Factors associated with geographic tongue
Clinical, immunological and microbiological aspects

Amal Dafar

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

UNIVERSITY OF GOTHENBURG

Gothenburg 2016
Factors associated with geographic tongue
© Amal Dafar 2016
amal.dafar@odontologi.gu.se

http://hdl.handle.net/2077/47417

Permission to reprint Study II published by Journal of Immunological Methods was obtained from Elsevier

Printed by Ineko AB, Gothenburg, Sweden 2016
To my parents, Salwa and Osama

And my little family, Hesham, Reda and Omar
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>7</td>
</tr>
<tr>
<td>SAMMANFATTNING PÅ SVENSKA</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF PAPERS</td>
<td>11</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>13</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>15</td>
</tr>
<tr>
<td>1.1. Background</td>
<td>15</td>
</tr>
<tr>
<td>1.2. Epidemiology</td>
<td>16</td>
</tr>
<tr>
<td>1.3. Pathogenesis</td>
<td>17</td>
</tr>
<tr>
<td>1.4. Clinical Findings</td>
<td>18</td>
</tr>
<tr>
<td>1.5. Histopathologic Findings</td>
<td>19</td>
</tr>
<tr>
<td>1.6. Diagnosis</td>
<td>19</td>
</tr>
<tr>
<td>1.7. Factors associated with GT</td>
<td>20</td>
</tr>
<tr>
<td>1.8. Management</td>
<td>25</td>
</tr>
<tr>
<td>2. SCIENTIFIC QUESTIONS</td>
<td>27</td>
</tr>
<tr>
<td>3. METHODOLOGY</td>
<td>29</td>
</tr>
<tr>
<td>3.1. Participants</td>
<td>29</td>
</tr>
<tr>
<td>3.2. Collection of clinical data</td>
<td>30</td>
</tr>
<tr>
<td>3.3. Laboratory analyses</td>
<td>32</td>
</tr>
<tr>
<td>3.4. Statistical analyses</td>
<td>34</td>
</tr>
<tr>
<td>3.5. Ethical considerations</td>
<td>36</td>
</tr>
<tr>
<td>4. MAIN FINDINGS</td>
<td>37</td>
</tr>
<tr>
<td>5. RESULTS</td>
<td>39</td>
</tr>
<tr>
<td>6. DISCUSSION</td>
<td>43</td>
</tr>
<tr>
<td>7. CONCLUDING REMARKS</td>
<td>55</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>57</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>59</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>69</td>
</tr>
</tbody>
</table>
Factors associated with geographic tongue
Clinical, immunological and microbiological aspects
Amal Dafar
Department of Oral Medicine and Pathology, Institute of Odontology, Sahlgrenska Academy
at University of Gothenburg, Gothenburg, Sweden

ABSTRACT

Geographic tongue (GT) is a common oral mucosal lesion. Research conducted on this lesion has been limited, and the etiology remains to be clarified. The aim of this thesis was to elucidate clinical, immunological and microbiological aspects of GT. In study I, the associations between GT and systemic diseases, the use of medication and tobacco, as well as the differences between referred and non-referred patients were investigated in a cohort of patients with GT. In study II, a novel methodology was developed to detect epidermal growth factor (EGF) and interleukin-8 (IL-8) in saliva using samples from healthy volunteers. This study opened new avenues to investigate these biomarkers, and vascular endothelial growth factor (VEGF) in saliva samples from patients with GT (study III). In study IV, the bacterial ecology of the tongue in patients with GT was explored using next-generation sequencing of DNA. Our results showed that:

- Hypertension, anti-hypertension medication and the use of snus (Swedish snuff) are factors potentially associated with GT. Furthermore, referred patients with GT represent a special group that consists mainly of young females with different clinical characteristics and more symptoms (Study I).
- Pretreatment of the saliva samples with sodium dodecyl sulfate (SDS) significantly improves the quantitative detection of IL-8 and EGF. It is important to consider age, gender, and the collection time of saliva sample when analyzing salivary biomarkers (Study II).
- Patients with GT are characterized by increased level of salivary IL-8 which is correlated with the severity of GT. Moreover, salivary levels of VEGF and EGF are altered in patients who were sub-grouped according to age, gender or presence of systemic diseases (Study III).
- The lingual microbiota is different in patients with GT than in healthy controls. We detected an under-abundance of Fusobacteria and an over-abundance of Spirochaetes members in patients with GT. We also showed that bacterial diversity is increased in GT lesions. In addition, the composition of the lingual microbiota is different at the lesions sites and at the healthy sites in patients with GT (Study IV).

In summary, this thesis links the clinical parameters of GT with new insights into the immunological and microbiological aspects. We present GT as a multifactorial disease, with several factors that have the potential to play important roles in its pathogenesis. We emphasize the importance of a multidisciplinary approach to clinical research that widens our knowledge and understanding of several aspects of the disease. This approach is of interest not only for researchers, but also for the clinicians who are meeting the patients and providing them with information about their disease.

Keywords: geographic tongue, benign migratory glossitis, tongue lesions, oral mucosal lesions, salivary biomarkers, lingual microbiota.

http://hdl.handle.net/2077/47417
Geografisk tunga (GT) är en av våra vanligaste orala slemhinneförändringar, men trots detta är etiologin fortfarande okänd. Syftet med avhandlingen är att belysa kliniska, immunologiska och mikrobiologiska aspekter av GT. I Studie I, studerades sambanden mellan GT och systemsjukdomar, medicinering och tobak. I Studie II, utvecklades en ny metod för att studera epidermal tillväxtfaktor (EGF) och interleukin-8 (IL-8) i saliv. Detta möjliggjorde i Studie III studier av dessa biomarkörer, inklusive vaskulär endotelial tillväxtfaktor (VEGF), i salivprover från patienter med GT. I Studie IV undersöktes den bakteriella sammansättningen hos patienter med GT med hjälp av nästa generations sekvensering av DNA. Våra resultat visade att:

- Hypertoni och anti-hypertensiva läkemedel är faktorer som är associerade med GT. Även användandet av svenskt snus är associerat med denna förändring (Studie I).
- Förbehandling av salivprov med natriumdodecylsulfat (SDS) förbättrade signifikant den kvantitativa analysen av IL-8 och EGF. Resultaten visade också att ålder, kön, och tidpunkten för salivprovsamling påverkade analyserna av dessa biomarkörer (Studie II).
- Det huvudsakliga fyndet i Studie III var de generellt signifikant ökade nivåerna av IL-8 i saliven hos patienter med GT vilket också korreleriade med svårighetsgraden av GT. Salivnivåerna av VEGF och EGF var beroende av ålder, kön och förekomst av systemsjukdomar.
- Den orala bakteriella sammansättningen hos patienter med GT skilde sig från friska kontroller. Resultaten i Studie IV visade på en minskning förekomst av Fusobacteria och en ökad av Spirochaetes. Studien visade också att den bakteriella mångfalden ökade i den orala floran hos patienter med GT i jämförelse med friska kontroller. Dessutom observerades att sammansättningen av den orala mikrofloran var olika beroende på om provet togs i tungförändringen eller i den kliniskt friska delen av tungan hos patienter med GT.

Sammanfattningsvis visar denna avhandling att GT är associerad med ett antal kliniska parametrar. Den ger också nya insikter när det gäller immunologiska och mikrobiologiska förhållanden. Dessa aspekter kan ha potentiell betydelse för etiologin till GT. Avhandlingen är inte bara av rent vetenskapligt intresse, utan också av betydelse för de kliniker som träffar patienterna med GT och som ska ge information om denna orala slemhinneförändring.
LIST OF PAPERS

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


III. **Dafar A, Bankvall M, Garsjö V, Jontell M, Çevik-Aras H.** Salivary levels of interleukin-8 and growth factors are modulated in patients with geographic tongue. *Submitted for publication.*

**ABBREVIATIONS**

Common abbreviations used in this thesis are listed in order of first appearance.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT</td>
<td>Geographic tongue</td>
</tr>
<tr>
<td>RAS</td>
<td>Recurrent aphthous stomatitis</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>FT</td>
<td>Fissured tongue</td>
</tr>
<tr>
<td>BMS</td>
<td>Burning mouth syndrome</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin-E</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>gp</td>
<td>General practitioners</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>TI</td>
<td>Tongue impressions</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-generation sequencing</td>
</tr>
<tr>
<td>OTUs</td>
<td>Operational Taxonomic Units</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin-A</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

The tongue is an organ of the human body that is engaged in several important functions. The ability to speak normally is crucial for the development of self-confidence. The joy of tasting food is largely mediated by the sensory and mechanical activities of the tongue, complemented by the normal masticatory and swallowing functions. When any of these functions are affected, social and physiologic well-being are also affected.

Geographic tongue (GT) is a neglected oral lesion. The scientific literature on GT is sparse compared to that on other oral mucosal lesions, for instance, recurrent aphthous stomatitis (RAS), despite the higher prevalence of GT (2.2%) compared to RAS (0.5%) (Robledo-Sierra et al., 2013). The main reason for this difference is presumably the usual asymptomatic and benign nature of GT. However, some patients with GT suffer from symptoms that affect the pleasure of eating food. Other patients are concerned about the esthetic appearance of their tongue. Most important are the patients who are worried and have a phobia of cancer because of their GT, knowledge of which is limited. This thesis is unique in studying the clinical, immunological, and microbiological features of GT, providing new insights into this disease through the use of non-invasive methods for sample collection and state-of-the-art techniques for examining the microbiota of the tongue and for detecting salivary biomarkers.

