CHAPTER 1

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PRIMARY SJÖGREN’S SYNDROME

Primary Sjögren’s syndrome (pSS) is a systemic inflammatory autoimmune disease characterized by symptoms of oral and ocular dryness (1). The sensation of a dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca) are the most frequently reported symptoms and are often the first symptoms experienced by pSS patients. Systemic manifestations occur in approximately 30-40% of pSS patients (Figure 1) (1). The prevalence of pSS is estimated at 0.3 to 1 in 1000 persons. Women are affected nine times more frequent than men (2).

Chronic inflammation of the salivary and lacrimal glands in pSS patients is characterized by periductal lymphocyte infiltration. Another important characteristic of pSS, which can be found in the majority of patients, is the presence of anti-Sjögren’s syndrome antigen A (anti-SSA/Ro) autoantibodies. Currently, the leading classification criteria to include pSS patients in studies is the 2016 American College of Rheumatology / European League Against Rheumatism (ACR/EULAR) Classification criteria (Table 1) (3). In the 2016 ACR/EULAR criteria, salivary gland lymphocyte infiltration and serum anti-Ro/SSA autoantibody presence are the most important criteria to classify a patient with oral and/or ocular dryness as having pSS.

**TABLE 1:** American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjögren’s syndrome (pSS) (3). The classification of pSS applies to any individual who meets the inclusion criteria*, does not have any of the conditions listed as exclusion criteria† and has a score of ≥ 4 when the weights from the five criteria items below are summed.

<table>
<thead>
<tr>
<th>Item</th>
<th>Weight/score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labial salivary gland with focal lymphocytic sialadenitis and focus score of ≥ 1 foci/4mm²</td>
<td>3</td>
</tr>
<tr>
<td>Anti-SSA/Ro-positive</td>
<td>3</td>
</tr>
<tr>
<td>Ocular Staining Score ≥ 5 (or van Bijsterveld score ≥ 4) in at least one eye</td>
<td>1</td>
</tr>
<tr>
<td>Schirmer’s test ≤ 5 mm/5 minutes in at least one eye</td>
<td>1</td>
</tr>
<tr>
<td>Unstimulated whole saliva flow rate ≤ 0.1 mL/min</td>
<td>1</td>
</tr>
</tbody>
</table>

*These inclusion criteria are applicable to any patient with at least one symptom of ocular or oral dryness, defined as a positive response to at least one of the following questions: (1) Have you had daily, persistent, troublesome dry eyes for more than 3 months? (2) Do you have a recurrent sensation of sand or gravel in the eyes? (3) Do you use tear substitutes more than three times a day? (4) Have you had a daily feeling of dry mouth for more than 3 months? (5) Do you frequently drink liquids to aid in swallowing dry food? or in whom there is suspicion of Sjögren’s syndrome (SS) from the European League Against Rheumatism SS Disease Activity Index questionnaire (at least one domain with a positive item).
†Exclusion criteria include prior diagnosis of any of the following conditions, which would exclude diagnosis of SS and participation in SS studies or therapeutic trials because of overlapping clinical features or interference with criteria tests: (1) history of head and neck radiation treatment, (2) active hepatitis C infection (with confirmation by PCR), (3) AIDS, (4) sarcoidosis, (5) amyloidosis, (6) graft-versus-host disease, (7) IgG4-related disease.
‡The histopathologic examination should be performed by a pathologist with expertise in the diagnosis of focal lymphocytic sialadenitis and focus score count.
§Patients who are normally taking anticholinergic drugs should be evaluated for objective signs of salivary hypofunction and ocular dryness after a sufficient interval without these medications in order for these components to be a valid measure of oral and ocular dryness.
ETIOLOGY OF PRIMARY SJÖGREN’S SYNDROME

A combination of genetic and environmental factors contributes to the etiology of pSS. Single nucleotide polymorphisms (SNPs) in human leukocyte antigen (HLA) regions are associated with pSS as well as SNPs in genes that are involved in innate and adaptive immunity (4). Many of the associated genetic variations associated with pSS are not specific for the disease, as significant overlap is observed between pSS patients and patients with systemic lupus erythematosus (SLE) (5). The environmental factors involved in the etiology of pSS are still largely unknown. In this respect, viruses – especially Epstein Barr virus – have been implicated as primary triggers for pSS (6), but the causal role of a viral infection in the etiology of pSS has still not been shown. In contrast to viruses, the role of bacteria in the etiopathogenesis of pSS is scarcely studied up to now and remains largely unknown thus far.

Mounting evidence indicates that the microbial composition in and on the human body – i.e. the human microbiome – plays an important role in human health and disease (7–9). Regulation, training and activation of the immune system is greatly influenced by the human microbiome (10). We hypothesized that a disturbed balance between the human microbiome and the host (i.e., dysbiosis) contributes to the chronic systemic inflammation observed in pSS patients.

