

Disorders of the Orofacial and Gastrointestinal Tract

A study with special reference to Orofacial Granulomatosis and Crohn's disease

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The oral cavity is of importance from a number of different aspects at various periods in life. For most people, social wellbeing is dependent on a functioning oral cavity whereby a malfunction often results in a feeling of social incapacity. The lips and the oral cavity are parts of the gastrointestinal tract and the only components that are exposed and used under social circumstances. Abnormalities in these components leave not only a detrimental loss of function in communication, but also compromised aesthetics, both of which are important factors for social wellness. Other key functions are the ability to enjoy as well as digest food. These functions are easily taken for granted but very debilitating when they are not properly operating.

This thesis comprises studies of some orofacial conditions where these functions are compromised. The majority of the studies focus on orofacial granulomatosis with special references to its association with Crohn's disease. Clinical similarities between orofacial granulomatosis and long-standing oral disorders in paediatric liver recipients, justifies also the inclusion of the latter condition in this thesis.

1.1 Characteristics of the disorders

1.1.1 Orofacial granulomatosis

Orofacial granulomatosis (OFG) is a rare granulomatous disease affecting the oral and facial mucosa causing lesions such as lip/facial swelling, angular cheilitis, cobblestone phenomenon, tag formation, mucosal ridges and full thickness gingivitis (1). It usually affects children and young adults (1, 2). Oral biopsies from the lesions may display non-caseating granuloma as well as lymphangiectasia. However, sometimes the only indication of

histological abnormality is an inflammatory infiltrate dominated by lymphocytes (3-5). OFG is a chronic condition that can precede CD, which is why it is of the utmost importance that these patients are followed over an extended period of time.

In 1985 Wiesenfeld was the first to describe OFG with clinical features such as diffuse facial swelling, enlargement of lips, oral ulcerations, gingival overgrowth and mucosal tags. At The Clinic of Oral Medicine the diagnosis of OFG was established using the criteria described by Wiesenfeld. Most of the OFG patients at our clinic had several of these features but in some rare cases the patients had only lip swelling together with one more of the criteria.

Lip swelling with angular cheilitis



Cobblestone phenomenon



Tag formation



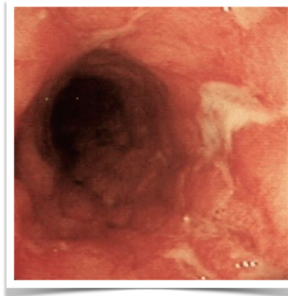
The clinical picture together with the anamnestic history will usually guide oral medicine practitioners to the correct diagnosis but there are other conditions similar to that of OFG. This similarity can be deceiving. Lip swelling can also be displayed in patients with angioedema. Although patients with angioedema often report that the symptoms appear quite rapidly, within a few hours. OFG patients often describe the lip swelling as a slow process over a few days. In patients with OFG the biopsies from oral lesions often display epithelioid cell granulomas with giant cells and this histopathological feature is not seen in patients with angioedema. It is also important to consider that the granulomas detected in biopsies from oral lesions could be a part of a systemic disease such as sarcoidosis. It is therefore necessary to rule out sarcoidosis by radiographic chest examination, serum levels of angiotensin converting enzyme, pulmonary function test or bronchoscopy.

1.1.2 Crohn's disease

Crohn's disease (CD) is a chronic inflammatory bowel disease presenting with segmental and transmural intestinal inflammation that may involve any area of the gastrointestinal tract. The symptoms of CD include persistent diarrhoea, abdominal pain and cramps, fever and fatigue. The onset of disease during childhood may result in impaired growth when untreated. The presence of granuloma in the intestinal mucosa is one of the hallmarks for the disease, thus CD belongs to the granulomatous disease.

In our studies the diagnosis of CD was determined by using the Porto criteria based on clinical findings and symptoms, endoscopy, histopathology and radiology (6). To characterise the phenotype of CD several clinical parameters were used as well as the usage of the Paris classification (7).

Crohn's disease



Healthy mucosa



1.1.3 Orofacial granulomatosis with Crohn's disease

The association between OFG and CD has been known for several decades as the two conditions share both clinical and histopathological features, but the exact connection is as yet unclear (2). Opinions differ regarding whether OFG should be referred to as a separate entity or as a part of the CD spectrum when it is present with CD. In fact, there has been an ongoing debate as to what constitutes the correct terminology. In some studies OFG, when it appears in association with intestinal CD, is referred to as oral Crohn's disease while in other studies OFG is considered as a separate entity. In 1969, Dudeney published a case report on a patient with CD who later developed swelling of the oral mucosa which had histopathological features such as oedema, chronic inflammation and granulomas containing epithelioid giant cells (8). Since then several reports have described the connection between the two granulomatous conditions but the common denominator with regards to the pathogenesis is still unknown (2, 9, 10).

1.1.4 Long-standing oral disorder in paediatric organ recipients

Lip swelling is one of the most common features in OFG and also a feature seen in some paediatric liver recipients who also develop other OFG like features such as angular cheilitis, cobblestone phenomenon and hyperplastic gingivae (11). Thus, the oral mucosal lesions in paediatric liver recipients are very similar to those seen in OFG but in contrast, the biopsies from these lesions do not display any granulomas. The condition also presents with a nodular tongue which is a unique clinical appearance not seen as apart of any other mucosal disorder. This novel finding has led us to coin the term nodular tongue syndrome (NTS) for this condition. The questions that come to mind are why it only seems to affect children and why mainly the liver transplanted children. The similar clinical picture to that of OFG and that our multidisciplinary team was the first to report this condition in paediatric liver recipients made it obvious to include NTS as part of this thesis.

Nodular tongue syndrome



Lip swelling with angular cheilitis



1.2 History

1.2.1 Orofacial granulomatosis

Granulomatous disorder in the orofacial mucosa was first referred to by Märt in 1859 (12) where he describes facial palsy and facial swelling. Then Melkersson in 1928 (13) reported the link between relapsing facial palsy and transient facial oedema. In 1931, Rosenthal (14) described three patients with fissured tongue, facial palsy and facial oedema which later became known as Melkersson-Rosenthal syndrome. Cheilitis granulomatosa is a recurrent enlargement of one or both lips and is histopathologically characterised by non-caseating granulomas, oedema as well as lymphangiectasia. This condition was described in 1945 by Miescher (15), which is why it is also referred to as Miescher's cheilitis. It was not until 1985 that the concept of OFG that is currently used was described by Wiesenfeld (1) with clinical entities such as diffuse facial swelling, enlargement of lips, oral ulcerations, gingival overgrowth and mucosal tags.

1.2.2 Crohn's disease

Inflammatory bowel disease (IBD) was first described by the English pathologist Matthew Baillie (16) in his book "The morbid anatomy of some of the most important parts of the human body" in 1793 where he reports that some diseases only cause morbid actions and other diseases cause structural changes. Although already in 1612, Gullielmus Fabricius Hildenus (17) reported following an autopsy of a boy who had died from persistent abdominal pain and diarrhoea causing an ulcerated caecum to contract into ileum. The first report on CD was in 1932 by Crohn *et al.* (18) from The Mount Sinai Hospital in New York in which they studied a group of 14 patients with "regional ileitis" and describing it as a sub-acute or chronic necrotizing and cicatrizing inflammation affecting mainly young adults. Dr Crohn often stated that CD was an inappropriate name for this condition and his preference was regional ileitis, regional enteritis or cicatrizing enterocolitis. Yet, CD is now an established name for the disease described by Dr Crohn and the term CD is used worldwide.

1.2.3 Nodular tongue syndrome in paediatric liver recipients

It has been a well-known fact for some time that solid organ recipients have a higher risk of developing immunoregulatory disorders such as immediate onset food allergy (19), IBD (20, 21) and autoimmune hepatitis (22, 23). Though, in 2010 our research team (11) was the first to report the entity of NTS in paediatric liver recipients and since then other studies of the same conditions have been reported by De Bruyne *et al.* and Vivas *et al.* (24, 25).

1.3 Epidemiology

1.3.1 Orofacial granulomatosis

There are no epidemiological data on OFG reported in Sweden and there are several reasons for this lack of reports. It is quite an uncommon disease, with a prevalence estimated well below 0,1% in the Swedish population. It has been suggested that the prevalence of OFG could be as high as 0.8% with the Celtic population having the highest prevalence (4). The terminology confusion makes it hard to compare data from one country to another. Some studies distinguish between OFG and OFG with concomitant CD while other reports do not make this distinction and herein lies the confusion. Additionally, the data collection differs from one geographic region to the next making it impossible to do comparisons between different regions.