1.1. Background

GT was first identified by the French physician Dr. Rayer in 1831 as a *wandering rash of the tongue* (Prinz, 1927). Wandering is defined as “moving about without a definite destination or purpose”. This definition describes the typical feature of GT lesions, which is that they often migrate across the tongue through healing on one edge while extending another edge. Thus, the name *benign migratory glossitis* is widely accepted. The term *geographic tongue* originates from the overall shape of the lesions, which resemble the continental outlines on a globe. These two terms are commonly used in the literature. Other terms that are used include: erythema migrans; exfoliatio areata linguae; superficial migratory glossitis; lingual dystrophy; pityriases linguae; transitory benign plaques of the tongue; marginal exfoliative glossitis; ectopic geographic tongue; and glossitis areata migrans (Assimakopoulos et al., 2002).
1.2. Epidemiology

The prevalence of GT varies greatly in the literature, ranging from 0.5% to 17.2%, as reported for Saudi and Libyan populations, respectively (Al-Mobeeriek and Al-Dosari, 2009; Byahatti and Ingafou, 2010). This discrepancy among the GT prevalences is largely dependent upon the study population, although methodological issues have also been implicated. For example, in the study of the Libyan population, the prevalence of 17.2% corresponds to 55 patients with GT of a total of 320 patients with ‘oral mucosal lesions (Byahatti and Ingafou, 2010). The correct prevalence that corresponds to 3460 total participants should be 1.5%. The same issue was encountered in a study of an Indian population, which presented a GT prevalence of 16.4% (N=98) among 595 patients with ‘tongue lesions’ (Patil et al., 2013). The correct prevalence that corresponds to 4926 total participants is 2.0%. Thus, overestimation of the prevalence appears be linked to methodologic issues. However, the relatively high GT prevalence reported for Israelis (12.7%) appears to be true (Yarom et al., 2004). Other studies have reported GT prevalences in the range of 1.0%–7.8% (Mumcu et al., 2005; Honarmad et al., 2013). In the Swedish population, GT prevalence was first reported in 1976, then in 1990, and recently in 2013 as 8.5%, 5.6%, and 2.2%, respectively (Axell, 1976; Salonen et al., 1990; Robledo-Sierra et al., 2013). The highest prevalence of GT in the study of Axell can be explained by: (i) the age limit (15 years) applied; and (ii) the diagnostic criteria used. Axell suggested two criteria for an acceptable diagnosis of GT even for those cases in which ‘migration’ was not documented: (i) well-demarcated zones that lack filiform papillae and show peripheral reddening; and (ii) white or yellowish, serpiginous lines that are partially surrounding red, depapillated areas or are that are seen in isolation. Thus, the lower age limit and the more flexible diagnostic criteria applied in that study may have led to the highest prevalence. In the study conducted by Salonen and co-workers, the reported GT prevalence was comparable to that listed in the study of Axell. This can be explained by the fact that these two studies: (i) covered almost the same age group; (ii) employed the same sampling method, which involved the collection of random samples from the general population; and (iii) involved diagnosis of oral mucosal lesions by oral medicine specialists. In contrast, the lower prevalence reported by Robledo-Sierra and colleagues study is attributed to: (i) a more specific population that included patients seen in general dental practitioners’ clinics; (ii) the recruitment of patients in a different age group (mean age of 61.5 ± 14.6 years); and (iii) the fact that diagnoses were made by general dentists, who may have under-diagnosed oral lesions.
The mean age of patients with GT reported to be around 30 years in several studies (Byahatti and Ingafou, 2010; Darwazeh and Almelaih, 2011; Honarmad et al., 2013). Nevertheless, GT is not uncommon in children (Figure 1), with reported prevalences of 5.2% for Brazilian preschool children (2–5 years of age) and 7.8% for Iranian school-children (7–18 years of age) (Vieira-Andrade et al., 2014; Rezaei et al., 2015).

![Figure 1. GT in an 18-month-old child](image)

The gender distribution of GT varies in the literature. Several studies have reported an equal gender distribution (Axéll, 1976; Salonen et al., 1990; Banóczy et al., 1993; Mumcu et al., 2005; Shulman and Carpenter, 2006), while others have shown predominance of GT in females (Darwazeh and Pillai, 1993; Honarmad et al., 2013).

### 1.3. Pathogenesis

The pathogenesis of GT has been suggested to be related to epithelial turnover, i.e., desquamation and keratinization processes (Hume, 1975). Normally, the rate of epithelial cell desquamation is equal to the rate of cell replenishment by the basal layers of the oral mucosal epithelium (Dawes, 2003). In this context, we assume that when the rate of epithelial desquamation is higher than the rate of replenishment, an ulcer-like lesion develops and keratin maturation decreases. If we consider that patients with GT have a defect in the mechanisms that control epithelial cell desquamation, regeneration, and keratinization, the above-mentioned mechanism may account for GT pathogenesis. However, it is not clear that inflammation in the connective tissue underlying the epithelium acts as a stimulus for the epithelial cells to desquamate or that the changes that occur in the tongue surface epithelium represent a trigger for inflammation, and thereby for epithelial desquamation. Further studies are needed to elucidate these mechanisms.
1.4. Clinical Findings

GT is classified as red and white lesions that affect the oral mucosa. GT is characterized by atrophy of the filiform papillae, leading to denuded lesions that appear as erythematosus areas surrounded by a white peripheral zone (Assimakopoulos et al., 2002) (Figure 2).

![Figure 2. Typical appearance of GT (erythematous areas surrounded by whitish peripheral zones)](image)

The number of lesions varies from single to several lesions that cover the entire surface of the tongue. Typically, GT lesions migrate in a map-like pattern with periods of exacerbation and remission. Exacerbation, which is the active phase of GT, occurs when the filiform papillae are lost and the keratin tips, which normally coat the filiform papillae, become matted to form spongiform pustules that exfoliate and expose the central area. Healing occurs when the active phase ends and the remission phase begins (Hume, 1975).

GT commonly affects the dorsal and lateral surfaces of the tongue. However, other sites of the oral mucosa, such as the buccal mucosa, labial mucosa, vestibule, mucobuccal fold, soft palate, floor of the mouth, lips, and gingiva, have been implicated and defined as ‘geographic stomatitis’ (Hume, 1975; Picciani et al., 2012; Brooks and Balciunas, 1987). Guidelines for the classification of geographic stomatitis were established by Hume and can be summarized as follows: Type 1, lesions that affect the tongue only; Type 2, lesions that affect the tongue and other parts of the mouth; Type 3, atypical Type 1 case with or without lesions in other parts of the mouth; and Type 4, no lesions on the tongue but lesions observed elsewhere in the mouth (Hume, 1975).

GT is often asymptomatic, although affected persons may complain of soreness, increased tongue sensitivity, and burning sensations elicited by the intake of spicy foods or acidic drinks (Jainkittivong and Langlais, 2005; Darwazeh and Almelaih, 2011; Banóczy et al., 1975).
1.5. **Histopathologic Findings**

Histopathologic examination of GT should cover the clinically apparent erythematous areas and white peripheral zones, since different histopathologic patterns were seen in these areas.

Electron microscopy examination of two biopsies collected from patients with GT showed that *the white elevated patches or margins* correspond to the actual lesion and are characterized by leukocyte infiltration, micro-abscess formation, intercellular edema, glycogen deposits, lack of keratinization, and increased exfoliation of necrotic surface cells. *The erythematous denuded areas* are healing areas that feature chronic inflammation with mononuclear cells, filament formation, and a lack of filiform papillae differentiation (Plackova and Skach, 1975).

In line with these findings, biopsies from thirteen patients with GT showed acute and chronic inflammatory infiltrates in the *white and erythematous areas*, respectively. This study has suggested that the final stage of the GT inflammatory process is the formation of new filiform papillae, which corresponds to the healing of GT (Marks and Radden, 1981).

A controlled study using scanning electron microscopy of 15 patients and 15 controls demonstrated that the surface of the GT contains 3 different types of mucosa: (i) *an atrophic area* on which the hairs of the filiform papillae are absent but the bodies are apparently typical; (ii) *a white margin that contains many desquamating cells*; and (iii) *an area of normal appearance* that contains filiform papillae with hairs attached. Micro-fissures are located between the atrophic mucosa and the mucosa of normal appearance (Kullaa-Mikkonen, 1986).

1.6. **Diagnosis**

GT is a clinical diagnosis that is based on the characteristic features of the lesions, presenting as a central erythematous area surrounded by a whitish peripheral zone, commonly on the dorsal and lateral surfaces of the tongue. The patient’s history of lesion migratory pattern is often pathognomonic. The frequency of agreement between oral medicine experts and general practitioners regarding GT diagnosis is the highest (96.0%) among all oral mucosal lesions that were diagnosed (Robledo-Sierra *et al.*, 2013). Since the clinical features of GT are typical, histopathologic confirmation is rarely required. However, such confirmation may be indicated in special circumstances, for example, in cases of doubtful diagnosis and when patients are anxious about their tongue lesion or have a cancer phobia.
1.7. Factors associated with GT

Association with other oral diseases

Fissured tongue (FT)
FT is characterized by the presence of grooves or fissures in the dorsal and lateral surfaces of the tongue (Axéll, 1976) (Figure 3a). Despite the macroscopic diagnosis of FT, microscopic examinations showed enlarged and smooth filiform papillae without hairs. Moreover, inflammatory cells infiltrates were identified in the epithelial and sub-epithelial layers (Kullaa-Mikkonen and Sorvari, 1986). The histopathologic evidence of inflammation and edema might justify the appearance of the clinically visible grooves (Järvinen et al., 2014). Whole saliva samples from patients with FT showed increased levels of sodium, lysozyme, myeloperoxidase, and immunoglobulins A, G, and M, owing to inflammation (Kullaa-Mikkonen et al., 1985) perhaps due to the accumulation of food and debris in the fissures.

The etiology of FT remains unclear. However, the association between FT and GT has been established (Honarmad et al., 2013; Shulman and Carpenter, 2006; Yarom et al., 2004) (Figure 3b). Indeed, the term ‘fissured tongue syndrome’ (FTS) was emerged based on the concept that GT and FT are different entities of the same inflammatory disease of the tongue (Kullaa-Mikkonen et al., 1987). This led to the proposal that FT is a sequel of GT, and that FT gradually increases with age (Hume, 1975; Yarom et al., 2004), which discounts the possibility of FT being a congenital anomaly.

Figure 3. (a) Fissured tongue (FT), and (b) concomitant GT and FT
Several studies have reported associations between FT and other diseases, such as psoriasis, orofacial granulomatosis, diabetes, asthma, Down syndrome, and Melkers–Rosenthal syndrome (Dawson, 1974; Marquardt et al., 2012; Guggenheim et al., 2000; Ghapanchi et al., 2015; Daneshpazhooh et al., 2007; Zimmer et al., 1992). The possibility that FT is inheritable has also been mentioned (Eidelman et al., 1976; Kullaa-Mikkonen, 1988).

**Burning mouth syndrome (BMS)**

The association between GT and BMS was first reported in 1998, where the authors reported that approximately 23% of patients with BMS also had other oral mucosal lesions, such as GT, erosive oral lichen planus, and oral candidiasis (Grushka et al., 1998). Several years later, the same research group demonstrated a more specific association of BMS with GT and FT (GFT). The authors pointed out a shift in the male-to-female ratio from 1:5 in BMS patients to 1:2 in BMS patients with GFT (Ching et al., 2012). The authors attributed this to local tissue changes, and that inflammation in GFT may predispose “male” patients with BMS to neuropathy, causing injury to the taste system and altered somato-sensation. However, it is not known if BMS is a consequence of GFT or that these diseases simply appear concomitantly. Usually, GFT is asymptomatic and often it is the case that the patients themselves do not know about their GFT until it is discovered by their dentist. On the other hand, BMS symptoms (mainly a burning sensation in the oral mucosa) are the main reason that patients seek professional help. In such scenario, it needs to be determined if this is a case of symptomatic GFT or if it is really BMS. Certainly, further research is needed in this area.

