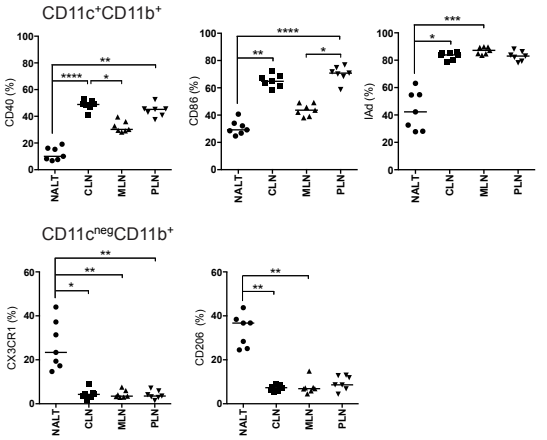

Figure 10. Expression of markers associated with cell activation and the capacity for tolerance induction in various antigen-presenting cell (APC) populations. *The tissues of BALB/c mice were analysed by flow cytometry for the expression of CD40, CD86, IAd, CX3CR1, and CD206 in the nose-associated lymphoid tissue (NALT), cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), and peripheral lymph nodes (PLN). The results are presented in representative dot plots. Each dot represents an individual mouse. The horizontal lines indicate the median values. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, as analysed using the Kruskal-Wallis test with Dunn´s correction.*

In general, the proportions of $CD4^+$ and $CD8^+$ T cells were lower in the NALT than in the lymph nodes, i.e., the CLN, MLN and PLN, which displayed similar proportions of these cells (Figure 11). The number of tTregs ($Helios^+FoxP3^+$) was lowest in the NALT and highest in the CLN, whereas the distribution of pTregs ($Helios^{neg}FoxP3^+$) was similar at all four sites (Figure 11). Interestingly, the NALT contained a high percentage of $Helios^+FoxP3^{neg}$ $CD4^+$ T cells, which was not observed for the other sites, which demonstrated similar (lower) percentages of these cells (Figure 11). This T-cell population has not been well-characterised in the literature but has been proposed to exert immunoregulatory functions.

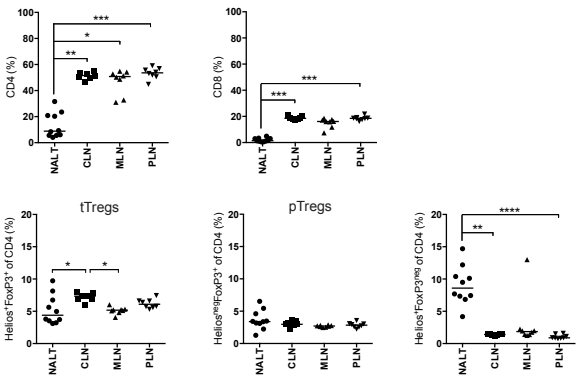

Figure 11. CD4, CD8, and regulatory T-cell (Treg) populations in various murine lymphoid tissues. *The tissues of BALB/c mice were analysed by flow cytometry for the expression of CD4, CD8, Helios, and FoxP3 in the nose-associated lymphoid tissue (NALT), cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), and peripheral lymph nodes (PLN). The results are presented in representative dot plots. Each dot represents an individual mouse. The horizontal lines indicate the median values. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, as analysed using the Kruskal-Wallis test with Dunn´s correction.*

Furthermore, the $CD4^+$ and $CD8^+$ T cells of the NALT expressed low levels of CD45RB, indicating that there are not many naïve T cells in this compartment in comparison to the other sites, where almost all the cells expressed this marker (Figure 12). Instead, the percentage of memory/effector T cells in the NALT was higher. In addition, the levels of expression of the homing markers α4β7, CCR4, and CCR9 on both the $CD8^+$ and $CD4^+$ T cells where higher in the NALT than in the other sites, which displayed low levels of these markers (Figure 12; only data for the $CD4^+$ T cells are shown, as the same trend was observed for the $CD8^+$ T cells). Overall, the results indicate that these cells have an effector T-cell function rather than a T-cell activation function, and that the homing capacity of the cells in the NALT is greater, with homing occurring to both the intestine and the skin.

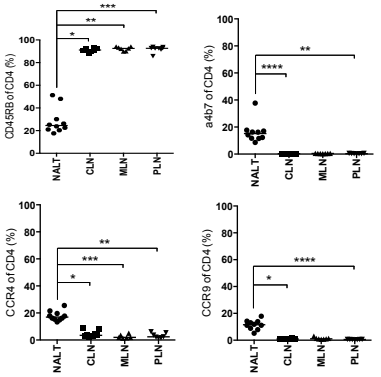

Figure 12. Expression of markers of activation and homing in the $CD4^+$ T-cell populations. *The tissues of BALB/c mice were analysed by flow cytometry for the expression of CD45RB, α4β7, CCR4, and CCR9 in the nose-associated lymphoid tissue (NALT), cervical lymph nodes (CLN), mesenteric lymph nodes (MLN) and peripheral lymph nodes (PLN). The results are presented in representative dot plots. Each dot represents an individual mouse. The horizontal lines indicate the median values. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, as analysed using the Kruskal-Wallis test with Dunn´s correction.*

Are there any differences in the cellular proliferative responses in vitro *between the NALT and CLN, as compared to the MLN and PLN?*

The cellular proliferation rates varied between the different sites; the NALT showed no proliferative response at all to stimulation with Con A, while the MLN showed the highest level of proliferation (Figure 13). The proliferation rates were similar for the CLN and PLN (Figure 13). The lack of induced proliferation shown by the cells in the NALT also indicates that this site is not of importance for T-cell activation. Instead, these cells may convey effector functions, whereby the low level of T-cell proliferation observed in the NALT reflects an on-going active suppression by cells with a regulatory capacity.

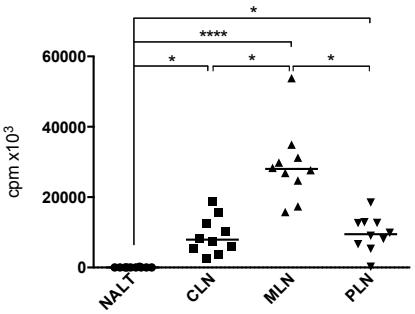

Figure 13. Cellular proliferation following *in vitro* stimulation with concanavalin A (ConA). *Single-cell suspensions (1×10^5 cells/well) derived from the nose-associated lymphoid tissue (NALT), cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), and peripheral lymph nodes (PLN) were cultured and stimulated with ConA (0.5 µl/well). Proliferation was estimated by radioactive thymidine incorporation. The results are presented in a representative dot plot (cpm = counts per minutes). Each dot represents an individual mouse. The horizontal lines indicate the median values. *$p<0.05$, and ****$p<0.0001$, as analysed using the Kruskal-Wallis test with Dunn´s correction.*

STUDY IV

In this study, both an oral tolerance model of induced airway hypersensitivity to OVA and a DO11.10 TCR transfer model were used.

Does the passage of an antigen through the oral cavity contribute to the overall immunological response and the degree of tolerance induced, as compared to the situation in which this passage is bypassed and the antigen is instilled by gavage directly into the stomach?

Naïve BALB/c mice were fed a semi-effective dosage of 1 mg OVA through the diet by either oral or gastric administration. A low dose of OVA was intentionally administered to avoid establishing complete oral tolerance, which would have made it difficult to compare the two different routes of administration. Six days after OVA administration, the mice were immunised intraperitoneally with 10 µg of OVA in Alum, and this was step was repeated 10 days after the first immunisation. Nine days after the last immunisation, a challenge was performed in which 100 µg OVA was administered nasally over four consecutive days, in order to evoke an airway hypersensitivity reaction. Twenty-four hours after the final challenge, BAL fluids, blood, and spleens were collected from the mice and analysed to determine if there was a difference in the immunological response between the two routes of antigen administration. Control mice were fed PBS, with half of them undergoing oral administration and half undergoing gastric administration, and then pooled to constitute a single control group that was used for both experimental set-ups. The experimental set-up is shown in Figure 14.

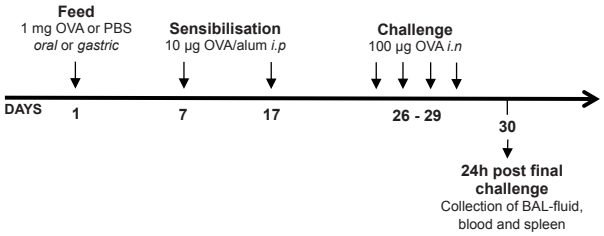

Figure 14. Experimental design of the oral tolerance model involving ovalbumin (OVA)-induced airway hypersensitivity in BALB/c mice.

The two routes of administration were equally efficient at inducing tolerance in the model of airway hypersensitivity, as both groups of mice displayed significantly lower levels of OVA-specific IgE in their sera, as compared with the PBS-fed mice (Figure 15). It should also be mentioned that there was a difference in total IgE levels for the two groups compared with the control group. When the Kruskal-Wallis test was used to compare the three groups together, the *p*-value for the gastric group versus the corresponding PBS-fed group was 0.03, and the *p*-value for the oral group versus the corresponding PBS-fed group was 0.059, which is close to the threshold of significance of $p<0.05$. When analysed with the Mann-Whitney U-test, there was a significant ($p=0.01$) difference between the oral group and the corresponding PBS group. Lastly, reduced numbers of eosinophils in the BAL fluids compared with non-tolerised PBS mice was observed (Figure 15). Our data indicate that passage of an antigen exclusively across the intestinal mucosa is sufficient to establish oral tolerance, and that the contribution of antigen passage through the oral cavity is negligible, at least in our experimental system for studying oral tolerance.

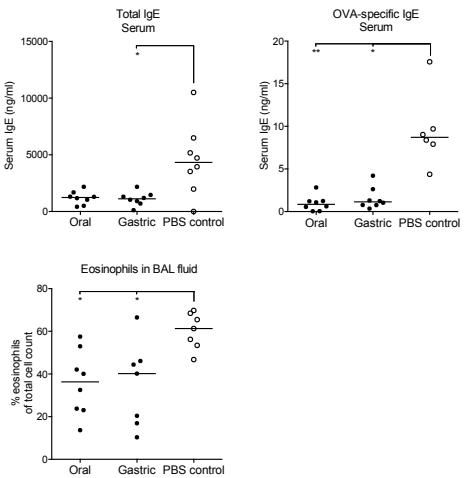

Figure 15. The oral tolerance model using ovalbumin (OVA)-induced airway hypersensitivity in BALB/c mice. *Hypersensitivity to OVA was measured in terms of the serum levels of total and OVA-specific IgE and the percentages of eosinophils in the broncho-alveolar lavage (BAL) fluids. Each symbol represents one animal (n=8 per group) and the horizontal lines show the median values for the group. *p<0.05 and **p<0.01, as analysed by the Kruskal-Wallis test with Dunn´s correction.*

Are the activation patterns of ovalbumin (OVA)-specific CD4$^+$ T cells and of Treg differentiation different when the OVA antigen is administered via *the oral route rather than the gastric route?*

Here, the DO11.10 TCR transfer model was applied, in which naïve BALB/c mice were adoptively transferred with OVA-specific CD4$^+$ T cells (labelled with CellTrace™ Violet, to allow detection of the cells) obtained from the spleens of DO11.10 TCR-transgenic mice (Figure 16). At 24 hours and 48 hours after the cell transfer, the mice were exposed to a high dose of OVA (50 mg), through either oral or gastric administration. This dosing regimen, through gastric administration, is known to induce tolerance, as evidenced by a reduced DTH response upon challenge with the tolerising antigen (Hadis et al., 2011). Mice that were fed PBS in the same manners served as the controls. Groups of mice were killed at 1 hour, 1 day, 4 days, 8 days or 13 days after the last OVA exposure, and lymphoid tissues from the NALT, CLN, MLN, and PLN were collected and analysed by flow cytometry to detect OVA-specific T-cell activation and Treg differentiation.

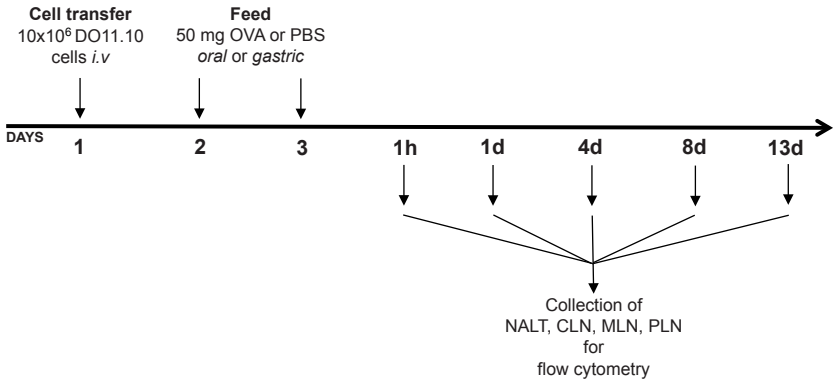

Figure 16. Experimental design for the DO11.10 TCR transfer model.

The overall immunological response in terms of the activation of OVA-specific CD4$^+$ T cells and Treg differentiation did not differ considerably between the two routes, even though slight differences were observed.

One hour after the last OVA exposure, the NALT displayed distinct proliferation of OVA-specific CD4$^+$ T cells, as compared to the other sites after oral but not gastric administration of the antigen (Figure 17). The other sites all showed insignificant levels of proliferation, irrespective of the administration route, with the exception of the CLN, which displayed a slight increase at this time-point after gastric administration. One day after the last OVA exposure the NALT displayed proliferation also after gastric administration, although the level was lower than for the orally fed group (Figure 17). Proliferation could now also be observed in the MLN for both routes of administration. For the CLN and PLN, the levels of proliferation were still negligible although a slight increase was observed for the CLN of the orally fed group. Lastly, on day 4, the majority of the cells had undergone cell division at all the sites in the mice that were fed OVA *via* the gastric route but not *via* the oral route; in the latter case, proliferation was lower, especially in the PLN (Figure 17). These results imply that the proliferation was initially slightly more rapid in the NALT after oral

administration. However, with time the gastric route of administration yielded the highest rates of proliferation.

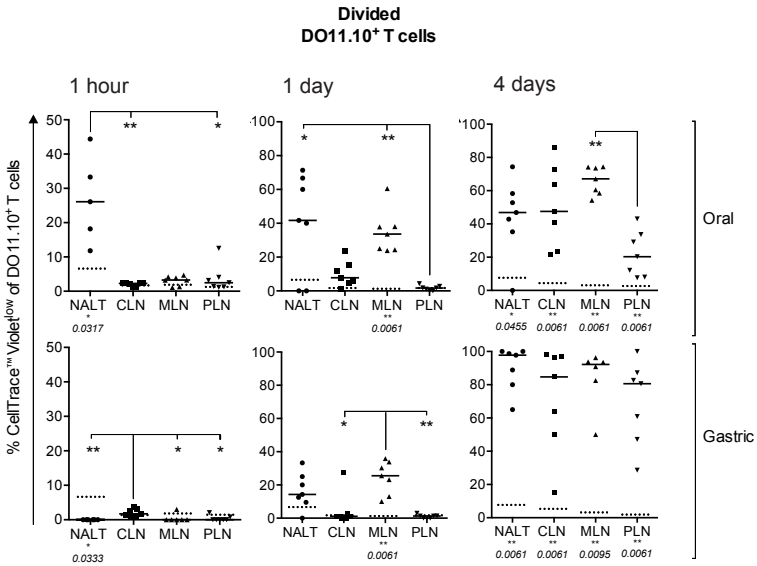

Figure 17. Proliferation levels of antigen-specific DO11.10$^+$ CD4$^+$ T cells 1 hour, 1 day, and 4 days after the last ovalbumin (OVA) exposure. *BALB/c mice that were adoptively transferred with DO11.10 spleen cells and fed OVA through oral or gastric administration were analysed by flow cytometry for the presence of dividing DO11.10$^+$ CD4$^+$ T cells in the nose-associated lymphoid tissue (NALT), cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), and peripheral lymph nodes (PLN) at 1 hour, 1 day, and 4 days after the final OVA exposure. The results are presented in representative dot plots from the flow cytometry analysis, showing the percentages of dividing (CellTrace™ Violet^{low}) DO11.10$^+$ T cells among the total CD4$^+$ T cells. Each dot represents an individual mouse. The horizontal solid lines indicate the median values for each group. Comparisons of the different sites analysed by the Kruskal-Wallis test with Dunn´s correction, *p<0.05 and **p<0.01, are shown in the graphs. The median values for the mice exposed to PBS are shown as horizontal dotted lines, and the p-values compared with the OVA-exposed mice for each site are shown underneath each graph in italics, *p<0.05 and **p<0.01. This latter analysis was performed using the Mann-Whitney U-test.*

One hour after the last OVA exposure, no clear induction of Treg cells was observed. The only significant observation was a slightly lower proportion of Treg cells in the NALT than in the MLN of mice fed OVA *via* gastric administration (Figure 18). The percentages of Treg cells (FoxP3$^+$CD69^{neg}) and activated Treg cells (FoxP3$^+$CD69$^+$) in the OVA-fed mice were similar to those in the PBS controls, irrespective of the administration route. At day 1, the induction of Treg cells was still low for both routes of administration, although slight increases in the percentages of activated Treg cells were noted in the CLN, MLN, and PLN of mice fed OVA by the oral route, as compared with those fed PBS (Figure 18). However, at this time-point, there was no clear induction of activated Treg cells in the mice fed OVA through the gastric route. Lastly, on day 4, there was a small increase in the percentages of Tregs, the increase being most pronounced in the NALT and CLN for both feeding regimens, although no significant differences were observed (Figure 18). In addition, there was an increase in the percentages of activated Treg cells in the mice that were fed OVA orally, as compared with PBS-fed controls. A slight increase in the number of activated Treg cells was noted also for the gastric route of administration. These results imply that induction of Tregs occurs over time at all the

different sites but that it is modest, and that the largest increase in activated Tregs is observable after oral administration of the antigen, although it is still low as that achieved *via* the gastric route.