1.3.2 Crohn's disease

Assessing the trends in incidence and prevalence of CD globally has been possible to do since the 1950s. Before 1950s it is not possible to rely on data due to the lack of collecting routines and although the data is reliable today it can still be difficult to compare different geographic regions as collection techniques differ. Most data is collected either from health administrative databases, medical records or disease registries and as non-homogenous data collection can give discrepancies the comparisons might not be accurate.

The recognition of higher CD incidence rate in urban areas compared to rural areas was reported already in 1958 by Houghton *et al.* (26). Additionally several studies show that developed countries with higher life quality standards have a higher rate of CD incidence compared to developing countries but there also seems to be a North-South gradient in incidence (27-30). In northern countries such as Denmark, Sweden, Scotland and Canada the incidence rate for CD is above 8/100 000 people per year whereas in southern countries in Europe such as Italy, Greece and Portugal the incidence rate is less than 4.2/100 000 people per year (31). Although this North-South gradient of CD incidence does not seem to be accurate for paediatric on-set of CD as regions like Corsica, Asturias and Croatia in southern Europe have reported higher incidence rate of paediatric CD compared to northern European regions such as Finland and northern France (32). The North-South gradient can be explained by both genetic and environmental factors. The differences in ethnicity between north and south regions may affect genetic predisposition to CD and in the northern regions less sun as well as improved hygiene could explain the North-South gradient. Most of the studies show a trend towards an increase in incidence rate in CD independent of the North-South gradient.

Regarding the prevalence of IBD, Sweden has together with Canada and Scotland, among the highest incidence rates in the world (31). In 2003 Hildebrand *et al.* (28) reported a study on the changing patterns of IBD in northern Stockholm between 1990-2001. In this study an IBD incidence rate of 7.4 per 100 000 was observed with CD being the disease of increase whilst UC was stable. Between 1990-2001 there was a boost in the incidence rate of CD from 1.7 to 8.4 per 100 000 (28) and since then CD has reached a level of 9.9 per 100 000 (33) in Sweden. This intensification of CD has led to a changed ratio of CD to UC, from 0.5 to 4.6. In Sweden a predominance of males has been observed in CD (34) and the increased CD:UC ratio has also resulted in males having a significantly higher incidence rate of IBD than females (28).

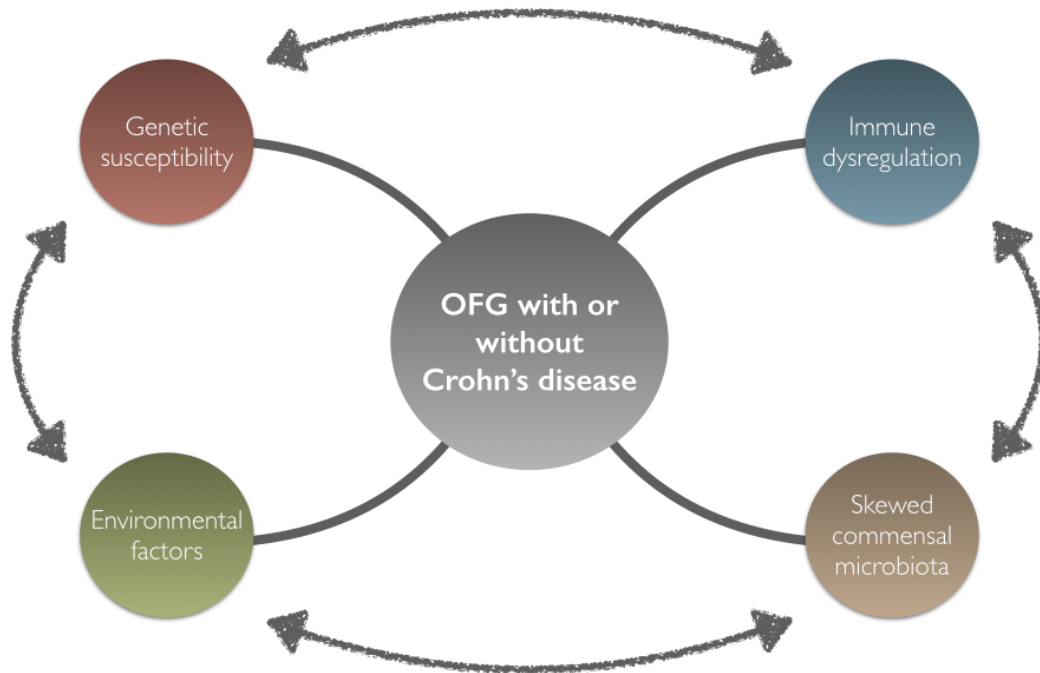
1.3.3 Oral lesions in paediatric liver recipients

Oral lesions observed in paediatric liver recipients were first reported in 2010 (11), which explains why it is hard to find any epidemiological data on this condition. Hitherto, there are only two reports on oral lesions in solid organ transplanted children (11, 25).

1.4 Aetiology

With regards to the aetiology of OFG and OFG in conjunction with CD, there is most likely not just one single factor causing these conditions but several factors in various combinations. In most studies, the frequently suggested elements are environmental influences, genetic susceptibility, immune dysregulation and skewed commensal microbiota. However, it is difficult to envision if any one of them is more important than the others, but we do know that IBD has been increasing since the 1950s. This increase began in the developed northern

countries but was later also seen in southern countries, possibly as a result of the transition from developing to developed countries. We have chosen to look into several plausible factors.



1.4.1 Environmental factors

Delayed type hypersensitivity to food constituents and food additives has been suggested in several reports as an aetiological cause for OFG as 60-68% of OFG patients report food allergy (35, 36). Successful treatment with elimination diets support this hypothesis (37). Benzoate and cinnamon are the most commonly suggested food substances but other substances such as dairy products (38), chocolate (39), oat (40), eggs, peanuts and monosodium glutamate (41) have been suggested as well (42). There are also reports suggesting that metals such as gold and mercury could be an aetiological agent of OFG (43).

Improved hygiene in developed countries has been a frequently suggested risk factor in the aetiology of CD. This risk factor is supported by the increased incidence rate seen in countries changing status from developing to developed country (31, 44). Countries like India and China, where the prevalence of CD has always been considered to be low, have vast populations with increased standard of life. This in turn has led to an increased incidence rate and with India and China having 40% of the world population it has been proposed that Asia in the future might possess the majority of global CD patients (31).

Early childhood infections have been put forward as having a protective effect on CD. These childhood infections are supposedly creating a balance between proinflammatory and tolerance-inducing mechanisms that in turn will protect against inflammatory responses to antigen stimuli (45).

According to the cold chain hypothesis the frequent usage of refrigerators has implied a change of the bacterial composition in our diet. Bacteria capable of survival at temperatures as low as -1°C to $+10^{\circ}\text{C}$ seem to have developed rapidly at the same time as the increased incidence rate of CD in 1960s and this coincides with the introduction of refrigerators. In 1937, 49% of American families had refrigerators, whilst in Europe as late as 1958 only 10% of French and 12% of British families had access to refrigerators. This coincides with the increased prevalence of CD starting in the 1940s in the USA, in the 1960s in the UK and even later in southern Europe. Interestingly, in Sweden, the home of Electrolux, half of Swedish families had refrigerators by the late 1950s and the increased prevalence for CD began in the 1950s in Sweden (44, 46).

Regarding tobacco usage, some reports show that smoking increases the risk of getting CD (44) whilst others state that there is no positive correlation with smoking (47). On the contrary, smoking has been suggested as having a protective effect on CD (48). In regards to OFG, in conjunction with CD, the patients are usually too young for the usage of tobacco. There are no reports on tobacco usage being a risk factor for OFG or having a protective effect on this disease.

1.4.2 Genetic susceptibility

The first report on familial inflammatory bowel disease (IBD) was suggested already at an IBD symposium in London in 1909 (49). Two of the three patients with ulcerative colitis (UC) reported that a father and a sibling had UC and the third patient had a brother and a sister with UC. These cases were considered to be coincidental. It took almost 50 years together with an increasing incidence rate until familial IBD was accepted. Familial IBD more often comprises of first-degree relatives, i.e. parents, siblings and children rather than second- or third-degree relatives. The discovery of familial IBD made it obvious that genetic susceptibility needed to be further investigated as an aetiological factor for CD.