**Association with systemic diseases**

**Psoriasis**

The association between GT and psoriasis remains a matter for debate. Several researchers have expressed the belief that GT is an oral manifestation of psoriasis, and the term “oral psoriasis” has been suggested. Furthermore, it has been reported that GT is a marker of psoriasis severity (Picciani et al., 2015; Zargari, 2006). Two hypotheses have driven the notion of this association. First, in a genetic association study conducted in a Brazilian population, it was shown that a human leukocyte antigen (HLA-Cw6) was significantly associated with both psoriasis and GT (Gonzaga et al., 1996). However, the B-allele antigens HLA-B58 and HLA-B57 were associated with GT and psoriasis respectively. The author suggested that some GT cases represent true oral psoriasis, while other cases are simply GT (Picciani et al., 2014). Second, a histopathologic similarity between GT and psoriasis has been suggested in several studies and summarized in a review by Picciani and co-workers (Picciani et al., 2016).
A study that compared 40 tongue biopsies from 20 patients with psoriasis and 20 patients with GT showed that 80% of the patients with GT showed features typical of psoriasis, while the remaining 20% raised the question as to whether GT is a single entity (Femiano, 2001). Currently, there are no established histopathologic criteria for achieving a conclusive diagnosis of oral psoriasis (Mattsson et al., 2015). An argument has been made that oral lesions associated with psoriasis are not necessarily GT but reflect instead geographic stomatitis that may affect any site of the oral mucosa and not necessarily the tongue (Yesudian et al., 2012).

In contrast, several studies have not found any association between GT and psoriasis (Costa et al., 2009; Rezaei et al., 2015). Costa et al. demonstrated that GT and FT are the most frequently observed oral lesions in patients with psoriasis, as well as in controls (Costa et al., 2009). Indeed, most patients with GT have no psoriasis, and the concomitant detection of psoriasis and GT is expected since both lesions are common in the population (Mattsson et al., 2015). It can be concluded that GT and FT are the most common, although not specific, oral lesions in psoriasis.

**Allergy and atopy**

Several studies conducted by Marks and colleagues have suggested that GT is a reaction pattern of the tongue to underlying conditions. Patients with GT show an increased serum level of IgE, which is a sign of atopy (Marks and Simons, 1979). Atopic patients suffer from tongue sensitivity, which appears clinically as fungiform papillary glossitis (Marks et al., 2005). Several studies have shown that GT is more common in patients with allergy (Järvinen et al., 1989; Miloglu et al., 2009; Honarmad et al., 2013) and in patients who have a tendency to develop recurrent acute inflammatory diseases, such as asthma and rhinitis (Marks and Czarny, 1984).

**Diabetes**

The first report of an association between GT and juvenile diabetes was established in 1987. The authors showed that the frequency of HLA-B15 was 28.6% in patients with diabetes and GT, as compared to 14.6% in patients with diabetes only (Wysocki and Daley, 1987).

However, few studies have identified GT as one of several other oral mucosal lesions that can be seen in diabetic patients (Bastos et al., 2011; Guggenheimer et al., 2000; Al-Maweri et al., 2013).
**Hypertension**

Hypertension has previously been reported as one of the most common systemic diseases in Indian and Libyan populations with tongue lesions (Patil et al., 2013; Byahatti and Ingafou, 2010). Although cases of GT were identified in both studies, the association with hypertension was not related to a specific tongue lesion. However, a more specific association between hypertension and three oral mucosal lesions, GT, lichenoid lesions, and snuff-related lesions, has previously been demonstrated (Robledo-Sierra et al., 2013).

**Tobacco use**

An inverse association between GT and cigarette smoking has been demonstrated in several studies (Salonen et al., 1990; Honarmad et al., 2013; Shulman and Carpenter, 2006; Avcu and Kanli, 2003; Miloglu et al., 2009). Nicotine affects the immune response under inflammatory conditions through the central nervous system. The nicotine activates the hypothalamus–pituitary–adrenal (HPA) axis to induce the production of glucocorticoids and activate the autonomic nervous system, so as to reduce the level of inflammation (Sopori, 2002). Additionally, nicotine activation of acetylcholine receptors on macrophages reduces the levels of pro-inflammatory cytokines, TNF-α, IL-1, and IL-6 (Wang et al., 2003). Consequently, nicotine reduces the inflammation, which might prevent the development of GT (Honarmad et al., 2013). The effect of smokeless tobacco was not studied as extensively as cigarette smoking. However, one study has demonstrated a direct association between GT and the use of tobacco snuff (Salonen et al., 1990).

**Psychologic factors**

Redman et al. examined 20 students with GT at times of severe stress, i.e., during their final examinations, and found that the majority of the students experienced worsening of their GT lesions during this period (Redman et al., 1966). Bancozy et al. demonstrated a connection between GT symptoms and stress (Banóczy et al., 1975). More recently, patients with GT have been investigated for an association with stress. Using the Perceived Stress Scale (PSS) questionnaire, the mean score for stress was found to be significantly higher for patients with GT (Ebrahimi et al., 2010). Salivary cortisol levels and anxiety scales were evaluated as measures of stress and it was shown that patients with GT have higher levels of salivary cortisol and higher anxiety scores (Alikhani et al., 2014).
**Hereditary and genetic factors**

The childhood onset of GT has raised questions about the hereditary and genetic background aspects of this disease. Interesting familial studies have investigated patients with GT and their corresponding families (parents and siblings) and have shown a strong tendency for familial occurrence of GT among first-degree relatives (Redman *et al.*, 1972; Eidelman *et al.*, 1976).

In the genetic context, human leukocyte antigen (HLA) has been investigated in several studies. A study of Australian patients with GT showed a higher frequency of HLA-B15. However, after statistical correction and subgrouping, the author attributed the increases in HLA-B15 to underlying atopy and not to GT *per se* (Marks and Tait, 1980). As mentioned earlier, patients with GT and psoriasis or diabetes have an increased frequency of HLA-Cw6 and HLA-B15, respectively (Gonzaga *et al.*, 1996; Wysocki and Daley, 1987). The three previous studies were not able to demonstrate a specific association between GT and HLA, since the presence of other systemic diseases, atopy, psoriasis, and diabetes, influenced the results. In contrast, the association of HLA with GT without an influence of other diseases was established for Greek patients, revealing increased frequencies of HLA-DR5 and HLA-DRW6, which suggests a genetic predisposition for GT (Fenerli *et al.*, 1993). Moreover, Picciani *et al.* have identified HLA-B*58* as being associated with GT in patients with different ethnic backgrounds (Picciani *et al.*, 2014). Apart from HLA, genetic polymorphisms in the genes for IL-1β, IL-6, and TNF-α have been assessed among patients with GT. A significant difference in IL-1β genotypes (CC, CT, and TT) was observed for patients with GT, and the CT genotype was significantly associated with a high risk (*p*=0.02, OR 2.76) of developing GT (Guimarães *et al.*, 2007).

**Hormonal factors**

A study of 26 pregnant women (13 with GT and 13 without GT) was not able to demonstrate an association between GT and the levels of estrogen and progesterone in the saliva (Ghalayani *et al.*, 2013). In contrast, Waltimo studied the effect of oral contraceptive use for 1 year on a young woman who has GT. The results suggested a hormonal association with GT. The total number of lesions peaked on the 17th day of the contraceptive pill cycle, and the subjective symptoms reached the maximum on days 16–20 of the cycle (Waltimo, 1991).
**Microbiological factors**

An observation of *Candida* species in cultures was made for 14% of patients with GT (Banóczy *et al.*, 1975). Similarly, *Candida* species were detected more often in patients with GT than in controls or patients with other tongue lesions, such as FT, filiform atrophy, and hairy tongue (Kullaa-Mikkonen and Kotilainen, 1983). Moreover, histologic examination of GT biopsies showed *Candida* hyphae (Kullaa-Mikkonen, 1986). Recently, GT, FT or both conditions have been associated with the presence of fungi, commonly *C. albicans*, on the tongue surface (Dudko *et al.*, 2013). To our knowledge, no studies have evaluated bacteria as a possible etiological factor for GT.

### 1.8. Management

Commonly, patients with GT are unaware of the presence of lesions on their tongue, and the dentist is usually the one who first discovers the lesions. In many cases, GT is asymptomatic, so management should be focused on providing reassurance and information about the benign nature of the condition, especially for those patients who suffer from cancer phobia. Although symptomatic GT lesions are not common, some patients suffer from sensitivity, a burning sensation, and soreness in the mouth, which can affect their quality of life, given that these symptoms interfere with eating and drinking. In this situation, treatment of GT is needed.

A study conducted in the late 1970’s showed that treatment of GT with 0.1% topical Retin-A solution, applied with a swab once or twice daily, resulted in complete resolution of the GT lesions in three patients (Helfman, 1979). In 1980, Henricsson and Axell tested local application of salicylic acid (7%) in alcohol (70%) on the tongue for 16 patients with GT. The results showed that 4 patients had complete resolution of their GT, 11 patients experienced improvement of their symptoms, and 1 patient had no changes (Henricsson and Axéll, 1980). Four years later, Pimlott tested the 7% salicylic acid in 13 patients in a double-blind study and found no differences in the mean pain score or the mean number of pain days between the active and the placebo group (Pimlott, 1984). Recently, a case report of systemic treatment of GT with cyclosporine in a patient who suffered from persistent and painful lesions for 5 years showed satisfactory improvement of the patient’s symptoms (Abe *et al.*, 2007). The patient was treated with an initial dose of cyclosporine (3 mg/kg/d) for 2 months followed by a maintenance therapy with half of the initial dose. The length of time that the patient was on maintenance therapy is unknown. Another case report of two patients who were treated with 0.1% topical Tacrolimus twice daily for 2 weeks showed improvement of the symptoms and lesions (Ishibashi *et al.*, 2010).
Introduction

To date, there is no clearly defined, clinically verified, optimal treatment for symptomatic patients with GT. Nonetheless, the identification of etiological factors is valuable for the development of a clear treatment strategy for patients who complain of GT symptoms.
2. SCIENTIFIC QUESTIONS

This thesis was designed to study the following aspects of geographic tongue (GT): clinical (Study I), immunological (Studies II and III), and microbiological (Study IV). The aim was to find answers to the following scientific questions:

Study I

- What factors are associated with geographic tongue (GT) in an adult Swedish population?
- What are the differences between referred and non-referred patients with GT?

Study II

- How can we detect with high sensitivity epidermal growth factor (EGF) and interleukin-8 (IL-8) in whole saliva?
- Does collection time of saliva sample, and the age or gender influence the levels of EGF and IL-8 in whole saliva?