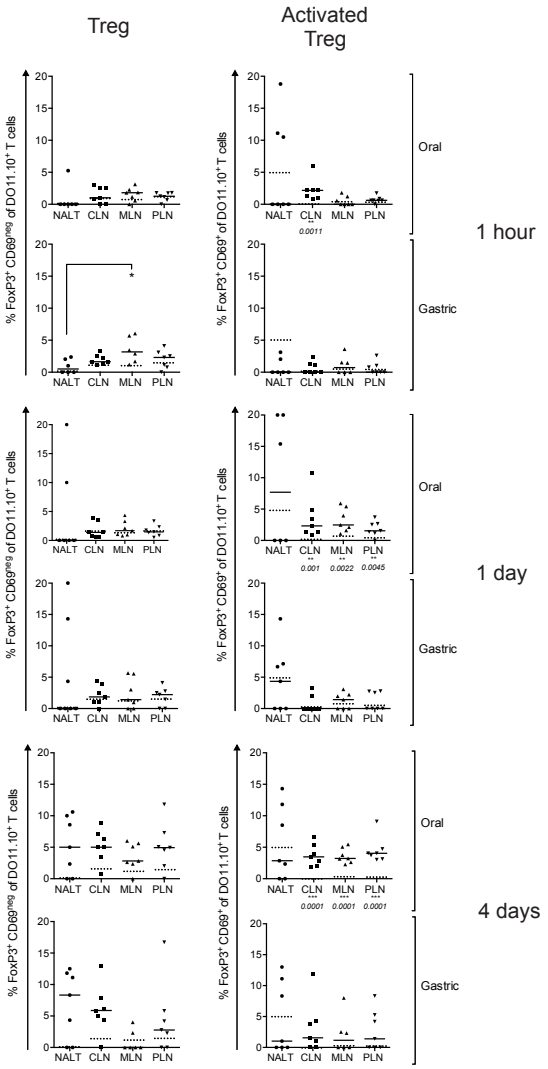

Figure 18. Expression of CD69 and FoxP3 by the antigen-specific DO11.10$^+$ CD4$^+$ T cells 1 hour, 1 day, and 4 days after the last ovalbumin (OVA) exposure. *BALB/c mice that were adoptively transferred with DO11.10 spleen cells and fed OVA via oral or gastric administration were analysed by flow cytometry for the expression of CD69 and FoxP3 in the DO11.10$^+$ CD4$^+$ T cells of the nose-associated lymphoid tissue (NALT), cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), and peripheral lymph nodes (PLN) at* 1 hour, 1 day, and 4 days *after the final OVA exposure. The results are presented in representative dot plots from flow cytometry analysis, showing the expression levels of CD69 and FoxP3 in the DO11.10$^+$ T cells among the total CD4$^+$ T cells. Each dot represents an individual mouse. The horizontal solid lines indicate the median values for each group. Comparisons between the different sites were analysed by the Kruskal-Wallis test with Dunn´s correction, *p<0.05. The median values for the mice exposed to PBS are shown with horizontal dotted lines, and the p-values compared with the OVA-exposed mice for each site are shown underneath each graph in italics, **p<0.01 and ***p<0.001. This latter analysis was performed using the Mann-Whitney U-test.*

Preliminary results are also provided regarding the activation pattern of the OVA-specific $CD4^+$ T cells in the oral mucosa and intestinal mucosa after oral administration of an antigen but not after gastric administration (Figure 19). The same lymphoid tissues were dissected as in **Study IV**, apart from the NALT, which was not dissected for these preliminary results. In addition, the time-points varied, with days 5 and 12 included in the preliminary results, whereas the results for 1 hour, 1 day, and 4 days were included in the original study.

The proliferation rates of the OVA-specific $CD4^+$ T cells in the lymphoid tissues (CLN, MLN and PLN) on day 5 after OVA exposure were similar to those seen on day 4 in the published study. The highest proliferation was observed in the MLN and the proliferation rates for the oral mucosa and intestinal mucosa were similar to those for the lymphoid tissues (Figure 19). Regarding the activation of the OVA-specific $CD4^+$ T cells, the lymphoid tissues showed strong activation (Figure 19). A similar degree of activation was observed in the oral mucosa, but not in the intestinal mucosa where the level of activation was lower (Figure 19). However, despite having a lower percentage of activated OVA-specific $CD4^+$ T cells, the number of activated $FoxP3^+$ Tregs was highest for the intestinal mucosa, whereas the other compartments displayed low numbers of these cells (Figure 19). Twelve days after OVA exposure, the rate of proliferation began to decrease, as did the percentage of activated OVA-specific $CD4^+$ T cells in all the compartments (Figure 19). The percentages of activated $FoxP3^+$ Tregs in the oral and intestinal mucosa increased markedly, especially in the intestinal compartment, compared to the lymphoid sites (Figure 19).

These results imply that a local expansion of $FoxP3^+$ Tregs occurs also in the oral mucosa after tolerance induction, as has previously been proposed for the intestine. However, this expansion of Tregs locally in the mouth appears to be delayed relative to the expansion that takes place in the intestinal compartment. Furthermore, our results support the notion that there are few activated cells within the lymphoid tissues, since they are inductive sites, and that these cells instead are found within the effector sites, such as the lamina propria. It is possible that the numbers of activated $FoxP3^+$ Tregs in the oral mucosa would have reached levels similar to those in the intestinal mucosa if a later time-point had also been included. Confirmation of these observations in repeated studies that include more mice and comparing the gastric and oral routes is required, especially if these studies would include the NALT as one of the lymphoid tissues. In addition, it would be of value to use the same time-points as were used in the original study.

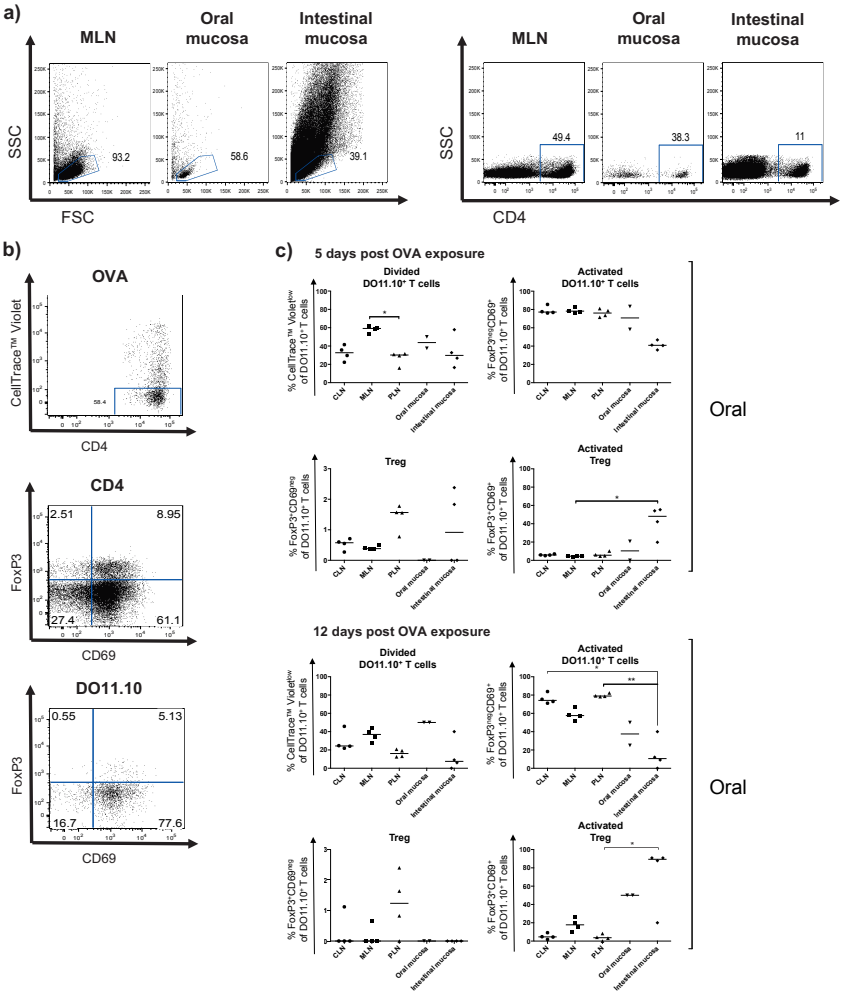

Figure 19. Proliferation of antigen-specific DO11.10$^+$ CD4$^+$ T cells and the expression levels of CD69 and FoxP3 in various lymphoid compartments and in the oral mucosa and intestinal mucosa. *Experimental design: BALB/c mice were injected intravenously with DO11.10 spleen cells. At 24 hours and 48 hours later, the mice were fed 50 mg ovalbumin (OVA) by oral administration. Mice were sacrificed 5 days or 12 days after the last OVA exposure, and cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), and peripheral lymph nodes (PLN) were collected along with the oral and intestinal mucosa and analysed by flow cytometry. a) The lymphocyte and CD4 gating strategy for MLN, oral mucosa, and intestinal mucosa. b) Gating strategies for the DO11.10$^+$ CD4$^+$ T cells stained with CellTrace^{TM} Violet, CD69, and FoxP3 from the MLN. c) Dot plots from the flow cytometry analysis showing the expression levels of CD69 and FoxP3 among the DO11.10$^+$ CD4$^+$ T cells. Each dot represents an individual mouse. The horizontal solid lines show the median value for each group. Comparisons between different sites analysed using the Kruskal-Wallis test with Dunn´s correction (* $p<0.05$ and ** $p<0.01$) are shown in the graphs.*

GENERAL DISCUSSION

The *'Discussion'* section of this thesis is based upon several questions that have arisen during the years that I have spent working on understanding the different aetiological aspects of RAS. As the questions are of a general character they do not provide any definite answers but they are nevertheless intriguing to explore and reflect upon. Serious challenges lie ahead in defining a set of suitable diagnostic criteria for RAS, and in performing appropriately designed research studies with well-defined, stringent cohorts of patients using suitable methods for uncovering the aetiology of RAS, and thereafter establishing accurate management strategies for this group of patients.

Is RAS a disease entity of its own that only manifests in otherwise healthy individuals, or can it also have an association with systemic factors?

This question remains unsolved. RAS is indeed found in patients who are otherwise completely healthy. However, there are also patients who present with recurrent oral ulcers and who suffer from a systemic disease or allergy or are on some kind of medication. Do these ulcers represent actual RAS or merely aphthous-like ulcerations? The answer probably involves a combination of these options in that some systemic conditions, allergies, and medications may be associated with RAS whilst others are not. For some of the patients who suffer from a systemic disease, RAS may manifest itself as part of the clinical picture, whereas in other patients RAS may be present in combination with a systemic disease but these two conditions should be regarded as separate entities. It has been suggested that RAS is not a single entity but instead presents with different phenotypes. The more common forms display discrete ulcers that typically commence in childhood or adolescence, abate to some degree with age, are not associated with any yet defined systemic disorder, and may be associated with a positive family history of recurrent oral ulcerations (Scully, 2012). However, the use of the term 'secondary complex aphthosis' has also been proposed, to describe associations with hematinic deficiencies, various systemic deficiencies and/or drug reactions (Baccaglini et al., 2011).

As long as we do not know for certain that RAS is found simply in patients who are not affected by any systemic disease, allergy or any type of medication, I consider it reasonable to include patients who present with RAS that may be due to systemic factors, as long as the cohorts of patients within the studies are well-defined and grouped accordingly into homogenous subgroups. It may well be that there are different phenotypes of RAS, as is the case for several other diseases, such as Crohn´s disease (Gasche & Grundtner, 2005). Furthermore, in my clinical experience, those patients who suffer from RAS without any other clear underlying cause are not the dominant group and they respond well to treatment with for example, topical steroids. A larger fraction of the patients, who often suffer from a more severe form of RAS and are more likely to be resistant to the available treatment strategies, show some degree of association with systemic disorders.

It is also important to acknowledge that RAS could be a pathological reaction pattern of the oral mucosa rather than a specific disease. This condition could be looked upon in the same way as a headache or fever, i.e., a general symptom that is manifested when there is a disruption of the homeostasis of the human body. As the range of possible pathological appearances in the oral mucosa is limited, different underlying immune mechanisms may result in similar types of lesions, such as ulceration, making it difficult to distinguish between RAS and aphthous-like ulcerations. Clearly, RAS is a multi-factorial condition in which numerous endogenous and exogenous factors contribute to

establishing an inflammatory process in the oral mucosa. Therefore, it is crucial to delve in details into the patient history, so as to understand which aetiological factors are of importance in that specific patient, since they may not be exactly the same as those in another patient with similar symptoms. Nonetheless, the patients may share certain common features, as has been explained in *'The RAS model'* (Figure 5).

What role does the genetic composition of an individual play in the development of RAS?

From **Study I**, which is the first GWAS study of RAS, we could confirm the results of previous studies showing that RAS is not a monogenic disease but is instead polygenic due to complex inheritance patterns, in which interactions between multiple host genes and possibly also environmental factors influence the risk of development of RAS (Ship, 1965, Miller et al., 1977, Chavan et al., 2012). Candidate-gene studies on RAS have been carried out previously. However, in such studies it is difficult to determine which specific genes ultimately play roles in the disease. GWAS allows analysing the entire human genome, across all chromosomes, at high resolution. In this particular study, the chosen approach was to combine linkage analysis with association analysis, whereby the linkage analysis was dependent upon an association. This allowed assessments of the genetic variants passed down to the offspring within each family (linkage) and between the different families (association). Furthermore, we identified signalling pathways that are involved in RAS and the genes responsible for the various pathways. The results, however, give only an indication of the genes and signalling pathways involved in RAS, and studies that include larger cohorts will have to be conducted, preferably still within families. An advantage that family-based case-control studies have over conventional case-control studies is that family members are more genetically similar than individuals who are randomly selected from a population. Family members without the disease are more likely to differ from those family members who have the disease with respect to aspects of importance for the disease, and the probability of finding differences related to a disease between healthy and diseased individuals within a family should be greater than when one compares random individuals. Therefore, family-based association testing, by conditioning on the family structure, is able to remove potential biases due to population stratification and environmental effects. However, this comes at the price of reduced power relative to population-based association studies. Although, the *p*-values generally are less significant, we also expect fewer false-positive findings due to population stratification and environmental effects. Thus, the choice of study design has to be made according to the study goals and the available resources. Besides including a larger family-based cohort, the pattern of inheritance is also of value for investigating further as well as the roles of environmental factors. For complex diseases, such as RAS, non-genetic risk factors tend to be of greater importance for disease development than the genetic variants themselves. It would be of value to conduct large-scale international studies to assess differences in ethnicity, to answer the question as to why people of certain nationalities seem to be at greater risk of developing RAS than others. Does it first and foremost have to do with the genetic basis of the individual or with the environmental factors to which that individual is exposed?