In 2001, two reports were published in the same issue of *Nature*, both demonstrating the association between nucleotide-binding oligomerization domain-containing protein 2 (NOD2) variations and CD (50, 51). Since then there has been over 70 genes or loci associated with the susceptibility of CD. Hitherto, NOD2 variations seem to have the strongest linkage to paediatric CD and with most of our patients being children or young adults we chose to investigate the occurrence of NOD2 variations in some of our patients.

NOD2, also known as CARD15, is a protein encoded by the NOD2 gene, which is located on chromosome 16. NOD2 acts as a bacterial sensor recognising muramyl dipeptide, which is a specific structure in the wall of Gram positive and Gram negative bacteria. This recognition of muramyl dipeptide starts an innate immune activation of NOD2, which triggers various other adaptor proteins. In turn this leads to a stimulation of nuclear factor kappa B (NF- κ B) and secretion of proinflammatory cytokines such as IL-1 β , IL-6 and tumor necrosis factor alpha (TNF α). A NOD2 variation leads to a deficit in bacterial recognition (51). Reports show that NOD2 variations are associated with a more complicated CD, i.e. stenosing and penetrating behaviour with greater demands for bowel resection (48). Regarding CD, the three

major disease risk alleles in NOD2 are Arg702Trp, Gly908Arg and Leu1007fsinsC. A heterozygote variation in one allele increases the risk for CD two to four times, but a homozygote variation gives a 20 to 40 fold increase in the susceptibility for CD (52). There seems to be geographic differences in the NOD2 variations in the healthy population. Studies show that variations in one of the three major risk NOD2 alleles in a healthy population in European countries is as high as 11,0% whilst in the Swedish population it is as low as 2.6% (53, 54).

Interestingly, although there is a consensus on the genetic susceptibility for CD there is no evidence for a genetic susceptibility with respect to OFG.

1.4.3 Immune dysregulation

From the bone marrow derived hematopoietic stem cells there are two possible maturation lineages, the lymphoid or the myeloid lineage. The lymphoid lineage includes T cells, B cells and natural killer cells. The myeloid lineage includes monocytes such as macrophages and dendritic cells as well as neutrophils, eosinophils and mast cells. Both the lineages contribute to the innate immune system and the adaptive immune system. The cells of the innate immune system include natural killer cells, mast cells, dendritic cells and macrophages. The innate immune system is often referred to as the first line of defence. Pathogens capable of penetrating the skin and mucous membranes are usually cleared by a rapid response of phagocytosis. The adaptive immune system serves as the second line of defence, which operates through a slower response executed by T cells and B cells through a cytotoxic action and generation of antibodies. The innate branch of the immune system has an unspecific response, while the adaptive branch provides an immunologic memory, enabling protective immunity against pathogens.

When Wiesenfeld first described orofacial granulomatosis in 1985 the histopathological features comprised dilated lymphatic vessels, oedema, lymphocyte infiltration and formation of granulomas. These histopathological characteristics could be suggestive of OFG being of immune dysregulation origin (1). Studies analysing the presence of T cells, B cells and macrophages have suggested that the immune response in OFG is of T helper 1 (Th1) type (3, 5). This is supported by the finding of Th1 associated cytokines such as interleukin 12 (IL-12) and interferon gamma (IFN- γ) in biopsies from OFG lesions (3).

In 1942 the first suggestion of IBD being a disease of immune dysfunction was reported in terms of an allergic reaction (55, 56) which led to an increased immunological interest in IBD with a focus on autoimmunity in the 1960s. In the 1970s studies suggested infants having a more permeable intestine and an immature intestinal defence allowing for antigens to penetrate gut mucosa (57). In the late 1980s and early 1990s increased intestinal permeability was reported both in CD patients and their healthy first-degree relatives (58) but there have also been reports that have not confirmed these findings (59, 60). The gut mucosa is constantly exposed to a barrage of microbial and environmental antigens, which is why a functioning barrier is of the utmost importance. Since the early 2000's there seems to be a consensus that IBD patients have an impaired gut barrier function (61, 62) but the reason and the consequences of this barrier leak are yet to be found. Both genetic (61) and environmental (63, 64) factors have been suggested to influence this leakage.

Apart from increased mucosal permeability, immune dysregulation has also been suggested to be a result of paneth cell dysfunction and endoplasmic reticulum stress. One of the functions

of paneth cells is to release antimicrobial peptides as a response to bacteria (65) and a dysfunction may have repercussions in microbial defence. Microbial and environmental antigens also stimulate cells to secrete excessive amounts of cytokines with endoplasmic reticulum stress as a consequence to this excess (66).

The inflammation may also be perpetuated by migration of leukocytes and an imbalance of effector and regulatory T cells (Tregs) (67). The effector T cells, such as T helper 1 (Th1) and T helper 17 cells (Th17) defend the mucosa against microbial and environmental antigens. The Tregs have a suppressor function on Th1 and Th17. An imbalance herein, as well as retention of leukocytes, may both mediate and sustain an inflammatory response.

An early priming of the gut mucosa and development of oral tolerance are important factors for distinguishing signals of dangerous and non-dangerous antigens. Oral tolerance is to actively suppress immune response to non-dangerous dietary antigens or commensal bacteria, which starts to develop already during infancy. T cells hyper-responsive to pathogenic microbial flora as well as commensal flora is a sign of defective oral tolerance and has been reported in IBD patients (68).

Failure in oral tolerance could also be an important factor to study in paediatric liver recipients developing oral lesions, as these lesions are not seen in adult liver recipients. The paediatric liver recipients have all had liver dysfunction during infancy and/ or early childhood. Most of the paediatric liver recipients have also had immune-regulating medication, which has affected immune response, potentially resulting in oral lesions.

1.4.4 Skewed commensal microbiota

The oral cavity hosts more than 700 microbes as commensal and transient flora developing from early childhood with a peak at puberty (69, 70). The commensal flora functions as a barrier against pathogenic microbes. Its composition varies at different sites of the oral cavity depending on the availability of oxygen, adhesion and protection from constant exposure to a barrage of saliva and foods. Studies have shown that salivary non-specific immunoglobulins IgA and IgG are raised in patients with OFG as well as serum IgA. The increased serum IgA may reflect mucosal inflammation anywhere in the gastrointestinal tract, including the oral mucosa (71). Increased serum levels of IgA against *Saccharomyces cerevisiae* have been reported in patients with OFG+CD and patients with only CD, whereas serum IgA against *Candida albicans* are detected in patients with OFG, CD and OFG+CD (71). *Staphylococcus aureus* has also been reported in patients with OFG and OFG+CD but *S. aureus* is also colonized in 30% of healthy individuals (70, 72). Studies on *Mycobacterium paratuberculosis* from oral tissue samples of patients with OFG solely (OFG-S) and OFG+CD have shown that this species is not associated with OFG (73). However, through polymerase chain reaction, *M. paratuberculosis* has been found in biopsies and samples of intestines removed at surgery from both adults and children with CD (74, 75).

Regarding CD there are several indications as to how the microbiota seems to effect the development of CD. Changes of microbes often lead to a more severe inflammation and animal models show that inflammation develops under non-sterile conditions but not in a germ-free milieu. However, no specific organism has been suggested as the key pathogenic microbe. Instead there seems to be a dysbiosis, i.e. microbial imbalance, affecting the host-microbe interactions (76). This dysbiosis seems to be an effect of the impaired ability of the gut mucosa to kill bacteria (77). The microbial imbalance can cause high concentrations of

microbes at different sites of the gut and these areas often correlate with the most common sites for inflammation in CD patients (78). When luminal stream is diverted away from inflamed segments of the gut mucosa this action seems to improve the disease activity (79). Notably, increased levels of *Mycobacterium paratuberculosis* and *Escherichia coli* and decreased levels of *Faecalibacterium prausnitzii* have been observed in CD (80-82).