Study III

- Are there differences in the levels of salivary interleukine-8 (IL-8), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) between patients with GT and controls?
- What are the factors that affect the levels of salivary IL-8, VEGF, and EGF among patients with GT?

Study IV

- Do patients with GT have different microbiota on the tongue in terms of abundance and diversity?
- What is the composition of the lingual microbiota in patients with GT?
3. METHODOLOGY

3.1. Participants

Study I

In this study, recruitment of the subjects was performed at two different types of dental clinics, general dental practices and oral medicine specialist clinics. At the general dental practices, represented by six general practitioners (gp) at six private dental clinics in Borås city, Sweden, a survey was conducted among 6448 patients who attended their annual dental examination in the period 2004–2006. Based on the results of this survey, non-referred patients who were examined by gp and who presented with geographic tongue (GTgp; N=130; 78 males and 52 females) were identified. From the same survey, 1029 individuals with a clinically healthy oral mucosa and no oral complaints were selected to serve as the control group.

At the oral medicine specialists’ clinics, referred patients were examined by oral medicine specialists (s) for GT (GTs; N=166; 82 males and 84 females), at either the Clinic of Oral Medicine, Public Dental Health, Gothenburg, Sweden between November 1997 and September 2013 or the Clinic of Oral and Maxillofacial Surgery and Hospital Dental Care, Central Hospital, Karlstad, Sweden between February 1998 and March 2011.

Study II

A total of twenty-eight (13 males and 15 females) healthy and non-smoking individuals from the staff members and laboratory personnel at the Clinic of Oral Medicine, Public Dental Health, Gothenburg, Sweden participated in the study. Each participant donated unstimulated whole saliva samples for assaying for salivary biomarkers and to establish a protocol for the analysis of saliva samples. To evaluate the effect of collection time, samples were collected on three occasions each day: in the morning (8:00 am – 10:00 am, N=28), after lunch (12:00 pm – 1:00 pm, N=7), and in the late afternoon (3:00 pm – 4:00 pm, N=7).

Study III

Thirty-four patients who were diagnosed with GT (20 males and 14 females) and thirty-eight age- and gender-matched controls (19 males and 19 females) donated unstimulated whole saliva samples for an investigation of salivary biomarkers. Patients were assigned to three different groups according to age: 24–39 years (N=11); 40–64 years (N=13); and 65+ years (N=10).
Methodology

Among the patients with GT, thirteen subjects suffered from systemic diseases, such as hypertension (N=7), rheumatoid diseases, e.g., fibromyalgia and rheumatoid arthritis (N=5), or psoriasis (N=3).

Study IV

A total of 35 patients with GT (19 males and 16 females) and 22 age- and gender-matched controls (12 males and 10 females) donated swab samples from the tongue for genetic identification of the colonizing bacteria.

For Studies III and IV, the participants were recruited from the Clinic of Oral Medicine, Public Dental Health, Gothenburg, Sweden and from a private dental clinic in Borås city, Sweden.

3.2. Collection of clinical data

Study I

Retrospective clinical data were collected using MedView, which is a computerized system for the formalized registration and subsequent analysis of clinical- and image-based information related to oral medicine. MedView operates with an input application that is focused on the collection and computerized storage of clinical data (Jontell et al., 2005). Both general practitioners (gp) and oral medicine specialists used the same software program (Medview) for the collection of the clinical data. Prior to the data collection, all gp undertook training by an oral medicine specialist for the diagnosis of oral mucosal lesions, the use of Medview and the intraoral photographing techniques. After the collection of the clinical data, the information was gathered in a single database and exported to the MedVisualizer program for visualization of the information (Jontell et al., 2005). Finally, the data that were selected for evaluation were transferred to the Microsoft Excel for Mac 2011 software for subsequent statistical analysis.

In this study, we evaluated systemic diseases, medications, tobacco use habits, the clinical criteria for GT lesions, and symptomatology. Systemic diseases were grouped according to the International Classification of Diseases and Related Health Problems, 10th revision (ICD-10) (WHO 2010). Medications were classified according to the Anatomical Therapeutic Chemical Classification System (ATC) (WHO 2011) and the Swedish Medicines Compendium for Physicians (http://www.fass.se). The use of tobacco products (cigarettes and Swedish snus) was registered as ‘yes’ or ‘no’.
Methodology

Regarding the clinical criteria, clinical examinations and clinical images were used to classify GT. Accordingly, the severity of the GT lesions was defined as: *mild*, i.e., a single lesion; *moderate*, i.e., 2–5 lesions; or *severe*, i.e., $\geq 6$ lesions. Lesions were classified as active when they exhibited well-demarcated white or red borders, and as passive for those cases that had missing borders but still contained depapillated areas. The presence of concomitant fissured tongue (FT), tongue impressions (TI), and tongue-related symptoms was also evaluated.

**Studies II and III**

All the participants were asked to refrain from eating, drinking, and carrying out oral hygiene procedures for at least 1 hour prior to saliva collection. Unstimulated whole saliva samples were collected by asking the participants to spit for 10 minutes into a sterile 50-ml tube. All samples were collected on ice, which is essential when collecting and processing saliva samples for protein analysis (Schulz *et al.*, 2012). Twenty-five $\mu$l/ml of protease inhibitor cocktail (S8830; one tablet diluted in 4 ml of distilled water; Sigma-Aldrich), were added immediately to the saliva samples along with 40 $\mu$l/ml EDTA (2 mM Sigma-Aldrich), to prevent auto-degradation of the proteins by proteolytic enzymes (Thomadaki *et al.*, 2011). The samples were then divided into 200-$\mu$l aliquots, to reduce the risk of the fluid being subjected to cycles of freezing and thawing (Francis *et al.*, 2000), and immediately stored at -80°C until analysis. For each individual, the salivary flow rate (ml/min) was calculated.

**Study IV**

The participants were requested to avoid brushing their teeth or consuming any food or beverages for a minimum of 1 hour before sampling of their lingual mucosa. All the samples were collected by the same dentist. Sterile swabs (Isohelix DNA Buccal Swab; Isohelix/Cell Projects Ltd., Harrietsham, Kent, UK) were used to collect bacterial samples according to the manufacturer’s instructions, with the exception of the site of sampling. We collected samples from the lingual mucosa rather than the buccal mucosa, as our aim was to identify the bacteria on the tongue surface, the same location as the GT lesions. In total, 91 tongue samples were collected from 35 patients with GT and from 22 controls. The samples were assigned to four groups: (i) GT-lesion sites (n=34); (ii) GT-healthy sites (n=35); (iii) from i and ii, paired samples, i.e., lesion and healthy sites of the same individual were (n=66) from 33 patients; and (iv) controls (n=22). One patient gave only one lesion sample since GT was covering almost the whole surface of the tongue; thus, the total number of GT-lesion samples was 34. One patient donated only one healthy sample as we could not detect any tongue lesions at the time of sampling. In addition, one patient gave both a lesion sample and a healthy sample but the lesion sample was excluded as it did not pass the DNA quality criteria. Thus, the total number of GT-healthy samples was 35.
3.3. Laboratory analyses

**Enzyme-linked immunosorbent assay (ELISA)**

In our project, we used the sandwich ELISA technique, which has the advantage of high specificity due to the use of two antibodies, i.e., one for capture and one for detection of the antigen. However, before the ELISA assay, several proteins require treatment with different reagents in order to make them suitable for analysis. Sodium dodecyl sulfate (SDS) is a reagent that has been shown to be effective in modifying small proteins so that they become suitable for ELISA detection (Lechtzier et al., 2002). The authors have tested 3 major requirements for SDS to be used for proteins treatment before the ELISA assay. Accordingly, the results have demonstrated that: (i) SDS has the ability to preserve the antigenicity of the proteins; (ii) SDS doesn’t cause unspecific binding of proteins to primary or secondary antibodies; and (iii) SDS doesn’t hinder the adsorbance of the proteins to ELISA microwells. Thus, SDS is an effective reagent for treating the small proteins making them suitable ligands in ELISA (Lechtzier et al., 2002).

**ELISA analyses of saliva samples (Studies II and III)**

Initially, saliva samples were pretreated with 0.4% SDS (Sigma-Aldrich). A sandwich ELISA was thereafter performed to determine the levels of biomarkers in whole saliva according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Briefly, ELISA microplate was coated with capture antibody (Mouse anti-human EGF/ IL-8/ VEGF) and incubated overnight. In the next day, the microplate was blocked to avoid non-specific binding. After that, SDS-treated saliva samples were assayed in duplicate dilution series. This was followed by adding the detection antibodies (Biotinylated goat anti-human EGF/ IL-8/ VEGF), and later, the enzyme-linked 2ry antibody (Streptavidin-HRP). Subsequently, substrate solution was added and yielded color changes in the samples. The previous reaction was stopped by the addition of a stop solution. Lastly, the optical density was determined using a microplate reader at 450-nm and the corresponding concentration of the proteins biomarker was calculated. The measurement of salivary biomarkers using concentration is affected by the dilution factor of the saliva, i.e., the more diluted the saliva, the lower the biomarker concentration. Therefore, the rate of secretion, i.e., output (pg/min), is a more-precise quantitative measure (McGurk et al., 1990), as it overcomes the individual variability of saliva secretion. Therefore, a standardized measure (flow rate in ml/min) was used to calculate the outputs of the salivary biomarkers.
Methodology

Next-generation sequencing (NGS)

Traditionally, bacterial identification has been performed using culture-dependent methods. However, it is estimated that only about 50% of oral bacteria are cultivatable (Dahlén et al., 2014). A revolution in bacterial identification has occurred with the development of culture-independent molecular methods using 16S ribosomal RNA (rRNA) genes. The 16S rRNA gene consists of 1500 base pairs and nine variable regions (V1–V9) (Figure 4). These variable regions have DNA sequences that are different in different bacteria, therefore the V regions can be used to identify bacterial taxa. Beside the nine variable regions, conserved regions have DNA sequences that are very similar between different bacteria.

![Figure 4. Schematic overview of the 16S rRNA gene. It consists of 1500 base pairs (bp). Hypervariable regions (V1–V9) are shown in gray, and conserved regions are shown in orange (Taken with permission from GATC Biotech)](image)

The gold standard of DNA sequencing methods is Sanger sequencing (first-generation sequencing). Sanger developed a method in 1977 for the determination of the nucleotide sequences in nucleic acids (Sanger et al., 1977). Next-generation sequencing (NGS) was developed in 2005 in response to the demand for faster and cheaper sequencing methods compared to Sanger’s method. NGS has the advantage of massive sequencing with higher throughput, as compared to Sanger sequencing. Different platforms are used for NGS (Siqueira et al., 2012), with the most commonly used platforms being 454 pyrosequencing / Roche and Illumina / Solexa, which was used in our project.