Prior to the commencement of our study, two larger-scale genetic studies had been performed in RAS cohorts, studying the up- and down-regulation of genes in RAS. In 2004, Borra *et al.* performed a cDNA microarray analysis to quantify the expression of 1,176 genes in patients with active ulcerations at the time of biopsy. The results revealed enhanced activity of the Th1 gene cluster as compared to the Th2 gene cluster (Borra et al., 2004). Presented here are the genes that were identified by Borra *et*

al. (in parentheses are the genes that we found that belong to the same gene family). Up-regulated genes in RAS: *MMP3* and *MMP11* (*MMP7*); *IL1B* and *IL5RA* (*IL4R* and *ILR6*); and *GNB1* (*GNB4*). Down-regulated genes in RAS: *PDGFA* (*PDGFD* and *PGDFRB*); *MAS* (*MAS1*); and *CSF1* (*CSF1R*). Furthermore, in 2011, Lewkowicz *et al.* analysed the expression of Th1/Th2/Th3 and Th17-related genes in biopsies from patients who presented with active lesions at the time of sampling (Lewkowicz et al., 2011). In total, 86 genes related to T-cell activation were identified. Also, in that study, skewness towards a Th1-like gene profile was identified. Presented here are the genes that were identified by Lewkowicz *et al.* as being up-regulated in RAS (in parentheses are the genes that we found that belong to the same gene family): *CCL7* (*CCL21*); *IFN-γ* (*IFNGR1*); *CSF-2* (*CSF1R*); *IL-6* and *IL-15* and *IL18R1* (*IL4R* and *IL6R*); and *TLR6* (*TLR2*).

In parallel with identifying the genes and signalling pathways that are involved in RAS, it is essential to study the RNA and protein expression profiles and the related biomarkers to understand the precise roles of these pathways. This can be achieved by, for example, transcriptome analysis, along with targeted gene expression and protein analysis. In addition, it is of interest to correlate the genetic compositions of individuals with RAS to the compositions of the microbiota in their oral cavities, as there is mounting evidence that the oral microbiota profile in patients with RAS differs from that in control individuals (Bankvall et al., 2014, Hijazi et al., 2015, Seoudi et al., 2015, Kim et al., 2016). RAS has been suggested to be a condition in which Toll-like receptors (TLRs) are of importance (Borra et al., 2009, Hietanen et al., 2012). These proteins are known to be significant for innate immune responses when microbes penetrate the physical barriers of the body, for example, the oral mucosa. The microbes express pathogen-associated molecular patterns (PAMPs), which are recognised by extracellular TLRs, expressed on the cell surfaces of immune cells, such as DCs, macrophages, neutrophils, and B and T cells, as well as by non-immune cells, such as epithelial cells, fibroblasts, and endothelial cells. When the PAMPs bind to the TLRs an immune response is initiated that can contribute to establishing the oral ulceration, since TLRs are known to mediate inflammation when stimulated by PAMPs. In addition, TLRs have been reported to control epithelial barrier integrity, which ultimately affects the permeability of barrier tissues (Johnston & Corr, 2016). Interestingly, the expression patterns of TLRs have been reported to differ between patients with RAS and healthy control subjects (Hietanen et al., 2012). In the oral mucosa of controls and at healthy mucosal sites in patients with RAS, the superficial epithelial layers did not show TLR expression but tended to do so in the supra-basal and basal cell layers, creating an upper *'non-responsive to PAMPs'* zone and a deeper *'reactive to PAMPs'* dormant defence zone. However, in the oral mucosa of active lesions in patients with RAS, the presence of immune cells that expressed TLRs was considerable, with TLRs 1, 2, 5, 7, and 8 being found throughout the epithelium. Furthermore, the expression of TLR 4, 6, and 10 was detected further down in the epithelium but in layers higher up than in the controls. Limited expression of TLR3 was observed. PAMPs also stimulate the production of pro-inflammatory cytokines, for example TNF-α, known to be increasingly expressed in RAS, which ultimately leads to the recruitment of TLR^+ leukocytes to the lamina propria, thereby further enhancing TLR reactivity. This indicates that there are changes in TLR expression during the disease process. While polymorphisms in the *TLR4* gene have previously been reported (Karasneh et al., 2015), there remain major gaps in our knowledge of the relative importance of TLRs for RAS development and of polymorphisms in these receptors that could contribute to disease. Furthermore, genetics influences mucin glycosylation (Marionneau et al., 2001, Linden et al., 2008a), and since we have reported potential differences between patients with RAS and control subjects with respect to glycosylation patterns, it is also of importance to elucidate the role of genetics in mucin glycosylation for this condition given that this could also increase the permeability of the oral mucosa. Overall, extensive research efforts remain to be made before we can achieve a thorough understanding of the

genetic basis of RAS, the role of heredity, and what effects that environmental factors play in this disease.

Does the oral microbiota play a role in the aetiology of RAS and if so, in what way?

From **Study II**, which is the first report conducted to analyse the oral mucosal microbiota outside of the actual lesion in patients with RAS, we were able to conclude that there was a difference in the oral microbiota profile of the buccal mucosa distant from the ulcer. Certain bacterial taxa were found to pre-dominate in the microbiota of patients with RAS, while other taxa were more pre-dominant in the control subjects. Unfortunately, using T-RFLP, we could not identify the specific bacterial taxa. The differences in the oral microbiota profile were most striking when patients with RAS were compared to healthy age- and gender-matched control subjects. However, the differences could also be attributable to the size of the lesions, i.e., that the patient suffered from minor or major RAS. No SIMCA analysis was carried out for the effects of medication on the oral microbiota profile of the patients with RAS, since it was not possible to gather all medications into a single variable. Instead, medications would have had to be divided into subgroups, where patients on medications with similar effects would be grouped together. The subgroups would ultimately have become too small to allow for a reliable analysis. In addition, the effects of medication on the oral microbiota composition have been shown to be of limited importance (Dahlén et al., 2012). Antibiotics are the one group of drugs that have a major impact, which is why the use of antibiotics was one of the exclusion criteria for the study. It would however have been useful to have known the patient´s history of intake of antibiotics within previous three months, not just during the last month.

Previously, research on the role of bacteria in RAS has taken a mono-causal approach, where it was believed that this condition was due to one bacterial species acting either as a direct pathogen or as a source of antigens that stimulated antibody production, which then cross-reacted with oral mucosal kerationocytes, resulting in the development of a lesion. In this context, several different bacterial species have been proposed, including *Streptococcus mitis*, *Streptococcus oralis,* and *Helicobacter pylori*. Although numerous articles on this topic have been published over the years, these theories remain unproven. The difficulties associated with resolving this issue, as well as the realisation within the medical research community that the complexity of many diseases lies in that they are multi-factorial where the composition of the microbiota may be an essential factor, lead us to hypothesise that the oral microbiota as a whole is a contributing factor of importance for RAS. Since the publication of our study (Bankvall et al., 2014) a couple of years ago, other research groups have worked according to the same hypothesis. In 2015, Hijazi *et al.* reported the first characterisation of the most abundant bacterial populations in patients with RAS, as compared to healthy controls, using 16S rRNA gene sequencing (Hijazi et al., 2015). A strength of that study is the homogenous cohort of patients, in that only patients with idiopathic RAS (i.e., without systemic diseases, allergies or medication) were enrolled using highly stringent methods. However, the sample size was small. The results showed no significant differences in overall bacterial diversity between the healthy sites of patients with RAS and the control subjects, which correspond well to the results of our study. Furthermore, no clustering within patient sample groups was identified using principle component analysis (PCA). Using PCA, the data-set is examined to see if there are any natural groupings of the material. For our data, we could not see any separations using PCA either. However, using PLS-DA, the SIMCA software found separations between the groups. Interestingly, in the study of Hijazi *et al.*, at the genus-level there was a greater abundance of total Bacteroidales in the healthy sites of patients with RAS, who did not present with active ulcerations at sampling, as compared to the healthy controls (Hijazi et al., 2015). They concluded that this could be a contributing factor to the initiation of

the disease. Later, in 2015, Seoudi *et al.* explored the oral mucosal microbiota and the salivary microbiota profiles of patients with Behçets disease, using patients with RAS as a disease control and applying the human oral microbe identification microarray (HOMIM) and matrix-assisted laser desorption/ionization time-of flight analysis (MALDI-TOF) (Seoudi et al., 2015). No clustering was observed within the different groups. However, certain differences in the composition of the oral microbiota were reported, with increased colonization by *Rothia dentocariosa* being noted at non-ulcerative sites of the patients with RAS, as well as an increased number of *Neisseria* and *Veillonella* in the healthy controls compared to the patients with RAS. Lastly, in 2016, Kim *et al.* published a study in which both the oral mucosa and salivary microbiota profiles were studied in patients with RAS and in healthy controls using pyrosequencing of the 16S rRNA genes (Kim et al., 2016). Here, sampling of the oral mucosa was carried out only in the lesions and not in the healthy mucosa. No differences in bacterial diversity were observed between the patients with RAS and the healthy controls, either in the oral mucosal microbiota of the RAS lesions or the salivary microbiota. Furthermore, decreased numbers of healthy core microbiota species, such as *Streptococcus salivarius,* and increased levels of rare species, such as *Acinetobacter johnsonii,* were observed at the lesion sites of the patients. It is important to acknowledge that sequencing techniques identify all the types of bacteria present in a sample, not only those from the actual sampling site but also those present due to sample contamination, which makes interpretation of the data for separating the relevant taxa from the background noise more challenging.

The drawbacks of our own study are that only one microbiological technique was applied, and that the group of patients with RAS was highly heterogeneous in that some patients may have suffered from primary RAS, while others had secondary complex aphthosis or aphthous-like ulcerations (depending on which terminology is adapted). While all fours studies conducted in recent years conclude that a change within the oral mucosal microbiota is likely in patients with RAS it is currently not known to what extent and in what perspective this observation is of importance. Therefore, the question as to the role of the oral microbiota remains in part unanswered, especially with respect to causality, i.e., if the presence of a lesion alters the microbiota of the oral cavity or if a change in the oral microbiota triggers lesion development. Moreover, it is not clear when the change occurs. This is a crucial issue to clarify if one is to answer the question as to whether the changes in the oral microbiota give rise to the lesions or the other way round. To resolve this issue, it will be essential to follow patients over a longer period of time when they will intermittingly have lesions and lack lesions, as well as during the prodromal and healing phases, and to examine both the lesion itself and the surrounding healthy mucosa, to define when exactly the change occurs. Utilising different microbiological techniques will also be valuable, as different methods have different advantages and disadvantages and it is important to verify the results with more than one technique.

Changes in the composition of the saliva may also be of importance for the alterations of the oral mucosal microbiota profile. A change in the oral mucosal microbiota may affect the ability to clear bacteria from the saliva or *vice versa* that the reduced ability to clear bacteria in patients with RAS results in the selection of bacteria within the oral cavity that create a different oral mucosal microbiota composition. Preliminary data from our research group reveal that patients with RAS have a reduced aggregation capacity for both *Streptococcus sanguinis* and *Actinomyces naeslundii* compared with the control subjects when un-stimulated whole saliva samples were collected from patients with RAS who did not present with lesions at sampling (unpublished observations). These bacterial species were selected because they are early colonisers and support the adherence of pathogenic bacteria (Kolenbrander & London, 1993). Mucins and secretory IgA facilitate aggregation of bacteria by saliva. We have previously demonstrated that the pattern of glycosylation of the mucin MUC7 in patients

with RAS seems to be different, in terms of having a less complex type of *O*-linked glycans attached to the protein backbone (making up the core structure of the mucin), to that of healthy siblings who do not suffer from RAS (Zad et al., 2015). MUC7, which is a common mucin of the oral cavity, has a more stable *O*-linked glycan profile than the other common mucin in the oral cavity, MUC5B, which is why MUC7 was studied rather than MUC5B. The types of glycans on MUC7 did not differ between the patients and the controls. However, the terminal glycan structures, especially the proportions of sialic acid and sialyl-Lewis X structures were decreased in the patients (Zad et al., 2015). The sialyl-Lewis X epitope is thought to be of importance for the homing of leukocytes (Prakobphol et al., 1998, Prakobphol et al., 2005), specifically lymphocyte re-circulation in the HEVs of lymph nodes (Rosen et al., 1985). This may result in ineffective aggregation and attachment of bacteria to the mucin structure (Humphrey & Williamson, 2001, Linden et al., 2008b), which ultimately affects the capacity for clearance, since fewer leukocytes are able to enter the lymphoid tissues in the oral cavity. In fact, MUC7 has been shown to bind to oral streptococci, and this adherence is lost when sialic acid residues are removed from the MUC7 molecule (Murray et al., 1992). Therefore, besides identifying the bacterial species involved in the aetiology of RAS, it is also imperative to study the role of saliva by, for example, investigating the clearance capacity for bacteria and the structure and function of the mucin layer in these patients compared to healthy individuals. Furthermore, studies on possible alterations to the immunoglobulins, cytokine profile, and other signalling molecules in saliva are also warranted.

Patients with RAS may display a reduced capacity to clear bacteria, which results in the selection of bacteria within the oral mucosa that can degrade the mucin layer, which contributes to the formation of an aphthous lesion. Therefore, it would also be of interest to investigate further the roles of the bacteria and how they affect the oral mucosa in terms of the immunological responses that are initiated. This would identify specific intracellular signalling pathways of importance and fully elucidate the role of the oral mucosal microbiota in the development of the lesions of RAS, allowing us to compare the results with already published observations on the role of the immune system in RAS. Most probably the oral microbiota affects both innate and adaptive immune responses (Hietanen et al., 2012). In addition, it has been reported that certain bacteria are directly involved in the aethio-pathogenesis of RAS. As an example, *A. johnsonii*, which is a species associated with an increased risk of RAS given that the levels of this bacteria are increased in the ulcers, inhibits the proliferation of gingival epithelial cells and shows increased cytotoxicity towards the same cells *in vitro* as compared to *S. salivarius,* a bacterium that is also associated with an increased risk of RAS given that the levels of this bacteria are decreased in RAS lesions (Kim et al., 2016). In addition, the relative abundance of Streptococcus has been reported to be negatively associated with the concentrations of IL-1β and IL-8 in saliva (Said et al., 2014), and since the levels of *streptococci* have been shown to be decreased in patients with RAS and the levels of *A. johnsonii* increased, this could contribute to ulceration and delayed healing of the RAS lesions. Understanding the functionalities of the bacteria residing within the oral mucosa of patients with RAS is of greater importance than being able to identify the exact bacterial species by name. It is imperative to determine which metabolites these bacteria produce and how they affect the oral mucosa and surrounding environment. The intestinal microbiota has strong implications for the health of individuals, such that disruption of the gut microbiota, i.e., microbial dysbiosis, causes inflammation that results in, for example inflammatory bowel disease. Therefore, there is no reason to doubt the importance of the role of the oral microbiota in oral mucosal diseases. In light of all the findings presented above, it is essential to explore further the roles of the oral microbiota, the saliva, and local immunoregulatory networks in the oral cavity.

What role do the oral-associated lymphoid tissues play during induction of tolerance?

Despite the intestine representing the largest mucosal surface and harbouring the highest number of immune cells, it has become evident that other parts of the mucosal immune system may also play a role in the induction of tolerance. The oral and nasal cavities form the entrance points for both the gastro-intestinal and upper respiratory tracts and are continuously exposed to vast amounts of antigens from the external environment. This awareness has initiated the development of new immunotherapy strategies, as well as the adoption of novel routes for vaccine delivery. There are, however, many questions to be answered before a full understanding of the mucosal immune system and its role in tolerance induction, not least the details of the interactions, dependencies, and differences between the various compartments, is achieved.