1.5 Management

In the management of OFG there are several factors that need to be taken into consideration. At The Oral Medicine Clinic in Gothenburg it is mandatory to send all patients with OFG to a gastroenterology clinic to establish whether oral symptoms are in conjunction with signs of gastrointestinal inflammation. Studies show that 54% of patients with OFG and without gastrointestinal symptoms present with macro and/or microscopic inflammation in biopsies from ileocolonoscopy (2). Depending on the outcome of a gastrointestinal examination the patient will either be treated with local corticosteroids if there are no indications for CD or if a CD diagnosis is established the patient will follow the treatment regimen for CD. Patient history and clinical features raising suspicion of food allergy will also result in a referral to an allergy specialist. In the event of food hypersensitivity induced OFG the patient will be put on a strict elimination diet. In addition to local corticosteroid treatment, the patients are also treated with retapamulin (Altargo®), cortison (Daktacort®) or flucloxacillin (Heracillin®) when needed. In the UK, with the highest prevalence rate of OFG, a benzoate and cinnamon free diet has been reported to be a successful treatment of OFG (35, 83). Benzoate is a food additive often found in fast food and pre-fabricated food.

Scientific questions

2

The overall scientific questions that were asked prior to the commencement of this thesis were (i) do Crohn's disease and orofacial granulomatosis represent distinctive disorders when they appear separately or in combination and (ii) do oral lesions seen in paediatric liver recipients stem from the same disease spectrum as orofacial granulomatosis?

To answer these two general questions the following specific scientific questions were addressed.

Does orofacial granulomatosis have different clinical manifestations depending on the presence or absence of Crohn's disease?

Does orofacial granulomatosis have different histopathological characteristics depending on the presence or absence of Crohn's disease?

Does orofacial granulomatosis have different genetic variations of NOD2 depending on presence or absence of Crohn's disease?

Does orofacial granulomatosis in conjunction with Crohn's disease signal a distinctive phenotype of Crohn's disease?

Does orofacial granulomatosis have different histopathological characteristics compared to clinically similar oral lesions seen in paediatric liver recipients?

3.1 Patients

The Department of Oral Medicine and Pathology, at Sahlgrenska Academy, University of Gothenburg, has collaborated with The Department of Pediatrics, Sahlgrenska Academy, University of Gothenburg, for over a decade on oral manifestations of inflammatory bowel diseases. This collaboration has resulted in the establishment of a national resource for patients with OFG-S and OFG+CD with a multidisciplinary network of microbiologists, allergy specialists, immunologists and geneticists. In addition, the novel clinical entity displayed in solid organ-transplanted children has lead to collaboration with The Stomatology Department and The Department of Liver Transplantation, A.C. Camargo Hospital, Sao Paulo, Brazil.

For paper I, II and III, the patients were retrieved from these two departments at Sahlgrenska Academy as well as in the Stockholm region from Astrid Lindgren Children's Hospital, Stockholm and the Pedodontic Clinic, Eastman Institute, Public Dental Health, Stockholm. Additionally, some patients for paper II were also retrieved from The Department of Oral Pathology, Malmö University, Malmö. In the first and second study the clinical and histopathological features of patients with OFG-S and OFG+CD were investigated. The total numbers of patients were 29 and 22 in the first (OFG-S n=17, OFG+CD n=12) and second (OFG-S n= 11, OFG+CD n=11) study respectively. Seven of the OFG-S patients and three of the OFG+CD patients were used in both studies.

In the third study 21 patients with CD+OFG were compared with a reference group of 39 patients with only CD (CD-R) regarding clinical features, as well as using the Paris phenotype classification, microscopic inflammation and granuloma occurrence.

Eleven of the patients with OFG+CD from the first study were included in the third study however none of the patients in the CD-R group were included in earlier studies.

The fourth study was conducted in collaboration with The Stomatology Department and The Department of Liver Transplantation, A.C. Camargo Hospital, Sao Paulo, Brazil resulting in a report on a total of six children who had been subjected to solid organ transplantation, four from our Brazilian colleagues and two from the Department of Oral Medicine and Pathology and the Department of Pediatrics, Sahlgrenska Academy, University of Gothenburg.

3.2 Methods

3.2.1 NOD2

In the first study analyses of available allele and/or genotype frequencies for NOD2 variants Arg702Trp, Gly908Arg and Leu1007fsinsC, all linked to CD, were performed. Buccal epithelial cells were collected using Isohelix SK1 Buccal Swabs. Polymerase chain reaction (PCR) and genetic analyses were carried out at the Genomic Core Facilities, Sahlgrenska Academy, University of Gothenburg, Gothenburg. Amplification by Touch-Down-PCR (TD-PCR) was performed in GeneAmp® PCR System 9700. Forward and reverse primers were used for the polymerase chain reaction (PCR). The PCR products were purified using magnetic beads in the automated workstation Biomek® NX. Sequence-PCR was carried out in a GeneAmp® PCR System 9700. The sequence-PCR products were purified using magnetic beads, also performed in the automated workstation Biomek® NX. The sequence reaction products were loaded on a 3730 DNA Analyzer and the results were analysed using the software program Sequencing Analysis and SeqScape. Studies with available allele and/or genotype frequencies for NOD2 variants Arg702Trp, Gly908Arg and Leu1007fsinsC, all linked to CD, were included.

3.2.2 Immunohistochemistry

Immunohistochemistry was performed on biopsies from 22 patients in the second study and on biopsies from another six patients in the fourth study.

Antibodies: Anti-human CD1a (clone 010, isotype IgG1, kappa), CD20 (clone L26, isotype IgG2a, kappa), mouse-monoclonal primary antibody anti-CD68, (clone KP1, isotype IgG1, kappa) and mast cell tryptase (MCT; clone AA1, isotype IgG1, kappa) were purchased at Dako Sweden AB. Monoclonal mouse antibodies anti-human CD3 (clone F7.2.38, isotype IgG1, kappa) and monoclonal rabbit CD11c (clone EP1347Y, isotype IgG) was obtained from AbCam, Cambridge, United Kingdom. Monoclonal antibody CD4 (second study clone 4B12, NCL-CD4-368/ fourth study clone 1F6-SP35) and monoclonal antibody CD8 (clone 4B11, NCL-CD8-4B11) were purchased at Novocastra, Leica Microsystems AB, Sweden. For the fourth study monoclonal rabbit-anti-human FOXP3 (clone 22510) were obtained from AbCam, (Cambridge, UK) as well as mouse monoclonal CD15 (clone 115M) and mouse monoclonal CD138 (clone 138M-15) from Cell Marque (Rocklin, CA, USA).

Immunostaining second study: Paraffin-embedded tissue specimens were cut at 4 µm thick sections and mounted on Dako IHC microscope slides, deparaffinised and then re-dehydrated. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min, washed in buffer

(0.1% phosphate buffered saline (PBS) + Triton-X-100 0.05%) for 5 min and followed by block non-specific proteins with 10% normal serum in 0.5% PBS for 20 min. For CD4 antigen retrieval in Tris-EDTA pH 9.0 was done in microwave oven for 10 min followed by blocking with Background Sniper (Biocare, Concord, CA, USA, BS966M) for 15 min. Staining with primary antibody against CD1a, CD3 (dilution 1:100), CD4 (dilution 1:200), CD8 (dilution 1:100), CD11c (dilution 1:65), CD20 (dilution 1:200), CD68 (dilution 1:1000) and mast cell tryptase (MCT) (dilution 1:1500) in 1% biotinylated secondary antibody (BSA) was performed for 60 min and washed in buffer for 2×5 min, followed by an incubation with BSA (Vector, Burlingame, CA 94110, USA) for 30 min and washed in buffer for 2×5 min. For CD4 Mach3MouseProbe (Biocare, M3M532L) was used as secondary antibody. Incubation with avidin-biotin complex (Vector ABC: 2.5 ml PBS + 50 µl A + 50 µl B) for 30 min was performed followed by a buffer wash for 2×5 min. DAB (DAB: One set of Sigma tablets to 5 ml of distilled H₂O) was added to slides for 2 min followed by washing in distilled H₂O several times. For CD4 a tertiary step was performed using Mach3MouseAP-polymer (Biocare, M3M532L) for 30 min and Vulcan Fast Red substrate (Biocare, FR805S) for 2 min with levamisol (Vector, SP-5000). Background staining in Mayer's hematoxylin was carried out for 40 seconds followed by blueing in tap water for 4 min. The sections were finally dehydrated and mounted with Pertex (Histolab Products AB, Spånga, Sweden). All steps were done at room temperature.