NGS of bacterial 16S rRNA genes (Study IV)

**DNA extraction**

Extraction of DNA from saliva samples was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with some modifications. The most important modification was the bead beating step. In this step, five glass beads (3.0-mm diameter) and 0.25 g of zirconia beads (0.1-mm diameter) were added to the lysis buffer (ASL), and the suspension was homogenized twice at 6 m/s for 40 sec (FastPrep Cell disrupter; Thermo Savant, Holbrook, NY,
Methodology

USA).

We believe that the use of the Qiagen extraction kit is more efficacious than using any one of the four other available kits (McOrist et al., 2002). In addition, the use of glass bead beating increases the likelihood of disrupting the very thick and sturdy cell walls of Gram-positive bacteria (Sjöberg et al., 2013), yielding DNA of good quality from our samples. The obtained DNA was quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Sequencing procedure

PCR amplification of the V3–V5 region of the bacterial 16S rRNA gene was carried out using forward primer \(5’\)-CCTACGCGAGGAGGCAGCAG-3’ and reverse primer \(5’\)-CCGTCAATTTCMTTTRAGT-3’. Sequencing was performed using the Illumina HiSeq 2500 platform. The library was constructed according to the protocol of GATC Biotech (Konstanz, Germany). To achieve accurate Operational Taxonomic Units (OTUs) assignments, three steps were performed. First, read pairs were merged by overlapping the forward and reverse sequence reads. Second, clustering was carried out based on sequence similarity, with a minimum pair-wise identity of 99.0%. Third, singletons and possible chimeric sequences were removed; only those reads with a quality score of ≥25 and a read length of 200–500 nucleotides were retained for further analysis. Thus, OTUs assignment was performed for non-chimeric and unique clusters using a BLASTn analysis (Altschul et al., 1990) for non-redundant 16S rRNA reference sequences, which were obtained from the Ribosomal Database Project (Cole et al., 2009). Taxonomic assignment based on NCBI Taxonomy (Federhen 2012) was considered and used as a reference database.

3.4. Statistical analyses

Study I

Medical history, the use of medications, and tobacco use were compared between non-referred patients with GT (GTgp) and controls using Fischer’s exact test. Since there was an age difference between the GTgp individuals and the controls, a multiple logistic regression (MLR) model was used to eliminate the confounding effects of age and gender.

Clinical criteria (severity, activity, and concomitant FT or TI) and symptoms were compared between referred patients (GTs) and non-referred patients (GTgp) using Fischer’s exact test. For all the tests, a \(p\)-value <0.05 was the threshold for statistical significance. The statistical analysis was performed using the Statistical Analysis Software (SAS) ver. 9.1. (SAS Institute Inc., Cary, NC, USA).
Study II

A paired t-test was used to compare the effects of pretreatment of saliva samples with SDS to controls, and to compare the effects of collection time of the saliva samples.

In addition, an unpaired t-test was used to evaluate the effects of gender and age on the levels of salivary biomarkers. All the analyses were performed using GraphPad Prism, vers. 6.0 for Mac OS X (GraphPad Software, La Jolla CA, USA) and the level for statistical significance was p<0.05.

Study III

Based on our pilot study, a sample size of a minimum of 30 patients and 30 controls was calculated as being necessary to achieve a statistical power of 80.0% using the G*Power ver. 3.1 software.

An unpaired t-test was used to compare the salivary levels of IL-8, VEGF, and EGF between patients with GT and controls, including the effects of gender, age, and presence of systemic diseases. The associations between GT clinical criteria (severity, activity and concomitant FT or TI) and levels of salivary IL-8, VEGF, and EGF were evaluated using ordinary one-way ANOVA. These statistical analyses were performed using the GraphPad Prism, vers. 6.0 for Mac OS X software with a significance level of p<0.05.

Additionally, multivariate analysis was used to overview all the investigated factors that had potential associations with GT. Thus, all the data were pooled into one model, to include: age, gender, medical history, clinical criteria as well as laboratory parameters i.e. salivary biomarkers. This model is known as Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA), which identifies information in the X-data set (different factors) that relates to the Y-data (GT or controls) using the (SIMCA P+ ver. 14.1 software, Umetrics AB, Umeå, Sweden).

Study IV

Bacterial abundance and diversity were compared among the three groups of samples, i.e., GT-lesion, GT-healthy, and controls, using one-way ANOVA (alternatively, the Kruskal-Wallis test). The same comparisons were performed for paired samples, i.e., GT-lesion and GT-healthy, using a paired t-test (alternatively, the Wilcoxon signed-rank test) using the GraphPad Prism ver. 6.0 for Mac OS X software and SAS ver. 9.1 software.
Methodology

Multivariate analyses were conducted with the SIMCA P+ ver. 14.1 software. The overall pattern of the GT microbiota was obtained using Principal Component Analysis (PCA). The composition of the lingual microbiota, defined in terms of OTUs among the different groups, was determined using Orthogonal Partial Least Squares (OPLS) analyses. Finally, OTUs that showed the strongest contributions in the respective OPLS models were further tested using Fisher’s exact tests or a paired t-test to confirm unequal distributions between the groups. For all the tests, the level of statistical significance was set at \( p<0.05 \).

3.5. Ethical considerations

The Central Ethical Review Board in Gothenburg, Sweden approved Studies I–IV (dnr. 032-12). All the patients had to read and sign an informed consent form prior to the commencement of the studies.
4. MAIN FINDINGS

Study I

- Geographic tongue (GT) is associated with hypertension, anti-hypertension medication and the use of Swedish snus.

- Referred patients with GT represent a special group that differ from non-referred patients.

Study II

- Pre-treatment of saliva samples with sodium dodecyl sulfate (SDS) improves the detection of epidermal growth factor (EGF) and interleukin-8 (IL-8).

- Collection time of saliva samples, age and gender of participants affect the levels of EGF and IL-8 in saliva.

Study III

- GT is an inflammatory condition characterized by increased salivary IL-8 level.

- Male patients with GT demonstrate increased levels of salivary IL-8 and EGF. However, young female patients with GT show an increased level of salivary IL-8 while reduced levels of salivary EGF and VEGF.

- Patients with GT and hypertension are characterized by a dual increase of salivary levels of IL-8 and VEGF.

Study IV

- Patients with GT have different bacterial abundance and diversity of their tongue.

- The composition of the lingual microbiota of patients with GT is different at lesion sites and healthy sites.
5. RESULTS

Study I

What factors are associated with geographic tongue (GT) in an adult Swedish population?

The non-referred patients with geographic tongue (GTgp; N=130) reported hypertension (23.8%) and use of anti-hypertension medication (32.3%) significantly more often (13.3% and 20.1%, respectively) than the control subjects (N=1029). Evaluation of tobacco use habits showed that significantly more patients with GT used Swedish snus than the control subjects (10.1% vs. 3.8%). In contrast, cigarette smoking was significantly less frequent among patients with GT than among the controls (5.4% vs. 15.4%). Since we reported significant differences in age and gender between patients with GT (median age, 62 years; 60.0% males) and controls (median age, 56 years; 40.2% males), a regression model was used to eliminate the confounding effects of age and gender. Accordingly, the rates of hypertension (OR 1.7, 95% CI 1.05–2.75, p=0.029), use of anti-hypertension medication (OR 1.6, 95% CI 1.01–2.70, p=0.042), and use of snus (OR 2.1, 95% CI 1.10–4.35, p=0.025) were significantly higher among patients with GT than among the controls. Thus, the established associations were not confounded by age and/or gender.

What are the differences between referred and non-referred patients with GT?

The group of referred patients with GT (GTs, N=166) consisted of more females (50.6%) of a younger age (median, 49 years). These patients had lower frequencies of FT (30.7%) but had significantly more symptoms related to the tongue (47.0%) (p<0.001). These symptoms were significantly associated with active lesions (p<0.001), as well as the concomitant presence of FT (p<0.001) and TI (p<0.05). In contrast, the group of non-referred patients (GTgp, N=130) comprised 40.0% females of an older age (median, 62 years) who presented more often with concomitant FT (49.2%) and fewer symptoms related to the tongue (9.2%), as compared to the referred patients with GT.

Study II

How can we detect with high sensitivity epidermal growth factor (EGF) and interleukin-8 (IL-8) in whole saliva?

The sensitivities of detection of EGF and IL-8 were significantly improved when the saliva samples were treated with 0.4% sodium dodecyl sulfate (SDS) before analysis in the ELISA assay. We compared the concentrations of salivary EGF and IL-8 between SDS-treated (test) and PBS-treated (control) paired samples (n=14). We found that the concentrations of EGF and IL-8 were significantly increased by 293% and 346%, respectively, in the SDS-treated samples.
Results

**Does collection time of saliva sample, and the age or gender influence the levels of EGF and IL-8 in whole saliva?**

The collection time of saliva sample had an effect on the levels of salivary biomarkers. Saliva samples that were collected after lunch (n=7) showed the lowest outputs of EGF and IL-8, as compared to samples that were collected in the early morning (p<0.05) or late afternoon (non-significant).

The salivary EGF levels were significantly higher among older individuals (N=14; age range, 40–65 years) than younger individuals (N=14; age range, 20–39 years, p<0.001), and were higher among males (N=15, p<0.05). Similarly, the salivary IL-8 levels were significantly higher among males (N=13) than females (N=15, p<0.001), although no difference was found between the different age groups for the salivary IL-8.

**Study III**

**Are there differences in the levels of salivary interleukine-8 (IL-8), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) between patients with GT and controls?**

A comparison of the patients with GT (N=33) and controls (N=37) revealed that the salivary IL-8 output was significantly higher in the patients with GT (1486.0±230.2 vs. 577.7±72.9, p<0.001). The salivary VEGF and EGF levels were initially not different between the patients and controls.

**What are the factors that affect the levels of salivary IL-8, VEGF, and EGF among patients with GT?**

We found that gender affected the levels of salivary biomarkers. Male patients with GT (N=20) had significantly higher salivary levels of IL-8 (p<0.01) and EGF (p<0.05) than male controls (N=18). Female patients (N=13) had also higher salivary level of IL-8 (p<0.01) but had lower salivary levels of VEGF and EGF, although these levels of VEGF and EGF were not significantly different from those of the female controls (N=19). Further analyses of the different age groups showed that young females (age range, 24–39 years) had significantly higher output of IL-8 (N=5, p<0.05) while significantly lower outputs of VEGF and EGF (N=6, p<0.05), as compared to young female controls (N=7).