The scientific questions posed in **Studies III** and **IV** were aimed at acquiring greater knowledge of the immune cells and immune mechanisms of the oral-associated lymphoid tissues, encompassing the NALT and CLN, as compared to the intestinal lymphoid compartment, the MLN, and the peripheral lymphoid compartment, the PLN. The overall question was to what extent and in which ways these two compartments contribute to the induction of tolerance. However, due to the extensive and intricate nature of this question, it has not been possible to answer it in full through these two studies alone, which was also not the intention. Thereby, many subsequent questions remain unanswered. In **Study III**, we investigated potential differences in the phenotypes of the APC and T-cell populations between the oral-associated lymphoid tissues, and studied the *in vitro* proliferative responses of the cells in the various compartments. Furthermore, in **Study IV,** we explored whether the passage of an antigen through the oral cavity contributes to the overall immunological response and degree of tolerance induced, as compared to a situation in which this passage is bypassed and the antigen is instilled by gavage directly into the stomach. In addition, we wanted to determine whether there are differences in the activation pattern of OVA–specific $CD4^+$ T cells and in Treg differentiation when oral administration was compared to gastric administration.

The general conclusion of **Study III** is that the oral-associated lymphoid tissues display differences in the phenotypes of the immune cell subsets found therein and the markers that they express, as compared to the intestinal and peripheral counterparts. In addition, there are differences in the *in vitro* proliferative responses between the different compartments. The greatest differences were observed between the NALT and the other sites, i.e., the CLN, MLN, and PLN, although the CLN also demonstrated certain differences compared to the MLN and PLN, displaying features characteristic of both. First, the NALT displayed a greater abundance of APCs, as well as differences in the proportions of the different subsets compared to the other sites, which showed percentages that were more similar to each other. Differences in the activation patterns of the APC subsets were also apparent between the different compartments, whereby the APCs of the NALT again showed a greater deviation from the cells of the other sites, since the APCs were less activated here. The APC populations of the NALT also seemed to possess a greater tolerance-inducing capacity compared to the other sites. Second, the NALT displayed lower proportions of T cells than the lymph nodes, which displayed similar proportions to each other. The number of tTregs was the lowest in the NALT and highest in the CLN, whereas the distribution of pTregs was similar for all four sites. The NALT also contained a greater abundance of $Helios^+FoxP3^{neg}$ $CD4^+$ T cells, which was not observed for the other sites, which between them demonstrated more similar proportions. This T-cell population has not been well characterised in the literature, even though it has been proposed to possess immunoregulatory functions (Abd Al Samid et al., 2016). Few naïve T cells were found in the NALT in comparison to the other sites, where almost all the cells were naïve. Instead, the proportion of memory/effector T cells in the NALT was higher. Furthermore, the expression of homing markers on T cells was higher

in the NALT than in the other sites, indicating that the homing capacity of the cells in the NALT is greater and that these cells have an effector T-cell function rather than a T-cell activation function. Lastly, the proliferation rates varied between the different sites, in that the NALT showed no proliferative response at all to stimulation. The lack of proliferation of the cells in the NALT also indicates that rather than being of importance for T-cell activation the cells at this site convey effector functions, despite the NALT being regarded as an inductive site.

In **Study IV**, the two routes of administration were found to be equally efficient for inducing tolerance in a model of airway hypersensitivity, since both groups of mice displayed significantly decreased OVA-specific IgE levels in their sera, as compared with the PBS-fed mice. In addition, there were fewer eosinophils in the BAL-fluid compared with non-tolerised mice. Concerning the overall immunological response, the levels of activation of OVA-specific $CD4^+$ T cells and Treg differentiation did not differ between the two routes, even though slight differences were observed. The rate of proliferation was initially slightly more rapid in the NALT after oral administration. However, with time, the gastric route of administration resulted in the highest rates of proliferation. Regarding the activation of OVA-specific $CD4^+$ T cells, the results imply that despite the NALT being the first site to display proliferation of the OVA-specific $CD4^+$ T cells, the degree of activation of these cells is low and remains low over time. In addition, an increase in the number of proliferating cells with time resulted in a decrease in the number of activated cells, irrespective of the feeding regimen used. Lastly, regarding the differentiation of Tregs, the results imply that there is induction of Tregs over time at all the different sites but that it is modest and that the largest increase in activated Tregs is observable after oral administration of the antigen, although it is still low compared to that achieved by antigen administration *via* the gastric route.

That the NALT represents a MALT structure and is not a lymph node is probably the most significant reason why this compartment differs the most from the other sites. The NALT lacks afferent lymphatics, which leads to an exposure of antigen that is not as comprehensive as that seen in lymph nodes, since antigens do not reach the NALT through HEVs. Instead, sampling of antigens through M cells is more selective than through HEVs, whereby the presentation of antigens to T cells is not as extensive. MALT structures and lymph nodes are, however, all classified as inductive sites in which primary immune responses are initiated. Therefore, it was surprising to find that the NALT had more of an effector function than one for activation. This feature is supported by the following observations: *(i)* the patterns of markers expressed on the lymphocytes, whereby despite high levels of $CD11c^+CD11b^+$ DCs, the expression of activation markers was low, the expression of CD45RB was low (indicating few naïve T cells), and the expression of homing markers on the T cells was high; *(ii)* the low rate of proliferation *in vitro*; and *(iii)* despite the initial rapid proliferation of the DO11.10 $CD4^+$ T cells, the rate of activation remained low over time. Therefore, there are several lines of evidence to support the idea of the NALT being more of an effector site. This may be controversial, as it is generally considered to be an inductive site. Instead, it may be a site that displays features that have nothing to do with activation or effector functions but instead has all to do with tolerogenic activities. It has been demonstrated that immature DCs that ultimately express low levels of activation markers stimulate naïve T cells to become Tregs (Maldonado & von Andrian, 2010). However, the NALT did not contain many Tregs. Instead, the $CD4^+FoxP3^{neg}Helios^+$ cells pre-dominated at this site. These cells have been reported to produce IL-10, indicating that they possess a regulatory function (Abd Al Samid et al., 2016). Furthermore, the NALT has been reported to contain a significant proportion of $CD4^{neg}CD8^{neg}CD3^+$ T cells (Rodriguez-Monroy et al., 2007). These cells may comprise part of the Treg population, as well as naïve T cells with the potential to differentiate into different effector cells upon antigenic stimulation (Rharbaoui et al., 2005). Therefore, the naïve T cells may not

be found within the $CD4^+$ and $CD8^+$ T-cell fractions, as we could observe, but instead appear within this double-negative fraction. Resident macrophages within the intestinal lamina propria have been shown to express high levels of CX3CR1. These cells have been demonstrated to play important roles during the induction of oral tolerance, assisting with loading of the antigen onto migratory $CD11c^+CD103^+$ DCs and with the expansion of Tregs locally (Pabst & Mowat, 2012). We are able to show that the NALT displays the highest percentages of $CX3CR1^+$ APCs, suggesting a similar expansion of Tregs in the nasal lamina propria.

The rapid proliferation of DO11.10 $CD4^+$ T cells in the NALT after oral administration is perhaps not that surprising, since the nasal and oral cavities lie in close proximity to one another, which makes it easy for the antigen to access the NALT from the oral cavity. One could speculate that proliferation in the CLN would be increased as well, since this lymphoid compartment lies close to both the NALT and the oral cavity. Moreover, both these lymphoid compartments drain to this site. However, there could be an aspect of time, in that drainage to the CLN does not occur as rapidly. Furthermore, it was surprising to discover that the rate of proliferation in the NALT soon increased also after gastric administration. This could be the result of the antigen after gastric administration reaching the blood-stream rapidly and disseminating through the circulation to the NALT. One would suspect that a similar scenario would apply also to the CLN and PLN, although the proliferation rates at these sites remained low. Another explanation could be that the antigen-specific T cells induced in the MLN home to the lamina propria of the nasal cavity as effector T cells or potentially as Tregs, although this does not explain why increased proliferation rates were not observed in the CLN and PLN, since they also lie in close proximity to effector sites. Overall, despite the initially more rapid proliferation rate in the NALT after oral administration, with time the gastric route of administration resulted in the highest rates of proliferation. This could be due to a higher concentration of antigens in the gut after administration by gavage. With oral administration, antigens can disseminate on their way to the intestine and the intestine has a larger mucosal surface, which may facilitate a more efficient uptake of the antigen. Another interesting finding was that the NALT despite demonstrating rapid proliferation of the DO11.10 $CD4^+$ T cells demonstrated a lower degree of activation of these cells as compared to the CLN, MLN, and PLN. This could be due to the fact that CD69 is an early activation marker and that it had already been down-regulated for the cells of the NALT that had proliferated. The rapid induction of antigen-specific T cell proliferation in the NALT might suggest that it is an optimal site for interactions to occur between APCs and T cells. However, support for this is lacking in the results presented for **Study III,** where the NALT was shown to display effector functions or perhaps tolerogenic functions rather than a strong capacity for activation.

The two routes of administration were equally efficient for inducing tolerance in a model of airway hypersensitivity, which implies that the oral-associated lymphoid tissues do not contribute in any additional way to the induction of oral tolerance. This may seem contradictory, since recent research efforts point to important roles for other parts of the mucosal immune system. Again, this could be due to the fact that the intestine comprises a larger part of the mucosal immune system. Nevertheless, the other parts may also contribute to the induction of tolerance, albeit possibly not to the same extent as the intestinal mucosa. Through the experimental designs that we have applied, it has not been possible do elucidate merely the role of the oral mucosa, as we did not circumvent the intestinal mucosa during oral administration of the antigen, which could have been an option of choice.

Two main questions remain unanswered: *(i)* Is the same step-wise model for induction of oral tolerance in the small intestine fully applicable or applicable to some degree to the nasal and oral cavities to establish tolerance in these compartments?; and *(ii)* Does dissemination of antigens and

activated T cells from the inductive sites, i.e., the MALT structures and lymph nodes, occur to the circulation after the administration of an antigen nasally, orally or intra-gastrically resulting in systemic tolerance? As shown both in the *'Introduction'* section of this thesis and here in the *'Discussion'* section, research studies have been performed and are on-going to answer these two questions. However, many details still remain unclear. For future reference, it is imperative to perform studies with a more stringent classification of the APCs and T cells. It is also necessary to include the B cells, since they play a significant role in mucosal immunity. More extensive studies of the epithelium and lamina propria of the nasal and oral cavities need to be performed to improve our understanding of the roles of the effector sites, and not only the inductive sites. Furthermore, it would be of interest to compare the oral and gastric routes of administration using three different groups of experimental animals, one where the antigen is administered by gavage, one where the antigen is administered only to the oral cavity thereby circumventing the passage to the intestine, and one where both the oral cavity and the intestine are exposed in the same feeding, resembling the natural route of an antigen. The role of the salivary glands requires additional research studies also, since they comprise effector sites of importance for both the nasal and oral cavities. Moreover, saliva, the secreted fluid from the salivary glands, plays an important role in immune defence within the oral cavity.

How can studies of tolerance contribute to understanding the role of food components in the development of RAS?

Ingested food antigens are exposed to the entire gastro-intestinal tract, from the oral cavity to the intestine. It has become clear that tolerance induction occurs also within the oral cavity and other mucosal sites, suggesting that abrogation of tolerance is possible also in the oropharyngeal compartment. The role of the oral cavity in tolerance induction is of particular interest for this thesis, as the abrogation of tolerance to certain food components has been proposed to underlie the development of RAS in a subgroup of patients suffering from this condition.

Atopic diseases have been proposed to initiate with atopic dermatitis in infants and thereafter proceed to food allergies, allergic rhinitis, and allergic asthma (Yu et al., 2016). Loss of tolerance can lead to responses to innocuous food antigens (allergic sensitisation), resulting in non-atopic conditions, such as coeliac disease, and atopic conditions, such as IgE-mediated, non-IgE mediated, and mixed-reaction food allergies (Yu et al., 2016). A limited number of studies have been conducted on the prevalence of atopy in patients with RAS, but they all point towards a possible association between the two (Wilson, 1980, Veller-Fornasa et al., 2003, Veller-Fornasa & Gallina, 2006). In addition, the occurrence of food allergies in patients with RAS has been suggested, as presented in the *'Introduction'* section of this thesis. Furthermore, IgA deficiency, which has been reported in patients with RAS (Porter & Scully, 1993), increases the tendency to develop allergies (Brandtzaeg, 2013).

Food allergies give rise to gastro-intestinal symptoms, urticarial and airway inflammation, which range in severity from mild to potentially life-threatening. It is not unreasonable to assume that symptoms such as oral ulcerations occur in the oral cavity due to a hypersensitivity reaction or allergy towards a food antigen, since other oral manifestations as well as other gastro-intestinal manifestations have been reported for patients with RAS (Sampson, 2016). As explained previously, the number of different reaction patterns within the oral cavity is limited. Therefore, oral ulcerations may be manifested as part of the clinical picture of food allergy. However, no study has reported the prevalence of oral ulcerations in patients with food allergies. It would therefore be of interest to

conduct a study on patients with diagnosed food allergies, to estimate the occurrence of such symptoms, since this information is currently unavailable.

How abrogation of tolerance is established in patients with food allergies is not well-understood, and why food tolerance is established in certain individuals and food allergy in others also remains an enigma. In the healthy state, tolerance is established, and during the disease state, sensitisation occurs. Treatment strategies for food allergies involve immunotherapy aimed at desensitisation. The mechanisms of desensitisation are not well-understood, and they may differ from those of immune tolerance in healthy individuals (Yu et al., 2016). Food allergies promote Th2-type immune responses (Yu et al., 2016). Damage to the epithelium occurs, which leads to an increased passage of antigens. This stimulates the secretion of epithelium-derived cytokines, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSPL), allowing DCs to mature and migrate to the lymph nodes. There, naïve T cells differentiate into Th2 cells, which home to the lamina propria and secrete Th2 cytokines, such as IL-4, IL5, and IL-13. This results in the accumulation of eosinophils and basophils and allows for antibody class switching of B cells to IgE. Patients with food allergies are known to have an increased gut permeability at baseline (Ventura et al., 2006). It seems likely that increased permeability of the oral mucosa also occurs in allergic individuals, which increases the risk of RAS lesions.

Currently, SLIT is being explored as a route for allergen-specific immunotherapy, creating an alternative to more conventional methods of immunisation. SLIT is becoming an alternative also for food allergens, for which subcutaneous immunotherapy (SCIT) was previously the only existing option. Compared to nasal immunisation, SLIT has the advantage that there is no risk that the antigen will be re-directed to the brain (Brandtzaeg, 2013). In general, this type of therapy is aimed at reducing or abrogating the sensitivity to a specific allergen by decreasing the levels of allergen-specific IgE and increasing the levels of IgG4, as well as reducing the response of effector $CD4^+$ T cells and inducing Tregs (Dioszeghy et al., 2016).

Until no association can be proven, food components remain as a possible aetiological factor in patients with RAS. The number of patients who display RAS as part of the clinical picture of a food allergy is probably low. However, since this condition is greatly debilitating, it is necessary to identify these individuals. Therefore, referral of patients with RAS and a suspected food allergy to an allergy specialist may be required as part of the clinical routine. However, it is a difficult task to identify these patients. Further research into this matter, in close collaboration with allergologists, is required to understand the role of food components in RAS.

Is there any scientific basis for the hypothetical classification of foods that may cause RAS into foods that potentially produce an etch wound of the oral mucosa, as compared to foods for which a hypersensitivity or an allergic reaction has been proposed?