Quantitative analysis was performed on a minimum of two areas per tissue section with an average of four areas per patient, apart from one patient where the biopsy was too small to count two different sections. Digitalised images from one to eight fields, dependent on biopsy size, at a magnification of ×80 were obtained using a light microscope (Leitz Wetzlar, Leica Microsystems, Wetzlar, Germany) equipped with a UC30 Olympus camera (Olympus Microsystems, Norcross, GA 30071, USA). The sections were then analysed using the computer software BioPix iQ 2.0 (BioPix, Gothenburg, Sweden), where the percentage of stained tissue area was calculated as previously described (84).

Immunostaining fourth study: Paraffin-embedded tissue specimens were cut at 3-4 µm thick sections and mounted on Menzel Superfrost plus object slides or silanized microscope slides, deparaffinised and then re-dehydrated. The following protocol was used on slides from the Swedish specimens. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min, washed in buffer (0.1% phosphate buffered saline (PBS) + Triton-X-100 0.05%) for 5 min and followed by block non-specific proteins with 1,5% normal serum in PBS for 20 min. For CD4 antigen retrieval in Tris-EDTA pH 9.0 was done in microwave oven for 10 min followed by blocking with Background Sniper (Biocare, Concord, CA, USA, BS966M) for 15 min. Staining with primary antibody against CD1a, CD3, CD8 (dilution 1:100), CD4, CD20, CD138 (dilution 1:200), CD15 (dilution 1:500), CD23 (dilution 1:250), CD25 (dilution 1:10), CD68 (dilution 1:1000), FOXP3 (1:8000) and mast cell tryptase (MCT) (dilution 1:1500) in BSA (Bovine Serum Albumin, Sigma Aldrich) was performed for 60 min and washed in buffer for 2×5 min, followed by an incubation with anti-mouse IgG /anti-rabbit IgG Biotinylated antibody PK 6102/PK 6101 Vectastain ABC kit from Vector for 30 min and washed in buffer for 2×5 min. For CD4 Mach3MouseProbe (Biocare, M3M532L) was used as secondary antibody. Incubation with avidin-biotin complex (Vector ABC: 2.5 ml PBS + 50 µl A + 50 µl B) for 30 min was performed followed by buffer wash for 2×5 min. DAB Peroxidase Substrate kit Vector (to 5ml distilled H₂O was added 4 drops of DAB Stock Solution and added 2 drops of Hydrogen Peroxide Solution) was added to slides for 5 min followed by washing in distilled H₂O several times. For CD4 a tertiary step was performed

using Mach3MouseAP-polymer (Biocare, M3M532L) for 30 min and Vulcan Fast Red substrate (Biocare, FR805S) for 2 min with levamisol (Vector, SP-5000). Background staining in Mayer's hematoxylin was carried out for 40 seconds followed by blueing in tap water for 4 min. Finally the sections were dehydrated and mounted with Pertex (Histolab Products AB, Spånga, Sweden). The slides from Brazil were processed using an automated immunohistochemical system (BenchMark XT, Ventana Medical Systems, Tucson, AZ, USA). All steps were done at room temperature.

Digitalized images at a magnification of $\times 40$ and $\times 100$ were obtained using a light microscope equipped with a digital camera.

3.2.3 Statistical analyses

Statistical analysis was conducted in the first, second and third study using the nonparametric Mann-Whitney U-test or Fisher's exact test (presence of NOD2 mutation) (GraphPad Prism; GraphPad Software, La Jolla, CA). *P* values < 0.05 were considered statistically significant.

Analyses of mast cell degranulation were performed in the second study with a semi-quantitative scale where the amount of released tryptase-positive granules was assessed: 0: no released granules, 1: low number of released granules (1–3), 2: moderate number of released granules (4–9) and 3: high number (10 or above) of released granules. The number of degranulated cells was expressed as a percentage of all tryptase-positive cells. For statistical analysis, data in scale steps 0 + 1 and 2 + 3 were merged.

For the fourth study the semi-quantification method was used to estimate the relative amount of positive inflammatory cells at the primary observation and to visualise the decline of these cells during the healing phase of nodular tongue symptom. A scoring procedure was used where 0 was set when no stained cells were observed; +: was scored when a low number of cells showing a scattered distribution and no foci or continuous cell infiltrate was observed; ++: moderate number of stained cells. The cells may be aggregated in foci but no continuous infiltrate of cells was present; +++: high number of positive cells commonly organised in infiltrates or networks. Two calibrated observers jointly scored the material.

To evaluate the clinical and demographic features in the first and third study a multivariate data analysis was performed using a principal component analysis - discriminant analysis (PCA-DA; SIMCA-P+ software, version 13; Umetrics, Umeå, Sweden). The clinical and demographic features were registered as X-variables. The diagnoses of the patients, i.e. OFG-S, OFG+CD, CD+OFG and CD-R were registered as Y-variables. This method was used in order to correlate the data matrices X and Y with one another and to see if the patient groups could be distinguished from each other.

3.3 Ethical considerations

All the patients in these studies have suffered severely from physical, social and aesthetical discomfort due to their disease. The suffering is not only limited to the patients, but is also effecting parents and siblings. Most of the patients have had to learn to deal with their disease, each in their own way. By the time they come to our department they are often tired of the health care system and resigned to their problems. Therefore in the treatment of these

patients it is of the utmost importance to understand their despair and minimise their discomfort. There is an extra burden on the patients when participating in the studies presented in this thesis. However, this is motivated as an increased knowledge and awareness could lead to an improvement in the treatment and care of this debilitating disorder.

In order to address the specific questions raised in these studies patients with orofacial granulomatosis solely (OFG-S), orofacial granulomatosis with concomitant Crohn's disease (OFG+CD or CD+OFG) and paediatric liver recipients were included for this thesis. Patients were mainly recruited from the Gothenburg and Stockholm region. Although, for the fourth study we collaborated with colleagues from Sao Paulo, Brazil and therefore included four patients from Sao Paulo, Brazil.

Does orofacial granulomatosis have different clinical manifestations depending on the presence or absence of Crohn's disease?

We compiled a number of variables including demographic data, clinical characteristics, NOD mutations, heredity for immunoregulatory diseases, clinical history of allergy, autoimmunity and self-reported complaints of OFG to find possible divergent data between the OFG-S and the OFG+CD group.

The gender distribution had a female/male ratio of 1:2.6. The median age at inclusion was 14 years with no significant differences between the two groups.

The main oral clinical features in the patient cohort were swelling affecting the lips (OFG-S/OFG+CD; 83 % vs. 83 %; $P=ns$), mucosal tag formation (OFG-S/OFG+CD; 71 % vs 75 %; $P=ns$), cobblestone phenomenon (OFG-S/OFG+CD; 71 % vs 42 %; $P=ns$) and facial swelling (OFG-S/OFG+CD; 76 % vs. 92 %). Angular cheilitis was observed in almost half of the patients (OFG-S/OFG+CD; 47% vs 42 %; $P=ns$) and a minority presented with perioral erythema (OFG-S/OFG+CD; 35 % vs 25 %; $P=ns$). Ulceration was the most rare of all clinical features observed in both groups (OFG-S/OFG+CD; 24 % vs 8 %; $P= ns$).

Six OFG patients had a first-degree relative with autoimmune disease. In the OFG-S group, one relative had diabetes, one had CD and one had ankylosing spondylitis, whereas in the OFG+CD group two relatives had diabetes and one had psoriatic arthritis. None of the OFG patients themselves presented with such autoimmune diseases. Three patients in the OFG-S group, but none in the OFG+CD group reported that they had first-degree relatives with OFG symptoms.

Twelve out of 29 OFG patients reported that they had a first-degree relative who suffered from allergy, seven patients in the OFG-S group and five patients in the OFG+CD group. Regarding allergies for all patients in the study, nine patients had a clinical history of food allergy (fish, shellfish, cow milk, cacao, nuts, and/or peanuts), five in the OFG-S and four in the OFG+CD group. Seven patients reported that they suffered from allergy induced by airborne allergens, predominantly rhino-conjunctivitis, four in the OFG-S and three in the OFG+CD group. Further, eight out of 29 patients reported that they suffered from eczema, six in the OFG-S group and two in the OFG+CD group.

Self-reported complaints of OFG were one of the variables where the two groups differed. All patients were asked to report various aspects of their perceived orofacial complaints using a structured form including a visual analogue scale (VAS). The OFG-S patients reported significantly higher levels of overall discomfort at the peak of their disease than did the OFG+CD patients (OFG-S vs. OFG+CD; $P=0.047$). The reported aesthetic problems (OFG-S vs. OFG+CD; $P=0.0003$) and social discomfort (OFG-S vs. OFG+CD; $P=0.026$) were also more pronounced in the OFG-S group compared to the OFG+CD group.