Medical status was found to be a factor affecting the levels of salivary biomarkers. Patients with GT who also had systemic diseases (N=13), such as hypertension, rheumatoid diseases, and psoriasis, were found to have significantly higher salivary levels of IL-8 than the controls. Since we established an association between GT and hypertension in Study I, we compared the patients with GT and hypertension (N=7) to control subjects (N=37). The salivary levels of IL-8 (1974.0±815.4 vs. 577.7±72.9, p<0.001) and VEGF (1084.0±199.6 vs. 681.0±67.7, p<0.05) were significantly higher among the patients with GT and hypertension compared to controls.
We investigated if there are associations between the levels of salivary biomarkers and the clinical presentation of GT. We found that the salivary levels of IL-8 correlated with GT severity, i.e., the higher the number of GT lesions, the higher was the level of salivary IL-8. However, active (n=17) and passive (n=10) lesions demonstrated significantly higher IL-8 levels ($p<0.05$ and $p<0.001$) than controls (n=37). Interestingly, passive lesions demonstrated higher levels of IL-8 than active lesions. The significant increase in IL-8 level among patients, compared to the controls, was not affected by the presence or absence of FT or TI ($p<0.001$). In contrast, the presence of FT (GT+FT; N=11) or TI (GT+TI; N=5) was associated with also a significantly increased level of VEGF, as compared to the controls ($p<0.01$ and $p<0.05$, respectively).

**Study IV**

*Do patients with GT have different microbiota on the tongue in terms of abundance and diversity?*

In this study, we investigated bacterial abundance in relation to the distribution of bacterial phyla. Eleven phyla were identified in total across all the swap samples. The majority of the microbiota examined across the three groups (GT-lesion, GT-healthy, and controls) belonged to the *Firmicutes* (69.0%). However, *Fusobacteria* were significantly less abundant in the GT-lesion and GT-healthy samples than in the controls ($p<0.001$). In contrast, *Spirochaetes* were significantly more abundant in the GT-lesion samples, as compared to the GT-healthy samples ($p<0.05$) and compared to the controls ($p<0.001$).

Microbial diversity is usually measured at the genus level by calculating the number of Operational Taxonomic Units (OTUs). Significantly higher diversity, i.e., higher number of OTUs, was found in the GT-lesion samples than in the GT-healthy samples ($p<0.01$) and the controls (non-significant). A comparison of paired samples gave the same result.

*What is the composition of the lingual microbiota in patients with GT?*

The microbial composition of the tongue in each group (GT-lesion, GT-healthy, and controls) was investigated by checking for the presence or absence of the 403 OTUs that were originally identified across all the samples. Accordingly, we identified two genera, *Acinetobacter* and *Delftia*, as being associated with GT. Those genera were identified at both lesion- and healthy-sites of GT. However, four taxa were identified specifically on the GT-lesion sites but not on the GT-healthy sites: *Microbacterium, Leptospira, Methylotenera, and Lactococcus*. Theses taxa may play a role in the development of GT lesions. Furthermore, the GT-healthy sites presented with two genera that were similar to those in the controls, i.e., *Mogibacterium* and *Simonsiella*, which indicates that GT-healthy sites may be in the process of restoring the normal bacterial flora of the tongue.
6. DISCUSSION

In this part of the thesis, we discuss the specific scientific questions posed at the outset, in light of the results obtained.

Study I

*What factors are associated with geographic tongue (GT) in an adult Swedish population?*

In this retrospective study, we evaluated the medical histories of patients with GT and the major finding was an association of GT with hypertension (OR 1.7, 95% CI 1.05–2.75, *p*=0.029). A limitation of this result is that hypertension was self-reported by the patients, as we did not conduct any blood pressure measurements. However, to overcome this limitation, a detailed evaluation of the medication profile for each patient was carried out. The results supported our major finding and revealed a significant increase in the use of β-blockers or calcium-channel blockers among patients with GT (OR 1.6, 95% CI 1.01–2.70, *p*=0.042). While we uncovered anti-hypertension drug use in 32.3% of the patients with GT, only 23.8% of these patients reported that they have hypertension. This may be explained by the fact that some patients consider themselves healthy because their blood pressure has been normalized through medication. Thus, we emphasize the importance of evaluating not only the medical history, but also the history of medication. Hypertension has previously been reported as one of the most common systemic diseases in patients with tongue lesions (Byahatti and Ingafou, 2010; Patil *et al.*, 2013). This association is general in nature, and not related to a specific tongue lesion. A more-specific association of hypertension with oral mucosal lesions, including GT, lichenoid lesions, and snuff-related lesions, has previously been demonstrated (Robledo-Sierra *et al.*, 2013). However, it is unknown if there is a common pathological pathway that mediate both GT and hypertension.

We evaluated two aspects of tobacco use in this study. First, GT and cigarette smoking showed an inverse association, i.e., GT was less prevalent among smokers. This finding is in line with those of other studies (Salonen *et al.*, 1990; Avcu and Kanli, 2003; Shulman and Carpenter, 2006; Miloglu *et al.*, 2009; Honarmad *et al.*, 2013). The immunomodulatory effect of nicotine is linked to activation of the hypothalamus–pituitary–adrenal (HPA) axis, which in turn induces the production of glucocorticoids and activate the autonomic nervous system, so as to reduce the level of inflammation (Sopori, 2002). Additionally, nicotine activation of acetylcholine receptors on macrophages reduces the levels of pro-inflammatory cytokines, TNF-α, IL-1, and IL-6 (Wang *et al.*, 2003). Consequently, nicotine reduces the inflammation, which might prevent the development of GT (Honarmad *et al.*, 2013). Second, GT and the use of snus showed a direct association, i.e., GT is more prevalent among the snus users. This finding has been demonstrated previously in a study of a Swedish population (Salonen *et al.*, 1990).
The effect of smokeless tobacco on increasing blood pressure has been established (Hergens et al., 2008). This effect is suggested to be due to sodium retention, as snus contains sodium, in addition to the nicotine and licorice, leading to poor blood pressure control (Gupta et al., 2004). It remains to be discovered if the use of snus affects the development of GT.

What are the differences between referred and non-referred patients with GT?
The referred patients with GT represent a special group that contains more females of a younger age (median age, 49 years) who are seeking specialist help for their symptoms and concerns about their tongue lesions. The symptoms related to the tongue are usually sensitivity, soreness, and a burning sensation. We found a significant association between the reported symptoms and the clinical presentation of GT, including the presence of concomitant fissured tongue (FT) and tongue impressions (TI), as well as the activities of the lesions. TI is a sign of parafunctional habits that may reflect the stress level of the patients when they are pressing their tongue against their teeth. Stress and GT have been correlated in previous studies (Banóczy et al., 1975; Ebrahimi et al., 2010; Alikhani et al., 2014). We identified active lesions as lesions that had a well-demarcated peripheral zone. It has been proposed that the peripheral zone represents the actual lesion where acute inflammation takes place (Plackova and Skach, 1975). Therefore, the more active the lesions, the more acute are the inflammation and symptoms. Accordingly, referred patients may suffer from anxiety due to symptoms related to the tongue. Furthermore, the appearance of the tongue may induce a cancer phobia in some patients. The management of such patients should be focused on providing reassurance and information about the benign nature of GT.

Non-referred patients with GT, who are often seen in general dental practices, are usually asymptomatic and are often unaware of their GT lesions. The typical patient is a male around the age of 60 years who may also present with FT. This observation supports the notion that GT transforms with age into FT (Hume, 1975; Yarom et al., 2004). The concomitant occurrence of GT and FT has been established previously (Shulman and Carpenter, 2006; Honarmad et al., 2013).

Study II

How can we detect with high sensitivity epidermal growth factor (EGF) and interleukin-8 (IL-8) in whole saliva?
The saliva is an indicator of the body health and it contains most of the biomolecules that are found in other biological fluids, such as the blood and urine (Schulz et al., 2012). The easy and non-invasive method of saliva collection has exponentially increased the use of salivary biomarkers for monitoring systemic and oral diseases (Javaid et al., 2016).
However, a lack of standardized laboratory protocols for the analysis of highly complex and viscous saliva samples has hindered the accurate and reliable detection of protein biomarkers (Schipper et al., 2007; Messana et al., 2008). The complexity of saliva is mainly due to the abundance of highly glycosylated proteins, mainly mucins. Mucins are composed of a backbone of proteins (10.0%-30.0%) and carbohydrates (70.0%-90.0%) (Bastardo et al., 2002), which are attached to the backbone in a brush-like projections (Wu et al., 1994), as illustrated in Figure 5. Microscopically, mucin aggregates can be seen to lead to the formation of clusters of particles, known as salivary micelles (Schipper et al., 2007; Young et al., 1999), which are formed by interactions between glycoproteins and ions (Schipper et al., 2007). The formation of micelles is a selective process that involves specific salivary proteins, including mucins, lactoferrin, amylase, salivary IgA, and proline-rich proteins (Soares et al., 2004). Small proteins, such as EGF and IL-8, bind readily to salivary micelles, thereby masking the real numbers of these small proteins (Kelly et al., 2002), as shown in Figure 5.

[Schematic structure of mucins](#)

Sodium dodecyl sulfate (SDS) breaks down the mucin aggregates into small molecules. The mechanisms for this have been suggested by Bastardo et al. and include: (i) breakdown of the physical bonding of the mucins; and (ii) a hydrophobic interaction between SDS and the glycan-side of the protein core, both of which are negatively charged (Bastardo et al., 2002). Thus, we hypothesized that SDS reduces saliva viscosity and thus improves the detection of EGF and IL-8. Neither saliva viscosity nor mucins were tested in this study, instead we looked at the effect of SDS treatment on the detection of EGF and IL-8 in saliva samples. SDS significantly improved the detection of EGF (by 293%) and IL-8 (by 346%). Similarly, previous studies have shown that SDS can be used effectively in preparing clinical samples for ELISA designed to detect soluble proteins (Lechtzier et al., 2002) and blood group substances (McCabe et al., 1988). To exclude the possibility that SDS degrades EGF and IL-8, we tested the effect of SDS on the corresponding standard for each biomarker. The concentrations of both standards were unchanged after SDS treatment, which means that using SDS has no effect on the antigenicities of EGF and IL-8 and that it is considered safe for the detection of EGF and IL-8 in saliva samples.
Discussion

*Does collection time of saliva sample, and the age or gender influence the levels of EGF and IL-8 in whole saliva?*

We evaluated the EGF levels at three time periods during the same day. Our results show that eating food significantly reduces the level of EGF in the saliva, which is similar to the result of a previous study (Ino *et al.*, 1993). Eating, together with taste and olfactory stimulation, initiate a reflex mechanism that promotes saliva secretion and that is mediated by the autonomic nervous system (Proctor and Carpenter, 2014). Two distinct mechanism are identified: (i) *parasympathetic*, involving the stimulation of cholinergic (muscarinic; M3) receptors that mediate the secretion of the fluid-phase of the saliva; and (ii) *sympathetic* stimulation, in which noradrenalin binds to the adrenergic receptors (mainly β1) and mediates the release of the protein contents (e.g., biomarkers) via exocytosis from the storage granules within the salivary glands (Proctor and Carpenter, 2014). This mechanism explains the finding that stimulated saliva, due to chewing, contains a higher level of EGF than unstimulated saliva (Konturek *et al.*, 1989). However, the powerful stimulation of eating on salivary glands and the long-acting reflex mechanisms, contribute to the release of proteins from the secretory granules while eating (Ekström *et al.*, 1998). In fact, the finding of the previous studies provides a good explanation for our result of reduced levels of EGF after eating. When the stimulation caused by eating is over, as in the case of our samples collected immediately after lunch, the level of EGF decreases because the protein contents have already been released. It appears that EGF synthesis in the salivary gland is a continuous process (Ino *et al.*, 1993), which also supports our finding of higher levels of EGF in the late-afternoon samples.