As presented in the *'Introduction'* section of this thesis, several studies on hypersensitivity and allergic reactions in RAS have been conducted. However, there are currently no studies that have been able to define exactly which foods are relevant or the exact mechanisms by which they potentially trigger lesion development. Anamnestic findings in patients with RAS suggest that acidic foods, such as citric fruits, pineapple, tomatoes, and vinegar, may cause RAS lesions (Hay & Reade, 1984). Patients with RAS in a Turkish population have been reported to have a higher intake of acidic foods than the general population (Gonul et al., 2007). In light of this, it may be necessary to distinguish between

foods that cause damage to the epithelium through an etch wound and foods that induce a hypersensitivity or allergic reaction. In *'The RAS model'* (Figure 5), these foodstuffs are classified into type I foods and type II foods respectively. The distinction between different food components in RAS and the reactions that they provoke has not been clearly established. For food components that cause a potential etch wound, only a few early studies have been published with contradictory results. It has been reported that the application of citric acid and acetic acid, acids commonly found in foodstuffs, directly to the oral mucosa of patients with a positive history of RAS caused ulcerative lesions in a number of patients with a history of allergy (the type of allergy was not reported, so it is not known if it was specifically a food allergy or not), as compared to patients who reported no allergy and in whom no lesions were reported despite a positive history of RAS (Tuft & Girsh, 1958). However, it has also been reported that no lesions developed in a group of patients with RAS after these two acids were applied (Kutscher et al., 1958). From that study, it is not clear whether the patients reported any allergies. In our clinic, patients frequently report that various food components aggravate their condition of RAS. However, it is difficult to interpret whether they mean that these foods cause the lesions or aggravate the symptoms. Naturally acidic foods will aggravate the symptoms if in contact with an ulceration of the oral mucosa. However, it could also be that acidic foods assist in the development of lesions in allergic patients as they already are sensitised to certain food components. This could be the reason why the same is not observed in patients without a history of allergy. Before this issue can be resolved, further studies need to be conducted, as the previous studies have been too few and have not been able to provide clarity regarding this issue.

What does the future hold for treatment strategies of RAS?

Currently, there is no consensus regarding the optimal treatment regimen for RAS. Furthermore, conclusive evidence for different treatment strategies cannot be drawn from the available systematic reviews. One of many reasons for this is that the methodologies used are poor. In an attempt to solve this problem, Tappuni *et al.* introduced an ulcer severity scoring (USS) system (Tappuni et al., 2013), so as to standardise the assessment of the severity of oral ulcers and their responses to treatment. This method has now been adopted at our department and in our clinical practice, to ensure a more consistent evaluation of disease severity and facilitate comparisons between our own cohorts of patients, and hopefully in the future, comparisons with cohorts from other studies conducted nationally and internationally.

In the management of patients with RAS, it is essential to rule out any other cause for the lesions, for example, hematinic deficiencies, hormonal imbalances or systemic disease, as well as any form of medication, local trauma or psychological factors as possible causative factors. Clinical management should therefore entail a systematic assessment of whether or not other factors could be associated with RAS, even though the putative associations with several of these factors have not yet been completely established. Close collaboration between the oral medicine specialists and other medical specialists is therefore crucial.

There is a constantly growing market for non-prescription products aimed at treating aphthous lesions symptomatically. However, in my experience, what the patient with RAS wants is a way to avoid treatment if possible but still prevent the development of lesions on a long-term basis. The only reasonable possibility to achieve this goal is through a complete understanding of the true aetiology of RAS. While a cure for RAS appears elusive based on what we currently know about this disease effective management could be achieved using prophylactic measures that counteract disease progression in patients with pre-disposing factors, and in this way relieve the patients of their

symptoms. For patients with severe forms of RAS, treatment strategies that involve prescription drugs will still be required. However, for many of the patients routinely treated with topical steroids (due to the of the lack of alternative treatments), measures that address the true aetiology in each and every patient could offer an alternative, more cost-effective strategy that ultimately would be beneficial for the patient, the clinician, and society at large.

In conclusion, this thesis is one of several studies with the focus on RAS. Compared to the other reports that have been written on this subject, this thesis most probably has the broadest approach, in that several different features of the disease are taken into account. The disadvantages of this strategy are that a detailed analysis of one particular factor is not feasible and it is a challenge to keep up with the literature and different methodologies when focusing on so many different aspects. However, to truly understand a condition and to decide which research studies need to be carried out, it is essential to penetrate every aspect of the disease. The focus in this thesis has been on host genetics, the oral microbiota, and immunoregulatory networks and their respective contributions to the development of RAS. While I cannot provide definite answers regarding the precise roles of these factors, it is fair to say that they are all of importance in RAS and that additional studies will have to be conducted to elucidate unambiguously their roles. Ultimately, these factors all influence each other, so the question is to what extent and how this is of relevance for the disease.

ACKNOWLEDGEMENTS

Supervisors:

Sofia Östman, Thank you for the hours that you have spent with me in the lab and the time put into writing, rewriting, and correcting the research articles. Personality-wise we are complete opposites, and while this has been challenging at times for both of us, I also believe that it has been one of the keys for completing this PhD project successfully. Spending time with you has given me, coming from a clinical environment, a great insight into pre-clinical research and for this I am grateful. It is something that I will always remember and treasure.

Mats Jontell, I wish to express my deepest gratitude to you for incorporating me into your research group, creating an inspiring, warm and supportive atmosphere. Thank you also for your endless knowledge, patience and guidance. When the going gets tough you always keep your cool, an invaluable quality for a supervisor. I will never forget my background and where I first became a researcher. I am also grateful for you sticking by me to the end and I wish you all the best for your retirement. I will miss you deeply.

Co-authors:

Agnes Wold, My thanks to you for providing a workspace for me in your lab and for the hours that you have spent reading, re-reading and correcting the manuscripts to do the work justice and exploit its full potential.

Åsa Torinsson Naluai, You have always treated me as an equal and never as just a PhD student, for which I am very thankful. I have thoroughly enjoyed working with you. Genetics is immensely complicated but you have been there all the way to guide me through it. I know that you have an enormous workload but you have never let that get in the way of our project and I hope that we can collaborate more in the future.

Fei Sjöberg, I would like to thank you for all your help with the microbiology study. Everything that I know about microbiological techniques is down to you. You have guided me through work in the lab and helped me with all the data analysis always with a smile and positive mind-set. I admire the patience and caution with which you work. I find you 100% trustworthy, which is a rare quality to find in a person and one that I appreciate so much about you.

Anna-Karin Östberg, I have really enjoyed working with you. You are so skilled and work with such caution. I hope that we can work a lot more together in the years to come. You are the nave of our lab and without you we would not manage. Thank you also for being so patient with me in answering all the questions that I have thrown at you throughout the years.

Gita Gale, Thank you for all the help with recruiting and sampling the patients and controls for the microbiology study, and for paving the way for me during the course of my thesis. Since you have always been one step ahead of me in your thesis work I have been able to rely a lot on you which has been so valuable for me.

Colleagues:

Special thanks to **the Swedish National Graduate School in Odontological Science** for providing me with the opportunity to pursue a PhD. I am proud of being part of the first research school in Odontological Science in Sweden and grateful for the opportunities this has given me. To **Anna**, **Lotta**, **Olivier** and **Claes-Göran**, my fellow PhD colleagues in Gothenburg, I am so glad that you were the ones to follow me on this adventure and thank you for all the lovely lunches we have had throughout the years. We all made it in the end! I also want to thank all my other PhD colleagues throughout Sweden who I would probably not have met if it had not been for the research school. Hopefully, our paths will cross again in the future. **Claes-Göran**, Thank you for being there for me in the beginning when I felt so lost and alone. You probably do not even know it but you helped me through some really tough times. I will never forget you for that. You are a really great person and I hope that life will treat you well in the future.

Special thanks to **the staff at the Clinic of Oral Medicine at Odontologen**. You are all so kind and helpful. Without you, collecting the patients´ samples would not have been possible and my lunches would have been so boring. I also want to send a thought to **Lisbeth** who unfortunately is not with us anymore. I will never forget our late Friday afternoon chats before going home. I miss you!

I also wish to thank **the staff at the Clinic for hospital dentistry in Borås**. It has been so valuable for me to be able to see patients within this field in parallel to my research. I have learned a lot and look forward to plunging even deeper into this area in the years to come. **Katarina** and **Marie-Louise**, You have both been so patient with me throughout the years and especially now at the end when the workload has been quite heavy. You have never put any pressure on me. **Vegard**, You have been a great supervisor, always making time for me and my many questions. I look forward to working with you even more in the future. You have been greatly missed at Odontologen but I hit the jackpot since I also work in Borås so I never had to say goodbye to you. **Anneli**, **Christin** and **Eta**, You have all been fantastic keeping me on track and up to date each and every Monday and I have loved working with you all.

Bengt Hasséus, I am so grateful to you, even though you have not been directly involved in my thesis, you have always been there in the background creating a positive research environment for our group. I have always felt that I could come to you with my questions and reflections whether it involves research, patients, teaching or career planning. You have such a positive and warm personality. I have in all the years that I have been at the department never seen you angry or having a bad day. That says a lot about the lovely person that you are.

Ulf Dahlgren, Thank you so much for your valuable discussions. You have taught me never to trust anything I read before critically dissecting it first and that research is never better than the person that performs it - a tough, but true insight. Thank you also for all your sessions in immunology. They have been great!

Gunnar Dahlén, I warmly thank you for your help with the microbiology study. I have greatly appreciated all our meetings and many discussions by e-mail and in person. You are such a level-headed and modest person. Despite your great achievements you are so down-to-earth, which is admirable.

Johan Carlsson, I have loved to work with you over the years. You are so nice and I love your sense of humour. You can´t kill a mouse with your bare hands but you are a god with the FACs machine. It has been a bit lonely here since you moved to Uppsala but I am so happy that you have settled in. I wish you the best of luck with the rest of your PhD and all your future races.

To **Gita**, **Jenny**, **Jairo**, **Amal**, **Mia and Magda**, It has been great sharing these years with you with all the ups and downs both at work and in life. It is amazing how many people you can fit into one single room from so many parts of the world. We have really become a close-knit family and I appreciate you all and the help, support, and comfort you have given me. **Jenny**, I admire you so much as a colleague, a mother and a friend and I hope that we will be able to support each other also in the years to come. **Amal** and **Jairo**, I will truly miss you when you go back home. The department will feel so empty without you. I hope that we can stay in touch and that we can continue supporting each other and collaborating in the future. **Mia**, You do not live in the same room as us any longer but you did for a number of years and I will always treasure the chats we had about everything and nothing. **Magda**, I am so happy that you have taken a seat in our room. You are lovely and I hope that we can get to know each other even better in the years to come.

Java, **Jonas** and **Angelica**, I am so glad that you have come and reinforced our department. I hope that I will be able to show you the same support that I have experienced during my time as a PhD student. I wish you all the best of luck with your projects.

Huliya, Finally, we can begin with the next part of the journey trying to find some more pieces of the puzzle behind RAS. I look forward to us working together in the coming projects.

To the ladies on the 6th floor at Guldhedsgatan 10A, **Ingela**, **Jolanta**, **and Eva.** You have all been so lovely to me showing me where things are and helping me with my lab-work. I have also enjoyed all the little chitchats we have had over the years.

I also want to thank warmly **Marita Nilsson** and **Anders Grip** for all the time and effort you have spent helping me with MedView and other technical hurdles. Unfortunately, I do not understand most of what it is you have done for me but it has been invaluable in helping me move forward with my research and thesis.

Eva Frantzich, There is only one thing I wish to say and that is: You are the best! You have helped me countless times with administrative hurdles, always with smiles and jokes and you are one of the quickest people I know at responding to e-mails. I never thought that Palasso and administering grants could be more complicated than finishing a PhD but it surely is, and I am so glad for your help over the years. Thank you also for all your assistance in preparing for the dissertation day. You are so well-organised!

Jennie Finnman and the **Genomics Core Facility**. Thank you for being so organised and efficient. You really took a large workload off me when you took care of all the DNA extractions of the patients´ samples.

To **Sara Alizadehgharib** and **Shikha Acharya**, Coming down to the lab is always nice when you are there. I hope that I will spend more time there with you when I get back from parental leave.

Christina Eklund, Thank you for introducing me to the lab and for being so positive and supportive. I have missed you a lot since you retired.

Vincent Collins, Thank you for proof-reading all the articles and this frame. You are so thorough and efficient and I have greatly appreciated working with you. I hope we can continue also in the future. Thank you likewise for the time you spent helping me in the manuscript-writing course and for supporting our research group with seminars to improve our writing even more.

Annika Herrström, Thank you for everything that you have done for me in preparing for the dissertation day. I would not have managed without you. With Eva and you by my side, I have been able to focus on preparing for the actual dissertation knowing that you have thought about all the rest.

I want to send a thought to **the former Shell refinery in Gothenburg**. I thoroughly enjoyed my summers at the lab working in such a warm and friendly atmosphere. I believe that was one of the reasons for me to want to return to a lab environment but I will never forget where I experienced it first!

Jette Lehrmann Madsen, I did not know you, but you let me come with you to the Gambia to conduct a study on caries prevalence in the adult population as my Master´s degree thesis. The love you feel for the Gambia and its people is extraordinary and I admire you for feeling so strongly about something. You helped to plant a little seed within me to be passionate about my profession and to feel pleasure in being able to help others. Thanks to you, I also had a positive first encounter with research.

Karin Sunnegårdh-Grönberg, You took Cilla and me under your wings when we so desperately wanted to go to the Gambia for our Master´s degree thesis. Nobody else would take us on and for this we will always be thankful to you. You had a hard time supervising us, steering us in the right direction, trying to make some Science out of our trip, but you managed and the research methodology that you taught me is something that I have used to great advantage during this PhD experience. Thank you so much for that and for creating a positive first research environment for me!

Patients:
To **all the Patients**, who have made this thesis possible. You suffer from a painful and greatly debilitating condition, yet you have showed up at your appointments and been so supportive of the work that we have done

and followed it with such interest. We appreciate you all so much and will continue to dig deeper into the true aetiology of RAS. We currently have a few pieces of the puzzle. Now, we just have to find the rest with the help of the world-wide research community and solve the puzzle correctly. Hopefully, you and I will live to see that day.

Friends and family:
Cilla, You are such a dear friend. Without you, I would probably never have gotten through dental school, as so I would never have completed my PhD either. Even though we are not roommates anymore I think of you each and every day and value our friendship so much and the experiences we have had, as well as the adventures that are yet to come.

Angelika, I am so glad that my sometimes whining about my research and thesis has not put you off wanting to pursue an academic career as well. If you do I hope to be able to support you in the same way that you have supported me throughout the years. It is so nice to have a friend who I have known since I was a baby and to know that we stick by each other no matter what the circumstances.

A special thank you to **all my friends**. You are all so lovely in your own ways and provide me with a good balance between work and normal life, which means so much.

To **Gudrun** and **Claes**, Thank you for all the support you have shown Lars, Sophie, and me throughout the years. It would not have been possible without you. I have also appreciated all the talks we have had about pursuing a PhD and academic life. It has been nice to talk to someone outside of the University who also shares these experiences.

Hannah and **Robert**, I have enjoyed feeling the support from you as siblings but also as fellow researchers because in that way we have been able to support and understand each other even more. I have felt comfort in sharing our ups and downs in life and in research. I am also grateful for the work you put in Robert in helping me with my RAS model. I am so pleased with how it turned out! I wish we all lived in the same city so that we could see each other more often but at the same time it is nice to know that we are always welcome for holidays.

Mum, Thank you for always being there for me and believing in me. You have never judged my (sometimes crazy) ideas but instead said: “Okay, if that is what you want to do how are you going to make it happen?” Thanks to you, I have always aimed for the stars but felt comfort in the fact that if I reach the tree-tops that is far enough. Thank you also for all the support that you have shown Lars, Sophie and me over the years. We could not have coped without you.

Sophie, There is nothing better than after a long and hard days work, when sometimes everything feels impossible and suffocating, to come home to your smiles and soft cuddles. I love you endlessly. I will never write a book again in my life - I promise! In a couple of weeks you will finally be a big sister!

Lars, There are not words to describe how much I love you. You have been my rock over the years and without you this thesis would never have been completed. The last six months have been a bit of a challenge but you have shown me nothing but support. You have been so patient and considerate. Now I cannot wait to have our new baby and focus on family life for a while!

When writing this acknowledgement I realised that I am blessed being surrounded by so many inspiring, warm and lovely people. You all make my life so enjoyable, thank you!

Gothenburg, February 2017
Maria Bankvall

REFERENCES

Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, Rudensky A, Sakaguchi S, Shevach EM, Vignali DA and Ziegler SF (2013). Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol* **14**: 307-8.

Abd Al Samid M, Chaudhary B, Khaled YS, Ammori BJ and Elkord E (2016). Combining FoxP3 and Helios with GARP/LAP markers can identify expanded Treg subsets in cancer patients. *Oncotarget* **7**: 14083-94.