Does orofacial granulomatosis have different histopathological characteristics depending on the presence or absence of Crohn's disease?

Biopsies from oral mucosa with granulomas were obtained from patients with OFG-S ($n=11$) and OFG+CD ($n=11$) and immunostained with antibodies against CD1a, CD3, CD4, CD8, CD11c, CD20, CD68 and mast cell tryptase (MCT), followed by a quantitative analysis using positively stained area method. Areas of positively stained cells within the connective tissue containing granulomas were registered. These areas of positive cells were then expressed as a percentage of the connective tissue area.

There was a significantly higher number of CD3-expressing T cells, as reflected by positively stained areas, in the connective tissues in patients with OFG-S compared to patients with OFG+CD ($P=0.005$). However, there was no significant difference in the presence of CD4-positive or CD8-positive cells between the OFG-S and OFG+CD groups.

CD11c-positive cells were significantly more abundant in the OFG-S group compared to the OFG+CD group ($P=0.007$). CD11c molecules were mostly expressed by non-dendritic multinucleated giant cells present in the granulomas. This marker could also be observed in other parts of the connective tissue, but then expressed by cells with a dendritic morphology.

The distribution of CD20 molecule-expressing B cells varied between biopsies; in some cases accumulation surrounding granulomas was observed, while in other cases a more homogeneous distribution was noted. No significant difference could be seen, although there

was a trend towards more B cells in the OFG-S group compared to the OFG+CD group ($P=0.087$).

As to the number of CD1a and CD68 molecule-expressing cells, there was no significant difference between the two groups. Neither was there any significant difference between the two groups in regards to MCT, although both groups showed a high degree of mast cell tryptase release.

Does orofacial granulomatosis have different genetic variations of NOD2 depending on the presence or absence of Crohn's disease?

Variation in the NOD2 gene has been shown to predispose for CD, which is why we decided to analyse NOD2 polymorphisms in our cohort. The three variants in NOD2 linked to CD are Arg702Trp, Gly908Arg and Leu1007fsinsC. Buccal swabs were used to collect DNA from the 29 patients with OFG. None of the 17 patients with OFG-S had any of the NOD2 variants previously shown to be associated with CD. In contrast, four out of 12 of our OFG+CD patients carried a single copy of the NOD 2 variation (Arg702Trp variant) revealing a significant difference in NOD2 variation between the two groups compared ($P=0.021$). Neither of the NOD2 variants Gly908Arg nor Leu1007fsinsC were found in any of the patient populations.

Does orofacial granulomatosis in conjunction with Crohn's disease signal a distinctive phenotype of Crohn's disease?

Twenty-one patients with CD and concomitant OFG (CD+OFG) and a reference group of 39 patients with CD (CD-R) and without OFG were compared for demographic data and clinical characteristics. In order to evaluate the clinical characteristics at a stage of untreated disease, the examinations were performed prior to any treatment. The two groups were compared using multivariate analysis as well as Fisher's exact test.

A clear predominance of males was seen in the children with CD+OFG (female/male ratio 1:4.25) as well as in the CD reference group (female/male ratio 1:2.5) with no significant differences between the two groups. The median age at CD diagnosis in the group with CD+OFG was 12.9 years and in the CD reference group the corresponding age was 10.3 years.

The extension of Crohn's disease was evaluated by taking into account the observed inflammation at endoscopy or imaging using the Paris classification as well as the histopathological distribution of the intestinal inflammation. A significantly higher proportion of CD+OFG patients had macroscopic involvement of the upper gastrointestinal tract than the CD references ($P=0.001$) as well as macroscopic ileocolonic inflammation ($P=0.013$). This coincides with a greater proportion of the patients in the CD+OFG group showing microscopic inflammation in parts of the upper gastrointestinal, i.e. oesophagus ($P=0.013$) and duodenum ($P=0.006$;) as well as in colon descendens ($P=0.016$) and sigmoideum ($P=0.032$).

At diagnosis, a significantly greater proportion of patients with CD+OFG displayed

granulomas throughout the entire gastrointestinal tract, from oesophagus to rectum, compared to the CD reference group ($P=0.023$) and as a result also showed more segments with both granulomas ($P=0.002$) and microscopic inflammation ($P=0.004$).

Perianal disease was in this study defined according to the Paris classification, i.e. including fistula, anal canal ulcers and/or abscesses. Perianal disease was significantly more common in the CD+OFG children than in the CD reference group ($P=0.033$).

Does orofacial granulomatosis have different histopathological characteristics compared to clinically similar oral lesions seen in paediatric liver recipients?

This is the first study exploring the immunohistological characteristics of a recently described disorder now denoted nodular tongue syndrome (NTS), which may develop in children following solid organ transplantation. The key feature of the condition is a nodular tongue appearance. This is a unique reaction pattern of the oral mucosa and we primarily describe this key feature of the condition.

Routine histopathology at the time of diagnosis

The nodular structures at the dorsum of the tongue represented swollen fungiform papillae. In areas where the inflammation was less intense, oedema was seen which was congruent with macroscopic swelling of fungiform papillae. The routine histology of the papillae was dominated by a marked subepithelial inflammation comprising mainly lymphocytes and macrophages. The lesions showed no signs of eosinophilic inflammation as no eosinophils were identified. As NTS has been considered as a potential granulomatous disorder due to clinical similarities with OFG, we performed an extensive search for granulomas. However, no granuloma formations were found in any of the biopsies. This scrutiny for granulomas was also performed on some buccal biopsies taken from intraoral cobblestone structures giving the same results. Thus, no features compatible with a granulomatous disease were observed in any of the lesions

Immunohistochemistry of NTS at the time of diagnosis

An intense infiltrate of evenly distributed CD3 positive T cells was found in the connective tissue. CD4 expressing helper T cells showed similar intensity and distribution but this antibody also visualised some intraepithelial dendritic cells. CD8 positive T cytotoxic cells were found less frequently and were estimated to about one third of the number of CD3 expressing cells. A substantial number of regulatory CD25 expressing cells were also observed. A few lymphocytes in some of the slides were also FOXP3 positive.

A substantial amount of CD20 expressing cells was observed in the connective tissues of all biopsies. CD20 is commonly used to detect B cells and this marker is not expressed by plasma cells. The CD20 positive cells did not display a typical B cell morphology but in general a more dendritic appearance. CD138 is a member of the heparan sulphate proteoglycan family and within the hematopoietic system it is expressed by plasma cells. Expression of CD138 is also typically observed on the surface of mature epithelial cells. This was confirmed in all our biopsies where pronounced staining of epithelial cells was observed. More interesting, a well-defined cellular infiltrate of CD138 expressing cells was also seen just beneath the subepithelial infiltrate of T cells.

Intraepithelial CD1a positive dendritic cells reflecting Langerhans cells were observed in numbers above the range normally displayed in healthy oral epithelium. A considerable number of CD1a expressing cells were also identified within the subepithelial infiltrate. In this location, the CD68 expressing macrophages outnumbered the CD1a positive cells but the number of intraepithelial cells was less than that observed for the CD1a.

Neutrophils are rarely seen in healthy oral mucosa. A few CD15 positive neutrophils were seen in the deeper part of the infiltrate. Scattered mast cells were also detected in the connective tissue at a range expected in the normal oral mucosa. In the deeper part of the subepithelial infiltrate a few CD23 expressing cells were seen. CD23, also known as Fc epsilon RII, or FcεRII, is the "low-affinity" receptor for IgE. The number of CD23 expressing cells was considerable less than the number of the CD20 positive cells.

The results presented in this thesis have answered the specific questions that were addressed and have given novel information regarding both orofacial granulomatosis (OFG) and Crohn's disease (CD). One of the important clinical consequences is whether orofacial granulomatosis in conjunction with Crohn's disease (CD+OFG) signals a more advanced disorder that demands an intensive treatment approach. This is of special importance as early intervention may be critical for effective treatment and favourable prognosis of Crohn's disease patients.

Does orofacial granulomatosis have different clinical manifestations depending on the presence or absence of Crohn's disease?