We found a higher level of EGF among older individuals, which agrees with the results of previous studies (Ino *et al.*, 1993; Dagogo-Jack, 1986). The physiologic regeneration of the oral epithelium reduces with age as the regenerative capacity of the tissues diminishes (Barakat *et al.*, 1969). Therefore, increased salivary EGF with age may plays a role in maintaining homeostasis in the oral cavity.

Regarding the gender, our results show that the levels of EGF are higher in males than in females. It has been suggested based on animal studies that the synthesis of growth factors in the salivary glands is androgen-dependent (Byyny *et al.*, 1974; Stern *et al.*, 2000). Stern *et al.* demonstrated that although female mice had lower concentrations of EGF, they had higher numbers of EGF receptors, which supports the notion that the availability of ligands and receptors is more important than the actual concentration of the ligand (Stern *et al.*, 2000). However, in human study, no difference in salivary EGF levels between males and females was observed (Dagogo-Jack, 1986).
Our results have revealed higher levels of salivary IL-8 than what we anticipated among healthy individuals. IL-8 is a chemotactic factor for neutrophils (Baggiolini et al., 1989), which are the hallmark of acute inflammation (Colgan, 2015). The majority of studies have focused on the roles of IL-8 and neutrophils in pathologic conditions. However, studies regarding the physiologic importance of IL-8 in the oral cavity are limited. A recent study demonstrated the crucial role of oral neutrophils in maintaining the oral ecosystem (Rijkscroeff et al., 2016). The oral cavity is a site of continuous mechanical trauma, as well as exposure to different pathogens. Neutrophils have the ability to phagocyte and digest pathogens and may then die by apoptosis (Mócsai, 2013). Thus, a steady supply of neutrophils to the oral cavity is needed. It has been shown that neutrophils are themselves able to secrete IL-8 (Gainet et al., 1998), which may play roles in the recruitment and maintenance of the steady supply of additional neutrophils.

We demonstrated higher levels of IL-8 in male than female participants. The effect of sex hormones on circulatory neutrophils chemotaxis has been investigated in vitro. It has been shown that progesterone is associated with increased neutrophil chemotaxis, whereas testosterone has no effect on neutrophil chemotaxis (Miyagi et al., 1992). However, the effect of sex hormones on salivary levels of IL-8 and neutrophil chemotaxis is questionable. We demonstrated also a reduction in salivary IL-8 levels after eating food. The extent in which IL-8 is present in secretory granules within salivary glands, and the effect of food intake on this cytokine are unknown.

**Study III**

*Are there differences in the levels of salivary interleukine-8 (IL-8), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) between patients with GT and controls?*

In this study, we found that IL-8 level was increased in the unstimulated whole saliva samples from patients with GT. The increased level of IL-8 was not dependent on age, gender or presence of systemic diseases. Furthermore, it was positively correlated with the disease severity, which we defined as the number of GT lesions. In vitro, a chemotaxis model has been used to demonstrate that neutrophil migration is increased towards areas of increased IL-8 concentration (Li Jeon et al., 2002), which corresponds well with our finding of increased levels of IL-8 as the number of GT lesions increased. Several studies have shown neutrophil infiltration of the sub-epithelium of GT lesions (Kulla-Mikkonen, 1986; Marks and Radden, 1981). Indeed, neutrophils play roles in the restitution of the broken epithelial barrier, wound healing, and homeostasis processes which are important for inflammation resolution (Colgan, 2015).
Discussion

Inflammation in GT may be caused by microorganisms that reside in the compromised tongue mucosa and stimulate cytokines production (Tlaskalová-Hogenová et al., 2004), thereby recruiting neutrophils to the site of inflammation (Agace et al., 1993). In addition to the chemotactic function of IL-8, angiogenic activity has been demonstrated (Qazi et al., 2011). The mechanism by which IL-8 regulates angiogenesis may involve the enhanced proliferation and survival of endothelial cells, as well as the activation of matrix metalloproteinases (Li et al., 2003).

Our studies initially found no differences in levels of VEGF and EGF between the patients with GT and the controls. However, this finding changed when the patients with GT were classified into subgroups, as described below. This highlights the importance of evaluating subgroups of patients in clinical studies, as patients mostly represent a heterogeneous population.

What are the factors that affect the levels of salivary IL-8, VEGF, and EGF among patients with GT?

Classification of patients with GT according to gender, age, systemic background, and clinical criteria of GT lesions did not change our results regarding IL-8 but did alter the results for VEGF and EGF.

The biological functions of EGF have been evaluated in experimental studies, which have demonstrated the importance of EGF in the maintenance of papillary morphology (Morris-Wiman et al., 2000), wound healing (Noguchi et al., 1991), and the chemotaxis of vascular endothelial cells during tissue repair (Grotendorst et al., 1989). As mentioned earlier in Study II, the observed effect of gender on EGF levels in murine models indicates that EGF synthesis is androgen-dependent (Byyny et al., 1974; Stern et al., 2000), such that male mice have higher salivary levels of EGF than female mice (Noguchi et al., 1991). We demonstrate in our study that males with GT have higher levels of EGF than both females with GT (45.0% higher) and male controls (25.0% higher). High concentrations of EGF are chemotactic for neutrophils and stimulator for IL-8 production (Lewkowicz et al., 2005). Therefore, we propose that increased levels of IL-8 and EGF may contribute to neutrophil chemotaxis in male patients with GT.

The physiologic roles of VEGF in the maintenance of homeostasis and the acceleration of wound healing in the oral cavity have been demonstrated (Taichman et al., 1998; Pammer et al., 1998). Several reports have shown that VEGF inhibition for cancer therapy is associated with the development of tongue lesions that resemble GT (Gavrilovic et al., 2012; Hubiche et al., 2013; Gilmore et al., 2016). In line with these findings, our results show that a subgroup of patients with GT that comprises young females (age range, 24–39 years) exhibits reduced levels of VEGF and EGF, together with higher level of IL-8. This finding bolsters our findings in Study I that this group of patients, i.e. young females, was referred to specialists due to more-symptomatic lesions (Dafar et al., 2016).
It is important to note that epithelial cells of the tongue have high mitotic activity (Barakat et al., 1969), and the reconstitution of epithelial cells requires a steady supply of VEGF and EGF to maintain epithelial integrity when the regeneration process is underway. Accordingly, we suggest that the pathogenesis of GT in young females is characterized by insufficient levels of VEGF and EGF for epithelial regeneration, which might be associated with more symptomatic GT lesions.

The medical status of the patients has an influence on our results. In Study I, we demonstrated an association between GT and hypertension (Dafar et al., 2016). This novel finding was explained in Study III by higher levels of IL-8 and VEGF in GT patients with hypertension, as compared to controls. Similar to our results, the serum levels of IL-8 and VEGF have been found to be higher among patients with hypertension (Marek-Trzonkowska et al., 2015). Anti-VEGF therapy has reported to be associated with the development of oral lesions that resemble GT (Gavrilovic et al., 2012; Hubiche et al., 2013; Gilmore et al., 2016) and the development of hypertension (Lankhorst et al., 2015). Therefore, we suggest that common immunological pathways and cellular events may be activated in both GT and hypertension, leading to increased levels of IL-8 and VEGF in the oral cavity. An association between GT and rheumatoid diseases or psoriasis cannot be established conclusively in this study, since very few patients with both diseases were identified.

In Study I, we created a set of guidelines for better understanding of GT clinical presentation. Subsequently, these guidelines were used in Study III to evaluate the levels of salivary biomarkers. Our severity scores, which were defined by the number of lesions, were correlated with the IL-8 levels. The higher the number of GT lesions, the higher was the level of IL-8 in the saliva. This observation suggests an augmented neutrophil chemotaxis and more inflammation when the number of lesions increased. Another suggested parameter was the activity of GT, which is defined as active if well-demarcated borders are seen, and passive if the borders are missing. We anticipated a higher level of IL-8 in patients with active GT. However, the levels of IL-8 were higher in the passive lesions. Thus, we suggest that the number of GT lesions is a better reflection than a detailed morphology of the GT lesions.

Fissured tongue (FT) is often seen in combination with GT lesions (Yarom et al., 2004; Shulman and Carpenter, 2006; Honarmad et al., 2013; Dafar et al., 2016). The combined GT+FT lesions were characterized by higher levels of IL-8 and VEGF, as demonstrated by our results. A previous study showed inflammatory infiltrates in the epithelium and sub-epithelium of FT lesions (Kullaa-Mikkonen and Sorvari, 1986), indicating an inflammatory process and edema formation (Järvinen et al., 2014). The possible triggering factor for inflammation might be the presence of fissures on the tongue surface, which may trap food, debris, and bacteria. Thus, high level of IL-8 is expected in GT+FT lesions.
It has been shown that IL-8 controls the expression of VEGF in endothelial cells, thereby promoting the up-regulation of VEGF receptors in an autocrine fashion (Martin et al., 2009). This might explain the dual increase of IL-8 and VEGF in GT+FT lesions as well as in GT lesions with concomitant tongue impressions (GT+TI). TI reflects the underlying parafunctional habits that result from pressing the tongue against the teeth. It has been suggested that parafunctional habits are associated with stress (Schiffman et al., 1992), which may increase levels of IL-1β, IL-6, and IL-8, and trigger the inflammation (Shields et al., 2015).

Study IV

Do patients with GT have different microbiota on the tongue in terms of abundance and diversity?