Abdulla Y and Lehner T (1979). *The effect of immune complexes on chemotaxis in Behcet´s syndrome and recurrent oral ulcers. In: Lehner T, Barnes CG, eds: Behcets Syndrome: Clinical and Immunological Features.,* Academic Press: London.

Adan A, Alizada G, Kiraz Y, Baran Y and Nalbant A (2016). Flow cytometry: basic principles and applications. *Crit Rev Biotechnol*: 1-14.

Adisen E, Aral A, Aybay C and Gurer MA (2008). Salivary epidermal growth factor levels in Behcet's disease and recurrent aphthous stomatitis. *Dermatology* **217**: 235-40.

Admyre C, Grunewald J, Thyberg J, Gripenback S, Tornling G, Eklund A, Scheynius A and Gabrielsson S (2003). Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid. *The European respiratory journal* **22**: 578-83.

Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, Neve EP, Scheynius A and Gabrielsson S (2007). Exosomes with immune modulatory features are present in human breast milk. *J Immunol* **179**: 1969-78.

Akintoye SO and Greenberg MS (2014). Recurrent aphthous stomatitis. *Dental clinics of North America* **58**: 281-97.

Al-Samadi A, Drozd A, Salem A, Hietanen J, Hayrinen-Immonen R and Konttinen YT (2015). Epithelial Cell Apoptosis in Recurrent Aphthous Ulcers. *J Dent Res* **94**: 928-35.

Albanidou-Farmaki E, Poulopoulos AK, Epivatianos A, Farmakis K, Karamouzis M and Antoniades D (2008). Increased anxiety level and high salivary and serum cortisol concentrations in patients with recurrent aphthous stomatitis. *Tohoku J Exp Med* **214**: 291-6.

Asanuma H, Thompson AH, Iwasaki T, Sato Y, Inaba Y, Aizawa C, Kurata T and Tamura S (1997). Isolation and characterization of mouse nasal-associated lymphoid tissue. *J Immunol Methods* **202**: 123-31.

Atkin PA, Xu X and Thornhill MH (2002). Minor recurrent aphthous stomatitis and smoking: an epidemiological study measuring plasma cotinine. *Oral Dis* **8**: 173-6.

Axell T (1976). A prevalence study of oral mucosal lesions in an adult Swedish population. *Odontologisk revy. Supplement* **36**: 1-103.

Axell T and Henricsson V (1985). The occurrence of recurrent aphthous ulcers in an adult Swedish population. *Acta Odontol Scand* **43**: 121-5.

Aydin S (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides* **72**: 4-15.

Baccaglini L, Lalla RV, Bruce AJ, Sartori-Valinotti JC, Latortue MC, Carrozzo M and Rogers RS, 3rd (2011). Urban legends: recurrent aphthous stomatitis. *Oral Dis* **17**: 755-70.

Bankvall M, Sjoberg F, Gale G, Wold A, Jontell M and Ostman S (2014). The oral microbiota of patients with recurrent aphthous stomatitis. *Journal of oral microbiology* **6**: 25739.

Banoczy J and Sallay K (1969). Comparative cytologic studies in patients with recurrent aphthae and leukoplakia. *J Dent Res* **48**: 271-3.

Bazrafshani MR, Hajeer AH, Ollier WE and Thornhill MH (2002a). IL-1B and IL-6 gene polymorphisms encode significant risk for the development of recurrent aphthous stomatitis (RAS). *Genes and immunity* **3**: 302-5.

Bazrafshani MR, Hajeer AH, Ollier WE and Thornhill MH (2002b). Recurrent aphthous stomatitis and gene polymorphisms for the inflammatory markers TNF-alpha, TNF-beta and the vitamin D receptor: no association detected. *Oral Dis* **8**: 303-7.

Bazrafshani MR, Hajeer AH, Ollier WE and Thornhill MH (2003). Polymorphisms in the IL-10 and IL-12 gene cluster and risk of developing recurrent aphthous stomatitis. *Oral Dis* **9**: 287-91.

Belenguer-Guallar I, Jimenez-Soriano Y and Claramunt-Lozano A (2014). Treatment of recurrent aphthous stomatitis. A literature review. *J Clin Exp Dent* **6**: e168-74.

Benson MJ, Pino-Lagos K, Rosemblatt M and Noelle RJ (2007). All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* **204**: 1765-74.

Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, Weissman IL, Hamann A and Butcher EC (1993). Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* **74**: 185-95.

Besu I, Jankovic L, Konic-Ristic A, Damjanovic A, Besu V and Juranic Z (2013a). Good tolerance to goat's milk in patients with recurrent aphthous ulcers with increased immunoreactivity to cow's milk proteins. *J Oral Pathol Med* **42**: 523-7.

Besu I, Jankovic L, Konic-Ristic A, Raskovic S, Besu V, Djuric M, Cakic S, Magdu IU and Juranic Z (2013b). The role of specific cow's milk proteins in the etiology of recurrent aphthous ulcers. *J Oral Pathol Med* **42**: 82-8.

Besu I, Jankovic L, Magdu IU, Konic-Ristic A, Raskovic S and Juranic Z (2009). Humoral immunity to cow's milk proteins and gliadin within the etiology of recurrent aphthous ulcers? *Oral Dis* **15**: 560-4.

Boras VV, Lukac J, Brailo V, Picek P, Kordic D and Zilic IA (2006). Salivary interleukin-6 and tumor necrosis factor-alpha in patients with recurrent aphthous ulceration. *J Oral Pathol Med* **35**: 241-3.

Borra RC, Andrade PM, Silva ID, Morgun A, Weckx LL, Smirnova AS and Franco M (2004). The Th1 /Th2 immune-type response of the recurrent aphthous ulceration analyzed by cDNA microarray. *J Oral Pathol Med* **33**: 140-6.

Borra RC, de Mesquita Barros F, de Andrade Lotufo M, Villanova FE and Andrade PM (2009). Toll-like receptor activity in recurrent aphthous ulceration. *J Oral Pathol Med* **38**: 289-98.

Brandtzaeg P (2010). Function of mucosa-associated lymphoid tissue in antibody formation. *Immunological investigations* **39**: 303-55.

Brandtzaeg P (2013). Secretory immunity with special reference to the oral cavity. *Journal of oral microbiology* **5**.

Brandtzaeg P, Kiyono H, Pabst R and Russell MW (2008). Terminology: nomenclature of mucosa-associated lymphoid tissue. *Mucosal Immunol* **1**: 31-7.

Brocklehurst P, Tickle M, Glenny AM, Lewis MA, Pemberton MN, Taylor J, Walsh T, Riley P and Yates JM (2012). Systemic interventions for recurrent aphthous stomatitis (mouth ulcers). *Cochrane Database Syst Rev*: CD005411.

Brozovic S, Vucicevic-Boras V, Mravak-Stipetic M, Jukic S, Kleinheinz J and Lukac J (2002). Salivary levels of vascular endothelial growth factor (VEGF) in recurrent aphthous ulceration. *J Oral Pathol Med* **31**: 106-8.

Bruce MG and Ferguson A (1986). The influence of intestinal processing on the immunogenicity and molecular size of absorbed, circulating ovalbumin in mice. *Immunology* **59**: 295-300.

Buno IJ, Huff JC, Weston WL, Cook DT and Brice SL (1998). Elevated levels of interferon gamma, tumor necrosis factor alpha, interleukins 2, 4, and 5, but not interleukin 10, are present in recurrent aphthous stomatitis. *Arch Dermatol* **134**: 827-31.

Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G and Bonnerot C (2005). Exosomal-like vesicles are present in human blood plasma. *International immunology* **17**: 879-87.

Calderon PE, Valenzuela FA, Carreno LE and Madrid AM (2008). A possible link between cow milk and recurrent aphtous stomatitis. *J Eur Acad Dermatol Venereol* **22**: 898-9.

Callery MP, Kamei T and Flye MW (1989). The effect of portacaval shunt on delayed-hypersensitivity responses following antigen feeding. *J Surg Res* **46**: 391-4.

Cha HR, Chang SY, Chang JH, Kim JO, Yang JY, Kim CH and Kweon MN (2010). Downregulation of Th17 cells in the small intestine by disruption of gut flora in the absence of retinoic acid. *J Immunol* **184**: 6799-806.

Chang SY, Song JH, Guleng B, Cotoner CA, Arihiro S, Zhao Y, Chiang HS, O'Keeffe M, Liao G, Karp CL, Kweon MN, Sharpe AH, Bhan A, Terhorst C and Reinecker HC (2013).

Circulatory antigen processing by mucosal dendritic cells controls CD8(+) T cell activation. *Immunity* **38**: 153-65.

Chase MW (1946). Inhibition of experimental drug allergy by prior feeding of the sensitizing agent. *Proc Soc Exp Biol Med* **61**: 257-9.

Chattopadhyay A and Chatterjee S (2007). Risk indicators for recurrent aphthous ulcers among adults in the US. *Community dentistry and oral epidemiology* **35**: 152-9.

Chaudhry A and Rudensky AY (2013). Control of inflammation by integration of environmental cues by regulatory T cells. *J Clin Invest* **123**: 939-44.

Chavan M, Jain H, Diwan N, Khedkar S, Shete A and Durkar S (2012). Recurrent aphthous stomatitis: a review. *J Oral Pathol Med*.

Chen Y, Inobe J, Marks R, Gonnella P, Kuchroo VK and Weiner HL (1995). Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature* **376**: 177-80.

Cooke BE and Armitage P (1960). Recurrent Mikulicz's aphthae treated with topical hydrocortisone hemisuccinate sodium. Double-blind controlled clinical trial. *British medical journal* **1**: 764-6.

Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y and Powrie F (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* **204**: 1757-64.

Dagalis P, Bagg J and Walker DM (1987). Spontaneous migration and chemotactic activity of neutrophil polymorphonuclear leukocytes in recurrent aphthous ulceration. *Oral Surg Oral Med Oral Pathol* **64**: 298-301.

Dahlén G, Fiehn NE, Olsen I and Dahlgren U (2012). *Oral Microbiology and Immunology,* Munksgaard: Denmark.

Dakin R (1829). Remarks on a cutaneous affection, produced by certain poisonous vegetables. *Am J Med Sci* **4**: 98-100.

Dioszeghy V, Mondoulet L, Puteaux E, Dhelft V, Ligouis M, Plaquet C, Dupont C and Benhamou PH (2016). Differences in phenotype, homing properties and suppressive activities of regulatory T cells induced by epicutaneous, oral or sublingual immunotherapy in mice sensitized to peanut. *Cellular & molecular immunology*.

Eguia-del Valle A, Martinez-Conde-Llamosas R, Lopez-Vicente J, Uribarri-Etxebarria A and Aguirre-Urizar JM (2011). Salivary levels of Tumour Necrosis Factor-alpha in patients with recurrent aphthous stomatitis. *Medicina oral, patologia oral y cirugia bucal* **16**: e33-6.

Embil JA, Stephens RG and Manuel FR (1975). Prevalence of recurrent herpes labialis and aphthous ulcers among young adults on six continents. *Can Med Assoc J* **113**: 627-30.

Eriksson K, Ahlfors E, George-Chandy A, Kaiserlian D and Czerkinsky C (1996). Antigen presentation in the murine oral epithelium. *Immunology* **88**: 147-52.

Eversole LR (1994). Immunopathology of oral mucosal ulcerative, desquamative, and bullous diseases. Selective review of the literature. *Oral Surg Oral Med Oral Pathol* **77**: 555-71.

Eversole LR, Shopper TP and Chambers DW (1982). Effects of suspected foodstuff challenging agents in the etiology of recurrent aphthous stomatitis. *Oral Surg Oral Med Oral Pathol* **54**: 33-8.

Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, Furtado GC, Lira SA and Shakhar G (2013). Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity* **38**: 581-95.

Faria AM, Maron R, Ficker SM, Slavin AJ, Spahn T and Weiner HL (2003). Oral tolerance induced by continuous feeding: enhanced up-regulation of transforming growth factor-beta/interleukin-10 and suppression of experimental autoimmune encephalomyelitis. *Journal of autoimmunity* **20**: 135-45.

Field EA and Allan RB (2003). Review article: oral ulceration--aetiopathogenesis, clinical diagnosis and management in the gastrointestinal clinic. *Aliment Pharmacol Ther* **18**: 949-62.

Francis TC (1970). Recurrent aphthous stomatitis and Behcet's disease. A review. *Oral Surg Oral Med Oral Pathol* **30**: 476-86.

Friedman A and Weiner HL (1994). Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc Natl Acad Sci U S A* **91**: 6688-92.

Furrie E, Turner MW and Strobel S (1994). Failure of SCID mice to generate an oral tolerogen after a feed of ovalbumin: a role for a functioning gut-associated lymphoid system. *Immunology* **83**: 562-7.

Furrie E, Turner MW and Strobel S (1995). Partial characterization of a circulating tolerogenic moiety which, after a feed of ovalbumin, suppresses delayed-type hypersensitivity in recipient mice. *Immunology* **86**: 480-6.

Gasche C and Grundtner P (2005). Genotypes and phenotypes in Crohn's disease: do they help in clinical management? *Gut* **54**: 162-7.

Gonul M, Gul U, Cakmak SK and Kilic A (2007). The role of the diet in patients with recurrent aphthous stomatitis. *Eur J Dermatol* **17**: 97-8.

Graykowski EA, Barile MF, Lee WB and Stanley HR, Jr. (1966). Recurrent aphthous stomatitis. Clinical, therapeutic, histopathologic, and hypersensitivity aspects. *JAMA* **196**: 637-44.

Gregerson DS, Obritsch WF and Donoso LA (1993). Oral tolerance in experimental autoimmune uveoretinitis. Distinct mechanisms of resistance are induced by low dose vs high dose feeding protocols. *J Immunol* **151**: 5751-61.

Griffin CJ (1981). The ultrastructure of T and B lymphocytes in recurrent apthous ulceration of the buccal mucosa. *Aust Dent J* **26**: 31-41.

Guilliams M, Crozat K, Henri S, Tamoutounour S, Grenot P, Devilard E, de Bovis B, Alexopoulou L, Dalod M and Malissen B (2010). Skin-draining lymph nodes contain dermis-

derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood* **115**: 1958-68.

Guimaraes AL, Correia-Silva Jde F, Sa AR, Victoria JM, Diniz MG, Costa Fde O and Gomez RS (2007). Investigation of functional gene polymorphisms IL-1beta, IL-6, IL-10 and TNF-alpha in individuals with recurrent aphthous stomatitis. *Arch Oral Biol* **52**: 268-72.

Hadis U, Wahl B, Schulz O, Hardtke-Wolenski M, Schippers A, Wagner N, Muller W, Sparwasser T, Forster R and Pabst O (2011). Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* **34**: 237-46.

Harkema JR, Carey SA and Wagner JG (2006). The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. *Toxicol Pathol* **34**: 252-69.

Hay KD and Reade PC (1984). The use of an elimination diet in the treatment of recurrent aphthous ulceration of the oral cavity. *Oral Surg Oral Med Oral Pathol* **57**: 504-7.

Hayrinen-Immonen R, Nordstrom D, Malmstrom M, Hietanen J and Konttinen YT (1991). Immune-inflammatory cells in recurrent oral ulcers (ROU). *Scand J Dent Res* **99**: 510-8.

Healy CM, Carvalho D, Pearson JD and Thornhill MH (1996). Raised anti-endothelial cell autoantibodies (AECA), but not anti-neutrophil cytoplasmic autoantibodies (ANCA), in recurrent oral ulceration: modulation of AECA binding by tumour necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma). *Clin Exp Immunol* **106**: 523-8.

Heritage PL, Underdown BJ, Arsenault AL, Snider DP and McDermott MR (1997). Comparison of murine nasal-associated lymphoid tissue and Peyer's patches. *American journal of respiratory and critical care medicine* **156**: 1256-62.

Hietanen J, Hayrinen-Immonen R, Al-Samadi A, Trokovic N, Koskenpato K and Konttinen YT (2012). Recurrent aphthous ulcers--a Toll-like receptor-mediated disease? *J Oral Pathol Med* **41**: 158-64.

Hijazi K, Lowe T, Meharg C, Berry SH, Foley J and Hold GL (2015). Mucosal microbiome in patients with recurrent aphthous stomatitis. *J Dent Res* **94**: 87S-94S.