To address this question, a Swedish cohort of 29 patients with OFG was investigated for their oral phenotype. All patients were diagnosed with orofacial granulomatosis, either solely OFG-S (n=17) or in conjunction with Crohn's disease OFG+CD (n=12). No significant differences in the clinical phenotype between the two groups were found.

There is an on-going debate as to whether OFG-S and OFG+CD represent different disease entities or whether they merely signify the same disease with different involvement of the gastrointestinal tract (2, 85, 86). There are studies that support OFG-S and OFG+CD to be different in various aspects as for example the study of Savage and co-workers who found that serum IgA antibodies to *S. cerevisiae* were raised significantly in the OFG+CD but not in the OFG-S group (71). However, there are no studies to support that OFG-S and OFG+CD present with different clinical characteristics. This does not exclude that the oral involvement of the two conditions represents different entities, but may merely indicate that oral tissues respond with the same clinical reaction pattern although instigated by different pathological stimuli. This concept is supported by the fact that OFG has similar clinical characteristics as

long-standing oral disorders in paediatric liver recipients although the aetiology of the two conditions is most likely different.

When our two patient groups were compared, the OFG-S patients reported their complaints significantly higher on the VAS than the patients in the OFG+CD group did. The main complaint was related to the disfigurement caused by the swollen lips, which is in accordance with a study by McCartan and co-workers (4). One explanation to the reported differences regarding complaints between the two groups may be that the OFG-S group had a more extended duration from onset to the time of diagnosis than the OFG+CD patients. Another possible explanation for the discrepancy in the VAS score may be that the symptoms from the gastrointestinal tract in the OFG+CD group could have overshadowed the oral symptoms in some of the cases.

In addition to the primary scientific question, the design of our first study enabled a detailed comparison with previously published results from other geographic areas such as Ireland and the UK (4, 87). The patients in our study were younger with a median age of 14 years (range 7-32 years) than reported from the UK (23 years, 2-73 years) (87) and Ireland (28 years, 5-84 years) (4). The clear predominance of males (female/male ratio 1:2.6) in our study is not supported by the studies from the UK and Ireland where a more equal gender distribution was shown.

The oral site involvements and inflammatory features of OFG in the Swedish and the UK patients differed as well. Regarding the sites involved, the lips and buccal mucosae were the most affected in both studies. However, the UK patients displayed far more gingival changes than the patients in our study, where more changes in the sulcus region were observed. As for inflammatory features, tag formation was more common in our study group compared to the OFG patients in the UK and Ireland. This difference could be a result of the Swedish patients being of a younger age, as a correlation between age and tag formation has been reported from the UK (87).

A high proportion of our patients with OFG, whether they had Crohn's disease or not, reported that they had allergies. This is in line with a recent report from the UK where a positive history of allergies was observed in about 80% of the OFG patients (36), although the Swedish cohort did not show a positive history of allergies to as high an extent as the UK report.

International comparison in disease may give important clues as to the aetiology, which is presumably composed of a mix of lifestyle, environmental, and genetic factors that may underlie variations in disease occurrence and characteristics across populations. Although there are obvious clinical differences in OFG patients between different geographic areas, a closer international collaboration is necessary to take full advantage of comparative studies of OFG.

Does orofacial granulomatosis have different histopathological characteristics depending on the presence or absence of Crohn's disease?

To answer this question, a total of 22 OFG patients (OFG-S [n=11] and OFG+CD [n=11]) were characterised and compared regarding the immune cell infiltration in the two groups. As

granulomas are one of the disease hallmarks, only OFG patients with granuloma formation in their oral biopsies were included.

CD3-positive T cells as well as CD11c-expressing dendritic cells (DCs) were present in significantly higher numbers in lesions from patients with OFG-S compared to patients with OFG+CD. Despite a significant difference in the percentage of CD3-positive T cells between the two patient groups, no differences in the percentages of the subsets of CD4-positive or CD8-positive cells were detected. This may be due to interference from CD4 molecules expressed by DCs, granulocytes and macrophages, making a valid comparison difficult.

CD11c-positive lamina propria resident DCs is one of the two major subsets of DCs residing in oral mucosa, the other one being CD1a-positive Langerhans cells (LCs) (88). Although the amount of CD1a-positive LCs did not differ between the groups in this study, our impression is that the number of CD1a-expressing cells is increased in both groups in comparison to healthy oral connective tissue. This is in line with our previous studies showing high numbers of CD1a-expressing LCs in the connective tissue of other oral inflammatory disorders, for example, oral lichen planus (89). The high amount of DCs in OFG-S patients may be a reflection of an oral reaction pattern involving an exposure to exogenous antigens such as food constituents or bacterial components. Specific food constituents have been attributed a triggering role in OFG (36, 40, 83) and a continuous exposure to food antigens may in a sensitised individual lead to the recruitment of DCs to oral tissues. There is however, no definitive evidence that OFG is related to food allergy (40), although occasionally patients may benefit from cinnamon and benzoate-free diets (90, 91).

B cells have previously been found in OFG (5) and here we show that CD20-expressing B cells are found in the connective tissue with a heterogeneous distribution. Thus, tissue lesions from patients with OFG-S seem to recruit more antigen-presenting cells, more T cells and a trend towards more B-cells than disease affected tissues from patients with OFG+CD.

A great number of mast cells with released granules were registered, although no significant difference between the two groups was recorded. Increased frequencies of mast cells have also been reported in the gut mucosa of patients with CD and these cells have been implicated in the pathogenesis of this disease (92). OFG-S and OFG+CD may represent two subcategories that could differ in various aspects, such as immune mechanisms and clinical characteristics but further investigations are needed with larger patient groups.

The observation that T cells and CD11c-expressing dendritic cells were present in higher numbers in lesions from patients with OFG-S compared to patients with OFG+CD, support the view that these two subcategories of OFG have immunopathogenic differences. However, an increase in the number T cells and CD11c-expressing dendritic cells in the cellular infiltrate of OFG-S patients is not reflected by any clinical differences in the oral phenotype of the two disorders.

Does orofacial granulomatosis have different genetic variations of NOD2 depending on the presence or absence of Crohn's disease?

To address this question, we examined the frequency of the three major NOD2 risk alleles for Crohn's disease (Leu1007fsinsC, Arg702Trp, and Gly908Arg). NOD2 has the strongest

linkage to paediatric CD, which prompted us to specifically investigate NOD2 variants in our cohort of OFG patients. None of the 17 patients in our cohort with OFG-S carried any CD-linked NOD2 variation. In contrast, four out of 12 patients (33.3 %) with OFG+CD displayed a NOD 2 variation. Thus, although this study comprises a limited number of patients, it is the first to support the concept of OFG-S and OFG+CD possibly being genetically different.

Our finding supports the hypothesis that OFG+CD may represent a subgroup of Crohn's disease with a different genotype. The allele frequency of Arg702Trp in the OFG+CD group was high and calculated to 16,7%. In comparison, a study of patients with paediatric Crohn's disease from Sweden showed an allele frequency for Arg702Trp calculated to 2.6% (93). Furthermore, in a Swedish CD twin study of CARD15 polymorphism, Arg702Trp variant was identified in 3 (heterozygotes) out of 38 CD patients making an allele frequency of 3.9% (54). In Swedish healthy controls, the Arg702Trp variant was even lower, 1.6% (54).

Table1 Showing phenotypic characterisation of Crohn's disease patients with concomitant orofacial granulomatosis and NOD2 variations.

Disease localisation/behaviour	Patient 1	Patient 2	Patient 3	Patient 4
Macroscopic location				
Ileal inflam (L1)				
Colonic inflam (L2)	x			
Ileocolonic inflam (L3)		x	x	x
Above ligament of Treitz (L4a)		x	x	x
Below ligament of Treitz (L4b)				
Microscopic inflammation				
Eosophagus		x		x
Ventricle		x	x	x
Duodenum		x	x	x
Ileum				x
Caecum		x		x
Colon asc		x		x
Colon transv		x	x	x
Colon desc	x	x	x	x
Sigmoideum	x	x	x	x
Rectum	x	x	x	x
Behaviour				
B1 non-strict., non-penetrating	x	x	x	x
B2 stricturing disease				
B3 penetrating disease				
P perianal disease		x	x	x
Occurrence of granuloma		x	x	x

Carriage frequency of the three-pooled NOD2 variants (Leu1007fsinsC (n=0), Arg702Trp (n=4), and Gly908Arg (n=0)) in our CD+OFG patients (n=12) was calculated to 33.3%. The corresponding figure in the Swedish paediatric Crohn's disease cohort (n=58) was reported to be 8.6 % (Leu1007fsinsC (n=2), Arg702Trp (n=3) and Gly908Arg (n=0)), (93) and in a

population of Swedish healthy controls to be 5.2 % (54). Studies of children from Germany and the United States have shown results closer to ours with a prevalence of pooled carriage frequency of 60% and 29%, respectively (94, 95). An obvious explanation to the striking difference between the observations made by Ideström *et al.* (93) and our observations is the concomitant presence of OFG in our CD+OFG group. Thus, not only earlier onset of disease is associated with NOD2 mutations (96-98) but perhaps also the presence of OFG.