Bacterial abundance is an ecological concept that refers to the relative representation of species in a particular ecosystem (Wikipedia, 2016). It has been estimated that the oral microbiota contains more than 600 species distributed across 13 phyla, in which six represent major phyla that contain 96.0% of all the taxa and include: Firmicutes (37.0%); Bacteroidetes (17.0%); Proteobacteria (17.0%); Actinobacteria (12.0%); Spirochaetes (8.0%); and Fusobacteria (5.0%) (Dewhirst et al., 2010). In our study, the species were identified by their DNA sequences and assigned to their corresponding phyla, and the abundance was compared for each phylum. We show that patients with GT have significantly fewer Fusobacteria. The role of Fusobacteria in the induction of human β-defensin-2, which exerts antimicrobial activity for maintaining a healthy mucosal surface, has been established (Yin and Dale, 2007). Thus, a lower abundance of Fusobacteria among patients with GT may have implications for the development of the GT lesions. In contrast, our results show a significantly greater abundance of Spirochaetes in patients with GT. The role of Spirochaetes in oral infections has been established (Dahle et al., 1993). In contrast to our findings of fewer Fusobacteria and more Spirochaetes in cases of GT, several studies have shown that the populations of Fusobacteria and Spirochaetes increased in parallel in cases of acute stomatitis and acute ulcerative gingivitis (Heylings, 1967; Stephens, 1972).

Microbial diversity was measured at the genus level by counting the total number of the Operational Taxonomic Units (OTUs) in each group. Our results show that microbial diversity is significantly higher in the lesion sites of patients with GT, i.e., more bacterial taxa were identified in the GT-lesion group than in the GT-healthy group. The greater diversity of bacteria in the GT-lesions is probably due to the significantly higher number of sequence reads for Spirochaetes at the phylum level. At the genus level, Leptospira was significantly more common in the GT-lesions than in the GT-healthy and control groups (p<0.05 and p<0.001). Treponemas was also more common in the GT-lesions, albeit non-significantly.
We demonstrate that the bacterial diversity of the GT-healthy group is slightly lower than that of the controls, although not significantly so. Our explanation for this is that the healthy sites of GT display a normal tongue structure, which is suitable for colonization by “commensal” bacteria. However, the nearby lesion sites of GT lack this normal structure, which means that the microbiota is altered and that it becomes difficult to re-establish the normal ecology. It has been shown in several studies that the changes in microbial diversity underlie the dramatic and rapid increases in chronic inflammatory and autoimmune disorders (Feillet and Bach, 2004; Ling et al., 2015; Abrahamsson et al., 2012).

What is the composition of the lingual microbiota in patients with GT?

In this study, we hypothesized that no specific pathogen is associated with GT but rather that a compositional change in the whole lingual microbial community is linked with GT. Figure 6 shows the significant taxa that were identified across all the swap samples at genus level.

![Figure 6. Composition of the lingual microbiota. The significant genera detected across all the groups are listed](image)

Our results show that the number of significant OTUs belonging to the phylum *Firmicutes* was much lower at the GT-lesion sites than in the controls (1 vs. 6). A reduction in the diversity of *Firmicutes* has also been associated with the lesions of oral lichen planus and recurrent aphthous stomatitis (Choi et al., 2016; Hijazi et al., 2015).
In fact, the *Firmicutes* phylum normally predominates the oral cavity, since *Streptococci*, which belong to this phylum, constitute the majority of bacteria resident in the mouth (Kreth *et al.*, 2009). Thus, the reduced diversity of *Firmicutes* in GT should have an impact on lingual microbiota ecology.

We have showed that *Acinetobacter* and *Delftia* (both of which belong to the *Proteobacteria* phylum) are associated with GT, regardless of whether the samples are collected from lesions or from healthy sites. In line with our results, a greater abundance of *Acinetobacter* has been associated with oral lichen planus and recurrent aphthous ulcer (Choi *et al.*, 2016; Kim *et al.*, 2016). In contrast to our findings, *Delftia* has been found to be associated with healthy root surfaces (Chen *et al.*, 2015). However, root surfaces represent a different habitat than mucosal surfaces, so differences in bacterial composition are to be expected.

Our results point to four genera as being associated with the lesion sites of GT: *Microbacterium, Leptospira, Methylotenera*, and *Lactococcus*. *Microbacterium* has been found to be associated with refractory periodontitis (Colombo *et al.*, 1998). To the best of our knowledge, *Leptospira* has not previously been identified in the oral lesions. However, a genus that is closely related to * leptospira* has been recognized in periodontitis (Choi *et al.*, 1994). In our study, *Leptospira* was encountered in 88.5% of the GT-lesion sites. It has been previously demonstrated in an experimental animal model that the human saliva and the oral mucosal barrier provide a natural defense against oral infections by *Leptospira* (Asoh *et al.*, 2014). Therefore, breakdown of the mucosal barrier in GT due to the loss of a normal tongue structure may explain the greater abundance of *Leptospira* in the GT lesions. *Methylotenera* is a newly defined genus (Kalyuzhnaya *et al.*, 2006; Kalyuzhnaya *et al.*, 2012) and data regarding its clinical implications are lacking. *Lactococcus* has been demonstrated to play an indirect role in the prevention of caries by competing with cariogenic bacteria (Tong *et al.*, 2012).

We have demonstrated that two genera were identified in the healthy sites of GT and in the controls: *Mogibacterium* and *Simonsiella*. Interestingly, *Mogibacterium* is found in increased numbers in periodontal pockets and in root canals after chemico-mechanical preparation (Gomes *et al.*, 2015), which indicates an association with more-healthy structures. Similar to our results, *Simonsiella* has been found in 22.0% of healthy oral mucosa tissues, compared to only 8.0% of erosive lichen planus patients. The same study showed that 95.0% of *Simonsiella* colonized the dorsal surface of the tongue (Pankhurst *et al.*, 1988).
The current status and the future direction of studies of the oral microbiome are described in a recent review by Krishnan et al., who summarize the current status as the identification of bacterial composition in order to answer the question "who are there in a community?". Progress is expected to functional studies that explain “what are they doing there?” in the context of host-microbiome relationships (Krishnan et al., 2016).
7. CONCLUDING REMARKS

In summary, this thesis presents new insights into GT, which is a neglected but important oral lesion. We believe that GT represents a reaction pattern of the tongue in which several etiological factors play a role in its pathogenesis. Since we consider GT to be a multifactorial disease, a multidisciplinary approach was adopted to enrich our knowledge and understanding of this disease. Thus, we studied the clinical, immunological, and microbiological aspects for this project. Since GT is regarded as an inflammatory condition, it was not surprising that differences in the levels of salivary cytokines and growth factors were detected between the patients and healthy controls. However, it is interesting to note how the levels of biomarkers vary across the different subgroups of patients with GT. The disturbance of the microbial ecology of the tongue in patients with GT is a novel finding that warrants further investigation. It remains to be discovered whether the altered oral microbiota is a cause or a consequence of the GT.
ACKNOWLEDGEMENTS

All praise to Allah who granted me all the blessings and guidance to proceed successfully. I would like to express my sincere gratitude to all who helped me in the course of this thesis. In particular, I wish to thank:

Mats Jontell, my main supervisor. Words are not enough to express how thankful I am to you for giving me the chance to be a PhD student. From Day 1 and throughout the years, you were supportive and helpful, and always concerned about the health and well-being of your students. I attribute the strengths of this thesis to your encouragement and effort. Both research and clinical work are amazing under your supervision. From the bottom of my heart, THANK YOU.

Hülya Çevik-Aras, my co-supervisor, thank you for introducing me to the research environment and for your continuous advice.

Maria Bankvall, my co-author and friend, for making the space for lots of my papers on your desk and for translating and correcting my Swedish.

Jairo Robledo-Sierra, my co-author and friend, for solving my technical and statistical problems and for sharing the experience of the clinical education.

Fei Sjöberg, my co-author and friend, for introducing me to microbiology and genetics. I definitely enjoyed working with you.

My co-authors, Vegard Garsjö, Ulf Mattsson, Paula Rico, and Ayşegül Işık, for your contributions to the development of this project.

My research family, at the Department of Oral Medicine and Pathology, including Bengt Hasséus, Maria Bankvall, Jairo Robledo-Sierra, Gita Gale, Jenny Öhman, Java Walladbegi, and Jonas Sundberg, for always being their smiling, chatting selves and for helping me overcome both minor and major obstacles in my research.

My laboratory colleagues, Sara Alizadehgharib and Anna-Karin Östberg, for motivating me to keep going when everything went wrong in the lab.

Eva Frantzich, for taking care of all the administrative chores.

Maria Nilsson and Anders Grip, for your IT support.

My clinic family, Kerstin Bäckman, Margareta Fredriksson, Annica Tengborg, and all the staff members at the clinics of oral medicine, it is a great honor for me to be working with you. What I have learnt from you is like a beacon will guide me on my future journey in Oral Medicine.

Salwa and Osama, my parents, for your continuous love and endless support since I was a child. Despite the physical distance, you were always my guides through the phone and screen. This thesis is my reward for the days you are always counting for me to be back home.

Manal, my twin sister and my other half, for your love and support overseas even when my day was your night. You always give me the feeling that you are beside me. Thanks for always revising my papers, revising this thesis and for the beautiful cover design.

Walaa, my little sister, for making me laugh when I call you because nothing is working in my PhD.

Tamer, Ahmad and Rayan, my brothers, for believing that “brotherhood” is a friendship that is not affected by distance.
Acknowledgements

_Siham and Reda_, my mother-in-law and father-in-law, for treating me as your daughter with lots of love and support.

_Dalia_, my sister-in-law and best friend, for being a school friend, family member, and an academic fellow. You are the person with whom I love to share everything about Sweden.

_Khadija, Waad and Heba_, my friends, you are the joy of my life.

_Reda and Omar_, my precious boys, from whom I have spent long hours away in order to get this thesis done. Whenever I see you boys, I recognize that life is full of blessings. Thank you for the happiness and fun you have brought into my life. I Love You!

_Hesham_, my beloved husband, for our journey together. Your endless support throughout my education, especially while preparing this thesis, is priceless. Thank you for always believing in me and encouraging me to believe that it will be done perfectly. Literally, I would not have made it without you. I Love You!

Special thanks to the Ministry of Higher Education in **Saudi Arabia** and to the Cultural Bureau in Berlin, Germany for the scholarship that is the funding source of this project.
REFERENCES


References


References


References


APPENDIX


III. **Dafar A, Bankvall M, Garsjö V, Jontell M, Çevik-Aras H.** Salivary levels of interleukin-8 and growth factors are modulated in patients with geographic tongue. *Submitted for publication*.

IV. **Dafar A, Bankvall M, Çevik-Aras H, Jontell M, Sjöberg F.** Lingual microbiota profiles of patients with geographic tongue. *Submitted for publication*.