Hirschhorn JN and Daly MJ (2005). Genome-wide association studies for common diseases and complex traits. *Nature reviews. Genetics* **6**: 95-108.

Humphrey SP and Williamson RT (2001). A review of saliva: normal composition, flow, and function. *The Journal of prosthetic dentistry* **85**: 162-9.

Hunter IP, Ferguson MM, Scully C, Galloway AR, Main AN and Russell RI (1993). Effects of dietary gluten elimination in patients with recurrent minor aphthous stomatitis and no detectable gluten enteropathy. *Oral Surg Oral Med Oral Pathol* **75**: 595-8.

Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C and Song SY (2004). Retinoic acid imprints gut-homing specificity on T cells. *Immunity* **21**: 527-38.

Jang MH, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, Guo Z, Umemoto E, Ebisuno Y, Yang BG, Seoh JY, Lipp M, Kiyono H and Miyasaka M (2006). CCR7 is critically

important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol* **176**: 803-10.

Johnston DG and Corr SC (2016). Toll-Like Receptor Signalling and the Control of Intestinal Barrier Function. *Methods in molecular biology* **1390**: 287-300.

Jontell M, Mattsson U and Torgersson O (2005). MedView: an instrument for clinical research and education in oral medicine. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **99**: 55-63.

June CH (2007). Principles of adoptive T cell cancer therapy. *J Clin Invest* **117**: 1204-12.

Jurge S, Kuffer R, Scully C and Porter SR (2006). Mucosal disease series. Number VI. Recurrent aphthous stomatitis. *Oral Dis* **12**: 1-21.

Kalpana R, Thubashini M and Sundharam BS (2014). Detection of salivary interleukin-2 in recurrent aphthous stomatitis. *J Oral Maxillofac Pathol* **18**: 361-4.

Karakus N, Yigit S, Rustemoglu A, Kalkan G and Bozkurt N (2014). Effects of interleukin (IL)-6 gene polymorphisms on recurrent aphthous stomatitis. *Archives of dermatological research* **306**: 173-80.

Karasneh J, Bani-Hani M, Alkhateeb A, Hassan A, Alzoubi F and Thornhill M (2015). TLR2, TLR4 and CD86 gene polymorphisms in recurrent aphthous stomatitis. *J Oral Pathol Med* **44**: 857-63.

Karlsson M, Lundin S, Dahlgren U, Kahu H, Pettersson I and Telemo E (2001). "Tolerosomes" are produced by intestinal epithelial cells. *Eur J Immunol* **31**: 2892-900.

Kawashima H and Fukuda M (2012). Sulfated glycans control lymphocyte homing. *Ann N Y Acad Sci* **1253**: 112-21.

Kearney ER, Pape KA, Loh DY and Jenkins MK (1994). Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity* **1**: 327-39.

Keller AC, Mucida D, Gomes E, Faquim-Mauro E, Faria AM, Rodriguez D and Russo M (2006). Hierarchical suppression of asthma-like responses by mucosal tolerance. *J Allergy Clin Immunol* **117**: 283-90.

Kim DY, Sato A, Fukuyama S, Sagara H, Nagatake T, Kong IG, Goda K, Nochi T, Kunisawa J, Sato S, Yokota Y, Lee CH and Kiyono H (2011). The airway antigen sampling system: respiratory M cells as an alternative gateway for inhaled antigens. *J Immunol* **186**: 4253-62.

Kim KS and Surh CD (2015). Induction of Immune Tolerance to Dietary Antigens. *Adv Exp Med Biol* **850**: 93-118.

Kim YJ, Choi YS, Baek KJ, Yoon SH, Park HK and Choi Y (2016). Mucosal and salivary microbiota associated with recurrent aphthous stomatitis. *BMC microbiology* **16 Suppl 1**: 57.

Kiyono H (2013). *Principles of mucosal immunology; The nasopharyngeal and oral immune system,* Garland Science: U.S.

Kiyono H and Azegami T (2015). The mucosal immune system: From dentistry to vaccine development. *Proc Jpn Acad Ser B Phys Biol Sci* **91**: 423-39.

Kiyono H and Fukuyama S (2004). NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat Rev Immunol* **4**: 699-710.

Kleinman DV, Swango PA and Niessen LC (1991). Epidemiologic studies of oral mucosal conditions--methodologic issues. *Community dentistry and oral epidemiology* **19**: 129-40.

Kleinman DV, Swango PA and Pindborg JJ (1994). Epidemiology of oral mucosal lesions in United States schoolchildren: 1986-87. *Community dentistry and oral epidemiology* **22**: 243-53.

Kolenbrander PE and London J (1993). Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* **175**: 3247-52.

Kraal G, Samsom JN and Mebius RE (2006). The importance of regional lymph nodes for mucosal tolerance. *Immunol Rev* **213**: 119-30.

Kraus TA, Brimnes J, Muong C, Liu JH, Moran TM, Tappenden KA, Boros P and Mayer L (2005). Induction of mucosal tolerance in Peyer's patch-deficient, ligated small bowel loops. *J Clin Invest* **115**: 2234-43.

Kruisbeek AM, Shevach E and Thornton AM (2004). Proliferative assays for T cell function. *Curr Protoc Immunol* **Chapter 3**: Unit 3 12.

Kumar BP, Keluskar V, Bagewadi AS and Shetti A (2010). Evaluating and comparing phagocytic functions of salivary and blood neutrophils in patients with recurrent aphthous ulcers and controls. *Quintessence Int* **41**: 411-6.

Kunisawa J, Nochi T and Kiyono H (2008). Immunological commonalities and distinctions between airway and digestive immunity. *Trends Immunol* **29**: 505-13.

Kuper CF, Hameleers DM, Bruijntjes JP, van der Ven I, Biewenga J and Sminia T (1990). Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study. *Cell and tissue research* **259**: 371-7.

Kuper CF, Koornstra PJ, Hameleers DM, Biewenga J, Spit BJ, Duijvestijn AM, van Breda Vriesman PJ and Sminia T (1992). The role of nasopharyngeal lymphoid tissue. *Immunology today* **13**: 219-24.

Kutscher AH, Barbash R, Zegarelli EV and Amphlett J (1958). Citric acid sensitivity in recurrent ulcerative (aphthous) stomatitis. *J Allergy* **29**: 438-41.

Kümmel W and Mikulicz vJ (1898). *Die Krankheiten des Mundes,* Fischer: Jena.

Lamichhane A, Azegamia T and Kiyonoa H (2014). The mucosal immune system for vaccine development. *Vaccine* **32**: 6711-23.

Lee H, Ruane D, Law K, Ho Y, Garg A, Rahman A, Esterhazy D, Cheong C, Goljo E, Sikora AG, Mucida D, Chen BK, Govindraj S, Breton G and Mehandru S (2015). Phenotype and function of nasal dendritic cells. *Mucosal Immunol* **8**: 1083-98.

Lehner T (1969). Pathology of recurrent oral ulceration and oral ulceration in Behcet's syndrome: light, electron and fluorescence microscopy. *J Pathol* **97**: 481-94.

Lewkowicz N, Kur B, Kurnatowska A, Tchorzewski H and Lewkowicz P (2011). Expression of Th1/Th2/Th3/Th17-related genes in recurrent aphthous ulcers. *Archivum immunologiae et therapiae experimentalis* **59**: 399-406.

Lewkowicz N, Lewkowicz P, Banasik M, Kurnatowska A and Tchorzewski H (2005). Predominance of Type 1 cytokines and decreased number of CD4(+)CD25(+high) T regulatory cells in peripheral blood of patients with recurrent aphthous ulcerations. *Immunol Lett* **99**: 57-62.

Lewkowicz N, Lewkowicz P, Dzitko K, Kur B, Tarkowski M, Kurnatowska A and Tchorzewski H (2008). Dysfunction of CD4+CD25high T regulatory cells in patients with recurrent aphthous stomatitis. *J Oral Pathol Med* **37**: 454-61.

Li Z, Li D, Tsun A and Li B (2015). FOXP3+ regulatory T cells and their functional regulation. *Cellular & molecular immunology* **12**: 558-65.

Linden S, Mahdavi J, Semino-Mora C, Olsen C, Carlstedt I, Boren T and Dubois A (2008a). Role of ABO secretor status in mucosal innate immunity and H. pylori infection. *PLoS pathogens* **4**: e2.

Linden SK, Sutton P, Karlsson NG, Korolik V and McGuckin MA (2008b). Mucins in the mucosal barrier to infection. *Mucosal Immunol* **1**: 183-97.

Liu WT, Marsh TL, Cheng H and Forney LJ (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* **63**: 4516-22.

Lyszkiewicz M, Witzlau K, Pommerencke J and Krueger A (2011). Chemokine receptor CX3CR1 promotes dendritic cell development under steady-state conditions. *Eur J Immunol* **41**: 1256-65.

MacPhail LA, Greenspan D, Feigal DW, Lennette ET and Greenspan JS (1991). Recurrent aphthous ulcers in association with HIV infection. Description of ulcer types and analysis of T-lymphocyte subsets. *Oral Surg Oral Med Oral Pathol* **71**: 678-83.

MacPhail LA and Greenspan JS (1997). Oral ulceration in HIV infection: investigation and pathogenesis. *Oral Dis* **3 Suppl 1**: S190-3.

Maldonado RA and von Andrian UH (2010). How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol* **108**: 111-65.

Marionneau S, Cailleau-Thomas A, Rocher J, Le Moullac-Vaidye B, Ruvoen N, Clement M and Le Pendu J (2001). ABH and Lewis histo-blood group antigens, a model for the meaning of oligosaccharide diversity in the face of a changing world. *Biochimie* **83**: 565-73.

Mascarell L, Lombardi V, Louise A, Saint-Lu N, Chabre H, Moussu H, Betbeder D, Balazuc AM, Van Overtvelt L and Moingeon P (2008). Oral dendritic cells mediate antigen-specific tolerance by stimulating TH1 and regulatory CD4+ T cells. *J Allergy Clin Immunol* **122**: 603-9 e5.

Mascarell L, Saint-Lu N, Moussu H, Zimmer A, Louise A, Lone Y, Ladant D, Leclerc C, Tourdot S, Van Overtvelt L and Moingeon P (2011). Oral macrophage-like cells play a key role in tolerance induction following sublingual immunotherapy of asthmatic mice. *Mucosal Immunol* **4**: 638-47.

Matheu MP, Cahalan MD and Parker I (2011). General approach to adoptive transfer and cell labeling for immunoimaging. *Cold Spring Harb Protoc* **2011**: pdb prot5565.

Mazzini E, Massimiliano L, Penna G and Rescigno M (2014). Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1(+) macrophages to CD103(+) dendritic cells. *Immunity* **40**: 248-61.

Meyer-Martin H, Reuter S and Taube C (2014). Mouse models of allergic airway disease. *Methods in molecular biology* **1193**: 127-41.

Miller MF, Garfunkel AA, Ram C and Ship, II (1977). Inheritance patterns in recurrent aphthous ulcers: twin and pedigree data. *Oral Surg Oral Med Oral Pathol* **43**: 886-91.

Miller MF, Garfunkel AA, Ram CA and Ship, II (1980). The inheritance of recurrent aphthous stomatitis. Observations on susceptibility. *Oral Surg Oral Med Oral Pathol* **49**: 409-12.

Miller MF and Ship, II (1977). A retrospective study of the prevalence and incidence of recurrent aphthous ulcers in a professional population, 1958-1971. *Oral Surg Oral Med Oral Pathol* **43**: 532-7.

Mills MP, Mackler BF, Nelms DC and Peavy DL (1980). Quantitative distribution of inflammatory cells in recurrent aphthous stomatitis. *J Dent Res* **59**: 562-6.

Miyasaka M and Tanaka T (2004). Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat Rev Immunol* **4**: 360-70.

Mohammad R, Halboub E, Mashlah A and Abou-Hamed H (2013). Levels of salivary IgA in patients with minor recurrent aphthous stomatitis: a matched case-control study. *Clinical oral investigations* **17**: 975-80.

Mowat AM and Agace WW (2014). Regional specialization within the intestinal immune system. *Nat Rev Immunol* **14**: 667-85.

Murphy KM, Heimberger AB and Loh DY (1990). Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* **250**: 1720-3.

Murray PA, Prakobphol A, Lee T, Hoover CI and Fisher SJ (1992). Adherence of oral streptococci to salivary glycoproteins. *Infect Immun* **60**: 31-8.

Nacer A, Carapau D, Mitchell R, Meltzer A, Shaw A, Frevert U and Nardin EH (2014). Imaging murine NALT following intranasal immunization with flagellin-modified circumsporozoite protein malaria vaccines. *Mucosal Immunol* **7**: 304-14.

Najafi S, Firooze Moqadam I, Mohammadzadeh M, Bidoki AZ, Yousefi H, Farhadi E, Tonekaboni A, Meighani G, Amirzargar AA and Rezaei N (2014). Interleukin-10 gene polymorphisms in recurrent aphthous stomatitis. *Immunological investigations* **43**: 405-9.

Nakache M, Berg EL, Streeter PR and Butcher EC (1989). The mucosal vascular addressin is a tissue-specific endothelial cell adhesion molecule for circulating lymphocytes. *Nature* **337**: 179-81.

Natah SS, Hayrinen-Immonen R, Hietanen J, Malmstrom M and Konttinen YT (1998). Quantitative assessment of mast cells in recurrent aphthous ulcers (RAU). *J Oral Pathol Med* **27**: 124-9.

Natah SS, Hayrinen-Immonen R, Hietanen J, Malmstrom M and Konttinen YT (2000). Immunolocalization of tumor necrosis factor-alpha expressing cells in recurrent aphthous ulcer lesions (RAU). *J Oral Pathol Med* **29**: 19-25.

Natah SS, Konttinen YT, Enattah NS, Ashammakhi N, Sharkey KA and Hayrinen-Immonen R (2004). Recurrent aphthous ulcers today: a review of the growing knowledge. *Int J Oral Maxillofac Surg* **33**: 221-34.

Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR and Reinecker HC (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* **307**: 254-8.

Nolan A, Lamey PJ, Milligan KA and Forsyth A (1991). Recurrent aphthous ulceration and food sensitivity. *J Oral Pathol Med* **20**: 473-5.

Novak N, Haberstok J, Bieber T and Allam JP (2008). The immune privilege of the oral mucosa. *Trends in molecular medicine* **14**: 191-8.

Ogawa Y, Kanai-Azuma M, Akimoto Y, Kawakami H and Yanoshita R (2008). Exosome-like vesicles with dipeptidyl peptidase IV in human saliva. *Biological & pharmaceutical bulletin* **31**: 1059-62.

Ogura M, Yamamoto T, Morita M and Watanabe T (2001). A case-control study on food intake of patients with recurrent aphthous stomatitis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **91**: 45-9.

Ohmichi Y, Hirakawa J, Imai Y, Fukuda M and Kawashima H (2011). Essential role of peripheral node addressin in lymphocyte homing to nasal-associated lymphoid tissues and allergic immune responses. *J Exp Med* **208**: 1015-25.

Okada H, Kuhn C, Feillet H and Bach JF (2010). The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clin Exp Immunol* **160**: 1-9.

Ostman S, Taube M and Telemo E (2005). Tolerosome-induced oral tolerance is MHC dependent. *Immunology* **116**: 464-76.

Ott J, Kamatani Y and Lathrop M (2011). Family-based designs for genome-wide association studies. *Nature reviews. Genetics* **12**: 465-74.

Oxford Dictionaries. "Aphtha". Oxford University Press. Available at: https://en.oxforddictionaries.com/definition/aphtha (last accessed 6th December 2016).

Pabst O and Mowat AM (2012). Oral tolerance to food protein. *Mucosal Immunol* **5**: 232-9.

Pabst R (2015). Mucosal vaccination by the intranasal route. Nose-associated lymphoid tissue (NALT)-Structure, function and species differences. *Vaccine* **33**: 4406-13.

Peng HJ, Turner MW and Strobel S (1990). The generation of a 'tolerogen' after the ingestion of ovalbumin is time-dependent and unrelated to serum levels of immunoreactive antigen. *Clin Exp Immunol* **81**: 510-5.

Pisitkun T, Shen RF and Knepper MA (2004). Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* **101**: 13368-73.