NOD2 variations have also been suggested to be associated with a disease phenotype driven by ileal involvement (99) and in our study three out of four patients with NOD2 variants displayed ileocolonic involvement, see table 1, page 25.

Does orofacial granulomatosis in conjunction with Crohn's disease signal a distinctive phenotype of Crohn's disease?

The third study was performed to answer this scientific question. Paediatric CD patients with and without OFG were examined for a number of clinical parameters including age at diagnosis, gender distribution and disease localisation. Macroscopic as well as microscopic inflammation was assessed with special focus on granuloma formation.

Children with CD and concomitant OFG (CD+OFG) seem to have a more extensive disease than paediatric CD patients presenting with intestinal inflammation only. This is shown by a significantly more macroscopic involvement of the upper gastrointestinal tract as well as an increased level of ileocolonic inflammation in CD+OFG patients compared to the patients in the CD reference group. The presence of a more extensive disease in the CD+OFG group is also supported by the histopathological examination, displaying more frequent microscopic inflammation in segments of the upper gastrointestinal, i.e. oesophagus, ventricle and duodenum. This finding is in accordance with a previous study by Pittock *et al.* (100) reporting more frequent inflammatory involvement of the upper gastrointestinal tract in patients with CD+OFG than patients with intestinal CD only. However, in a more recent study the same research group was not able to confirm this finding (101). The patients in the OFG+CD group also present more frequently with a microscopic inflammation in colon descendens and sigmoideum. In conclusion, patients with CD+OFG have to a greater extent a pan-enteric disease, which may represent a prognostic factor signalling a more severe outcome.

Granuloma formation is one of the key features of CD as well as of OFG and a high proportion of our CD+OFG patients (92%) showed granulomas in their oral lesions. Notably, the proportion of patients with granuloma formation in the intestinal mucosa was much higher in the CD+OFG group than in the CD reference group. Furthermore, in patients where granuloma formation was present, they often appeared in several intestinal segments.

In this study perianal disease definition was based on the Paris classification standards for anal disease (7) including fistula, anal canal ulceration and/ or abscess. Thus, simple fissures and isolated skin tags were not considered as perianal disease. This study displayed perianal disease in 48% of CD+OFG patients compared to only 18% in the CD-R patient group, which is in accordance with Harty *et al.* (101) where perianal disease was found in 50 % of the patients with CD+OFG. As for gender distribution, there was a predominance of males in both the OFG+CD and the CD-R group, which has been reported in other studies as well (93, 100-102).

The CD+OFG group exhibits a number of clinical characteristics such as extensive disease, perianal disease and upper gastrointestinal involvement. Thus, our data suggest that findings of oral lesions patients with CD could be used as a signal, indicating an extensive intestinal inflammation, perianal involvement and pronounced granuloma formation. The granulomatous condition of OFG+CD and CD observed in different segments of the orofacial mucosa and gastrointestinal tract has been suggested to consist of separate entities (87). This study shows that CD with orofacial involvement is more extensive and severe, which may result in a complicated disease demanding special treatment. However, studies with a larger cohort of patients from various geographic areas are necessary to gain further insight and validation of the CD+OFG phenotype.

Does orofacial granulomatosis have different histopathological characteristics compared to clinically similar oral lesions seen in paediatric liver recipients?

Our research group has previously identified a novel mucosal disorder in paediatric liver recipients. This nodular tongue syndrome (NTS) appears in conjunction with other clinical features such as mucosal tags, cobblestoning, swollen lips, and angular cheilitis i.e. similar clinical features to those that characterise OFG. Thus, one of the rationales behind this study was to portray the immune histopathological characteristics of NTS and to compare those with the characteristics of OFG observed in paper II.

In contrast to OFG, which is a granulomatous disorder, no granulomas were identified either in the tongue or buccal biopsies from the patients with NTS. The absence of granuloma means that the recently proposed term, “OFG-like lesions”, for this long-standing mucosal lesion is not relevant from an immunopathogenic point of view. The partly clinical similarities between NTS and OFG may simply reflect that the repertoire of manifestations in the oral mucosa is limited and thus, a different underlying immune mechanism may result in similar types of lesions, such as cobblestoning. The nodular structures at the dorsum of the tongue represent swollen fungiform papillae, a hypertrophy most likely caused by an oedema due the high vascularity of the fungiform papillae.

A substantial number of CD20 expressing cells were observed in the subepithelial infiltrate of NTS. The morphological and immune reactive features of CD20 expressing cells have previously been reported in the subepithelial infiltrate of OFG (5). CD20 is a trans-membrane protein expressed on mature B cells through all stages of their development. Plasma cells do not express CD20 but CD138. There were only a few CD138 expressing cells in the infiltrate, which did not co-localise with CD20 expressing cells. The number of CD20 expressing cells also clearly outnumbered the CD138 expressing cells. Most CD20 expressing cells displayed an irregular morphology and some cells had a dendritic appearance, different from the round morphology of CD20 expressing cells generally seen in lymphoid tissues. The expression of CD23, which is the “low-affinity” receptor for IgE, was much more scattered than the expression of CD20 and we can therefore conclude that most CD20 positive cells were CD23 negative. The primary role of the CD20-expressing cells is unknown but it can be theorised that these cells may have an antigen-presenting function of importance for the instigation of NTS.

Food allergens may have the role as triggering factors in the disease process of NTS. To date, ours and other clinical experiences do not support the involvement of food allergens causing

the coexisting acute onset IgE-mediated reactions. Dietary elimination has not appeared to improve the lesions of NTS (11, 24). However, it could not be excluded that other non-identified food allergens can be involved. The coexistence of NTS and immediate-onset transient food allergy reactions in the same individual, support the view that NTS arises as a consequence of a dysregulated immune system (11, 24).

Young age seems to be a risk factor in the development of NTS (11, 25), but also coexisting acute-onset IgE-mediated food allergy (11, 24). These circumstances indicate that pre-transplant chronic liver disease as well as an immature immune system may contribute to the disease process of NTS. Animal models indicate that the liver is an important organ for the development of tolerance, especially to food antigens (103, 104). High rate of food sensitizing has also been reported in children with liver end-stage disease prior to liver transplantation (105). Taken together, our theory is that the liver failure per se, which precedes organ transplantation, is the primary cause of disturbed immune regulation instigating a lack of tolerance which paves the way for NTS development. The immunosuppressive treatment may constitute an additional risk factor. Thus, a number of factors may contribute to an antigen-driven inflammation where the triggering antigen is yet to be identified, however, food antigens, auto-antigens as well as antigens of commensal microbes should be taken into account. Although a common denominator between the long-lasting oral lesions seen in paediatric liver recipients and OFG has not been identified, it is intriguing that both conditions share clinical features and that the for-mentioned triggering antigens may be involved in the aetiology of both disorders.

Concluding remarks

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OFG patients with or without CD have similar phenotypic characteristics but seem to have genotypic differences in NOD2. A skewed gender distribution with a predominance of males has been observed. We show that CD3-positive T cells and CD11c-positive lamina propria resident DCs are present in higher amounts in tissue specimens from patients with OFG-S than those in patients with OFG+CD. Additionally, our findings display a great number of mast cells with released granules in both OFG-S and OFG+CD patients. Our results suggest that CD in conjunction with OFG represents a distinctive sub-phenotype of CD with clinical characteristics such as extensive intestinal inflammation, perianal involvement and pronounced granuloma formation. The nodular tongue syndrome (NTS) represents swollen fungiform papillae caused by an oedema due the high vascularity of the fungiform papillae. The cellular infiltrate in NTS is dominated by T lymphocytes and macrophages. As no granuloma formations were found, we conclude that NTS does not belong to granulomatous diseases.

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