Porter SR and Scully C (1991). Aphthous stomatitis--an overview of aetiopathogenesis and management. *Clin Exp Dermatol* **16**: 235-43.

Porter SR and Scully C (1993). Orofacial manifestations in primary immunodeficiencies involving IgA deficiency. *J Oral Pathol Med* **22**: 117-9.

Potter M (1985). *The BALB/c mouse. Genetics and immunology,* Springer Berlin Heidelberg: Germany.

Prakobphol A, Boren T, Ma W, Zhixiang P and Fisher SJ (2005). Highly glycosylated human salivary molecules present oligosaccharides that mediate adhesion of leukocytes and Helicobacter pylori. *Biochemistry* **44**: 2216-24.

Prakobphol A, Thomsson KA, Hansson GC, Rosen SD, Singer MS, Phillips NJ, Medzihradszky KF, Burlingame AL, Leffler H and Fisher SJ (1998). Human low-molecular-weight salivary mucin expresses the sialyl lewisx determinant and has L-selectin ligand activity. *Biochemistry* **37**: 4916-27.

Prescott SL, Pawankar R, Allen KJ, Campbell DE, Sinn J, Fiocchi A, Ebisawa M, Sampson HA, Beyer K and Lee BW (2013). A global survey of changing patterns of food allergy burden in children. *World Allergy Organ J* **6**: 21.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ and Sham PC (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* **81**: 559-75.

Qin T, Yin Y, Wang X, Liu H, Lin J, Yu Q and Yang Q (2015). Whole inactivated avian Influenza H9N2 viruses induce nasal submucosal dendritic cells to sample luminal viruses via transepithelial dendrites and trigger subsequent DC maturation. *Vaccine* **33**: 1382-92.

Rees TD and Binnie WH (1996). Recurrent aphthous stomatitis. *Dermatol Clin* **14**: 243-56.

Rharbaoui F, Bruder D, Vidakovic M, Ebensen T, Buer J and Guzman CA (2005). Characterization of a B220+ lymphoid cell subpopulation with immune modulatory functions in nasal-associated lymphoid tissues. *J Immunol* **174**: 1317-24.

Risch N and Merikangas K (1996). The future of genetic studies of complex human diseases. *Science* **273**: 1516-7.

Rivera-Hidalgo F, Shulman JD and Beach MM (2004). The association of tobacco and other factors with recurrent aphthous stomatitis in an US adult population. *Oral Dis* **10**: 335-45.

Robertson JM, Jensen PE and Evavold BD (2000). DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J Immunol* **164**: 4706-12.

Robledo-Sierra J, Mattsson U, Svedensten T and Jontell M (2013). The morbidity of oral mucosal lesions in an adult Swedish population. *Medicina oral, patologia oral y cirugia bucal* **18**: e766-72.

Rodriguez-Monroy MA, Rojas-Hernandez S and Moreno-Fierros L (2007). Phenotypic and functional differences between lymphocytes from NALT and nasal passages of mice. *Scand J Immunol* **65**: 276-88.

Rogers RS, 3rd (1977). Recurrent aphthous stomatitis: clinical characteristics and evidence for an immunopathogenesis. *J Invest Dermatol* **69**: 499-509.

Rosen SD, Singer MS, Yednock TA and Stoolman LM (1985). Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation. *Science* **228**: 1005-7.

Said HS, Suda W, Nakagome S, Chinen H, Oshima K, Kim S, Kimura R, Iraha A, Ishida H, Fujita J, Mano S, Morita H, Dohi T, Oota H and Hattori M (2014). Dysbiosis of salivary microbiota in inflammatory bowel disease and its association with oral immunological biomarkers. *DNA Res* **21**: 15-25.

Sallay K and Bánóczy J (1968). Remarks on the possibilities of the simultaneous occurence of hyperkeratosis of the mucous membranes and recurrent aphthae. *Oral Surgery, Oral Medicine and Oral Pathology* **25**: 171-175.

Salonen L, Axell T and Hellden L (1990). 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* **19**: 170-6.

Sampson HA (2016). Food allergy: Past, present and future. *Allergol Int* **65**: 363-369.

Savage NW, Seymour GJ and Kruger BJ (1985). T-lymphocyte subset changes in recurrent aphthous stomatitis. *Oral Surg Oral Med Oral Pathol* **60**: 175-81.

Schroeder HE, Muller-Glauser W and Sallay K (1983). Stereologic analysis of leukocyte infiltration in oral ulcers of developing Mikulicz aphthae. *Oral Surg Oral Med Oral Pathol* **56**: 629-40.

Schroeder HE, Muller-Glauser W and Sallay K (1984). Pathomorphologic features of the ulcerative stage of oral aphthous ulcerations. *Oral Surg Oral Med Oral Pathol* **58**: 293-305.

Scully C (2006). Clinical practice. Aphthous ulceration. *The New England journal of medicine* **355**: 165-72.

Scully C (2012). Myths or legends and RAS. *Oral Dis* **18**: 521; author reply 520.

Seoudi N, Bergmeier LA, Drobniewski F, Paster B and Fortune F (2015). The oral mucosal and salivary microbial community of Behcet's syndrome and recurrent aphthous stomatitis. *Journal of oral microbiology* **7**: 27150.

Shakya AK, Chowdhury MY, Tao W and Gill HS (2016). Mucosal vaccine delivery: Current state and a pediatric perspective. *J Control Release* **240**: 394-413.

Shanholtzer CJ, Schaper PJ and Peterson LR (1982). Concentrated gram stain smears prepared with a cytospin centrifuge. *J Clin Microbiol* **16**: 1052-6.

Ship, II (1965). Inheritance of aphthous ulcers of the mouth. *J Dent Res* **44**: 837-44.

Ship I (1972). Epidemiologic aspects of recurrent aphthous ulcerations. *Oral Surg Oral Med Oral Pathol* **33**: 400-6.

Ship JA, Chavez EM, Doerr PA, Henson BS and Sarmadi M (2000). Recurrent aphthous stomatitis. *Quintessence Int* **31**: 95-112.

Shulman JD (2004). An exploration of point, annual, and lifetime prevalence in characterizing recurrent aphthous stomatitis in USA children and youths. *J Oral Pathol Med* **33**: 558-66.

Shulman JD, Beach MM and Rivera-Hidalgo F (2004). The prevalence of oral mucosal lesions in U.S. adults: data from the Third National Health and Nutrition Examination Survey, 1988-1994. *J Am Dent Assoc* **135**: 1279-86.

Sibley WK (1899). Neurotic Ulcers of the Mouth: (Stomatitis Neurotica Chronica). *British medical journal* **1**: 900-1.

Sircus W, Church R and Kelleher J (1957). Recurrent aphthous ulceration of the mouth; a study of the natural history, aetiology, and treatment. *The Quarterly journal of medicine* **26**: 235-49.

Spahn TW, Fontana A, Faria AM, Slavin AJ, Eugster HP, Zhang X, Koni PA, Ruddle NH, Flavell RA, Rennert PD and Weiner HL (2001). Induction of oral tolerance to cellular immune responses in the absence of Peyer's patches. *Eur J Immunol* **31**: 1278-87.

Spahn TW, Weiner HL, Rennert PD, Lugering N, Fontana A, Domschke W and Kucharzik T (2002). Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. *Eur J Immunol* **32**: 1109-13.

Squier CA (1991). The permeability of oral mucosa. *Crit Rev Oral Biol Med* **2**: 13-32.

Stanley HR (1972). Aphthous lesions. *Oral Surg Oral Med Oral Pathol* **33**: 407-16.

Stenman G and Heyden G (1980). Premonitory stages of recurrent aphthous stomatitis. I. Histological and enzyme histochemical investigations. *J Oral Pathol* **9**: 155-62.

Stockel D, Kehl T, Trampert P, Schneider L, Backes C, Ludwig N, Gerasch A, Kaufmann M, Gessler M, Graf N, Meese E, Keller A and Lenhof HP (2016). Multi-omics enrichment analysis using the GeneTrail2 web service. *Bioinformatics*.

Strobel S, Mowat AM, Drummond HE, Pickering MG and Ferguson A (1983). Immunological responses to fed protein antigens in mice. II. Oral tolerance for CMI is due to activation of cyclophosphamide-sensitive cells by gut-processed antigen. *Immunology* **49**: 451-6.

Subramanyam RV (2011). Occurrence of recurrent aphthous stomatitis only on lining mucosa and its relationship to smoking--a possible hypothesis. *Med Hypotheses* **77**: 185-7.

Sun A, Chu CT, Liu BY, Wang JT, Leu JS and Chiang CP (2000). Expression of interleukin-2 receptor by activated peripheral blood lymphocytes upregulated by the plasma level of interleukin-2 in patients with recurrent aphthous ulcers. *Proc Natl Sci Counc Repub China B* **24**: 116-22.

Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR and Belkaid Y (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* **204**: 1775-85.

Sun M, Fu SM, Dong GY, Wu D, Wang GX and Wu Y (2013). Inflammatory factors gene polymorphism in recurrent oral ulceration. *J Oral Pathol Med* **42**: 528-34.

Sutton RL (1911). Periadenitis Mucosa Necrotica Recurrens. *J. Cutan Dis* **29**: 65-71.

Tappuni AR, Kovacevic T, Shirlaw PJ and Challacombe SJ (2013). Clinical assessment of disease severity in recurrent aphthous stomatitis. *J Oral Pathol Med* **42**: 635-41.

Taylor LJ, Bagg J, Walker DM and Peters TJ (1992). Increased production of tumour necrosis factor by peripheral blood leukocytes in patients with recurrent oral aphthous ulceration. *J Oral Pathol Med* **21**: 21-5.

Thomson AW and Knolle PA (2010). Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol* **10**: 753-66.

Truelove SC and Morris-Owen RM (1958). Treatment of aphthous ulceration of the mouth. *British medical journal* **1**: 603-7.

Tuft L and Girsh LS (1958). Buccal mucosal tests in patients with canker sores (aphthous stomatitis). *J Allergy* **29**: 502-10.

Tursi A, Giorgetti G, Brandimarte G, Rubino E, Lombardi D and Gasbarrini G (2001). Prevalence and clinical presentation of subclinical/silent celiac disease in adults: an analysis on a 12-year observation. *Hepatogastroenterology* **48**: 462-4.

Ueta E, Umazume M, Yamamoto T and Osaki T (1993). Leukocyte dysfunction in oral mucous membrane diseases. *J Oral Pathol Med* **22**: 120-5.

Veller-Fornasa C, Bezze G, Rosin S, Lazzaro M, Tarantello M and Cipriani R (2003). Recurrent aphthous stomatitis and atopy. *Acta Derm Venereol* **83**: 469-70.

Veller-Fornasa C and Gallina P (2006). Recurrent aphthous stomatitis as an expression of pathergy in atopics. *Acta Dermatovenerol Alp Pannonica Adriat* **15**: 144-7.

Ventura MT, Polimeno L, Amoruso AC, Gatti F, Annoscia E, Marinaro M, Di Leo E, Matino MG, Buquicchio R, Bonini S, Tursi A and Francavilla A (2006). Intestinal permeability in patients with adverse reactions to food. *Dig Liver Dis* **38**: 732-6.

Vignali DA, Collison LW and Workman CJ (2008). How regulatory T cells work. *Nat Rev Immunol* **8**: 523-32.

von Andrian UH and Mackay CR (2000). T-cell function and migration. Two sides of the same coin. *The New England journal of medicine* **343**: 1020-34.

Walker DM (2004). Oral mucosal immunology: an overview. *Ann Acad Med Singapore* **33**: 27-30.

Wang Q, Garrity GM, Tiedje JM and Cole JR (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**: 5261-7.

Watanabe H, Numata K, Ito T, Takagi K and Matsukawa A (2004). Innate immune response in Th1- and Th2-dominant mouse strains. *Shock* **22**: 460-6.

Wells HG and Osborne T (1911). The biological reactions of the vegetable proteins, I, Anaphylaxis. *J. Infect. Dis* **8**: 66-124.

Willard-Mack CL (2006). Normal structure, function, and histology of lymph nodes. *Toxicol Pathol* **34**: 409-24.

Wilson CW (1980). Food sensitivities, taste changes, aphthous ulcers and atopic symptoms in allergic disease. *Ann Allergy* **44**: 302-7.

Wolvers DA, Coenen-de Roo CJ, Mebius RE, van der Cammen MJ, Tirion F, Miltenburg AM and Kraal G (1999). Intranasally induced immunological tolerance is determined by characteristics of the draining lymph nodes: studies with OVA and human cartilage gp-39. *J Immunol* **162**: 1994-8.

Worbs T, Bode U, Yan S, Hoffmann MW, Hintzen G, Bernhardt G, Forster R and Pabst O (2006). Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med* **203**: 519-27.

Wray D (1981). Gluten-sensitive recurrent aphthous stomatitis. *Dig Dis Sci* **26**: 737-40.

Wray D and Charon J (1991). Polymorphonuclear neutrophil function in recurrent aphthous stomatitis. *J Oral Pathol Med* **20**: 392-4.

Wray D, Graykowski EA and Notkins AL (1981). Role of mucosal injury in initiating recurrent aphthous stomatitis. *Br Med J (Clin Res Ed)* **283**: 1569-70.

Wray D, Rees SR, Gibson J and Forsyth A (2000). The role of allergy in oral mucosal diseases. *QJM* **93**: 507-11.

Wright A, Ryan FP, Willingham SE, Holt S, Page AC, Hindle MO and Franklin CD (1986). Food allergy or intolerance in severe recurrent aphthous ulceration of the mouth. *Br Med J (Clin Res Ed)* **292**: 1237-8.

Wu HY, Nikolova EB, Beagley KW and Russell MW (1996). Induction of antibody-secreting cells and T-helper and memory cells in murine nasal lymphoid tissue. *Immunology* **88**: 493-500.

Wu-Wang CY, Patel M, Feng J, Milles M and Wang SL (1995). Decreased levels of salivary prostaglandin E2 and epidermal growth factor in recurrent aphthous stomatitis. *Arch Oral Biol* **40**: 1093-8.

Yamamoto T, Yoneda K, Ueta E and Osaki T (1994). Serum cytokines, interleukin-2 receptor, and soluble intercellular adhesion molecule-1 in oral disorders. *Oral Surg Oral Med Oral Pathol* **78**: 727-35.

Yamazaki S, Maruyama A, Okada K, Matsumoto M, Morita A and Seya T (2012). Dendritic cells from oral cavity induce Foxp3(+) regulatory T cells upon antigen stimulation. *PLoS One* **7**: e51665.

Yang R, Liu Q, Grosfeld JL and Pescovitz MD (1994). Intestinal venous drainage through the liver is a prerequisite for oral tolerance induction. *J Pediatr Surg* **29**: 1145-8.

Yasar S, Yasar B, Abut E and Asiran Serdar Z (2012). Clinical importance of celiac disease in patients with recurrent aphthous stomatitis. *Turk J Gastroenterol* **23**: 14-8.

Yu W, Freeland DM and Nadeau KC (2016). Food allergy: immune mechanisms, diagnosis and immunotherapy. *Nat Rev Immunol*.

Zad M, Flowers SA, Bankvall M, Jontell M and Karlsson NG (2015). Salivary mucin MUC7 oligosaccharides in patients with recurrent aphthous stomatitis. *Clinical oral investigations* **19**: 2147-52.

Zuercher AW (2003). Upper respiratory tract immunity. *Viral immunology* **16**: 279-89.

APPENDIX

I. **Bankvall M**, Östman S, Jontell M, Torinsson-Naluai Å. A genome-wide association study of recurrent aphthous stomatitis. *In manuscript*

II. **Bankvall M**, Sjöberg F, Gale G, Wold A, Jontell M, Östman S. The oral microbiota of patients with recurrent aphthous stomatitis. *J Oral Microbiol*. 2014 Oct 29;6:25739.

III. **Bankvall M**, Jontell M, Wold A, Östman S. Tissue-specific differences in immune cell subsets located in the oral-associated lymphoid tissues. *In manuscript*

IV. **Bankvall M**, Östberg AK, Jontell M, Wold A, Östman S. The engagement of oral-associated lymphoid tissues during oral versus gastric antigen administration. *Immunology*. 2016 Sep;149(1):98-110.