THE HUMAN MICROBIOME

A brief history on bacteria and next-generation sequencing

Ever since Antony van Leeuwenhoek discovered his ‘animalcules’ in the 17th century, we know that the human body is inhabited by many microorganisms (11). Van Leeuwenhoek described his discovery as: “the people in the United Netherlands are not as many as the living animals that I carry in my own mouth” (Figure 2). Since then, the microscope became the most important instrument to identify and classify bacteria and other microorganisms. Bacterial culturing, together with microscopic analysis, has long been the only method to identify bacterial species, but the discovery of DNA meant a start of a revolution in microbiology research.
In 1868, the Swiss doctor Friedrich Miescher was the first to describe ‘nuclein’ in the nucleus of white blood cells, which later became known as deoxyribonucleic acid, DNA (12). In 1953, Watson and Crick discovered the double-helix structure of DNA (13) and in 1985 the polymerase chain reaction (PCR) was described for the first time by Saiki et al. (14). PCR is an enzymatic DNA amplification of a specific gene or region of a DNA-strand, and remains one of the most important steps in DNA research to date. Not only human, but also DNA from microorganisms (e.g., bacteria and viruses) can be amplified by PCR.

In the early years of the second millennium, high-throughput sequencing, which is also described as massively parallel sequencing, second-generation sequencing or next-generation sequencing, started to become mainstream in laboratories investigating human and microbial DNA (15). In 2012, the Human Microbiome Project Consortium reported the first large-scale study in which next-generation sequencing (NGS) was used to describe the healthy human microbiome (16).

**Advantages and limitations of 16S ribosomal RNA sequencing**

The main advantage of NGS over conventional culturing techniques or a standard PCR approach, is that NGS can reveal the complete microbial composition in a sample, including microorganisms that have not yet been identified with other techniques (Figure 3). A disadvantage of next-generation sequencing is that the abundance of specific bacteria is
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always relative to that of the total bacterial composition. This limits the ability to gain insight in the relationship between the real bacterial load of a specific bacterial species and the host (17). Until now, 16S ribosomal RNA (16S rRNA) sequencing is worldwide the most used next-generation sequencing method to assess the complete bacterial composition in a sample. With 16S rRNA gene sequencing, one (or several) of the nine hypervariable regions in the 1500 base pair long 16S rRNA gene is/are amplified with universal primers. Downstream software technology allows researchers to identify all the different bacteria present in a sample, based on the genomic information in the sequenced 16S hypervariable region (Figure 3) (21,22). 16S rRNA gene sequencing is relative low-cost – compared to whole genome sequencing – and downstream bioinformatics pipelines are open-source and user friendly. 16S rRNA gene sequencing can reliably identify bacteria to the genus level, but not to the species or strain level. Thus, 16S rRNA gene sequencing provides a more distant view of the microbe-host interaction, compared to whole genome sequencing (WGS), which allows for bacterial species identification (Figure 3). 16S rRNA gene sequencing is useful to assess which bacteria are present (‘who is there?’), but is unable to identify the genetic functions of bacteria (‘what are they doing?’), which is also possible with WGS. Taken the limitations into account, 16S rRNA gene sequencing remains a good method for explorative research on the interaction between the human host and bacteria. When more detailed assessment of the host-microbe interaction is required, WGS, conventional culturing or targeted PCR techniques can be used.

The human microbiome and immunity

The human microbiome consists of all microorganisms (i.e., viruses, phages, bacteria, archaea and fungi) that live in and on the human body. The number of bacteria in the human microbiome was recently estimated to be 38 trillion (3.8*10^{13}), comparable to the number of human cells (30 trillion), and a cumulative weight of 200 grams (23). Furthermore, the number of bacterial genes in the gut microbiome is over 100 times larger than the number of human genes (24).

In a healthy situation, a homeostatic balance develops during life between the host immune system and the microbiota living in and on the human body. A well-balanced host immunity–microbiome relationship is characterized by the induction of protective responses to pathogens and regulatory responses to harmless microbiota. If pSS patients have a specific dysbiosis of the microbiota composition on mucosal surfaces, then this might contribute to the development of the disease. Disturbance of the host-microbiome homeostasis may also be a symptom of underlying disease, however. In pSS patients, dryness of epithelial surfaces (i.e., oral, eye, skin and vagina) is presumed to influence the microbial composition at these sites. A first step towards addressing the role of the human...
The 16S-based approach

Extract DNA

Microbial community sample

The shotgun metagenomic approach

Amplify and sequence 16S rRNA

Group similar sequences into OTUs

Use database to identify OTUs

Community composition: Which organisms are present?

Variant sequences and SNPs

GATTACA
GATTACA
GATTACA
GATTTCA
GATTTCA
GATTTCA

Relative abundance of OTUs in community

OTU phylogeny

Compare sequences to reference genomes

Community function: What can the community do?

Abundance

Functions

Relative abundance of gene pathways in community

Use database to identify sequences

KEGG
SEED
BLAST

NCBI
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**FIGURE 3:** Bioinformatic methods for functional metagenomics. First, community DNA is extracted from a sample, typically uncultured, containing multiple microbial members. The bacterial taxa present in the community are most frequently defined by amplifying the 16S rRNA gene and sequencing it. Highly similar sequences are grouped into Operational Taxonomic Units (OTUs), which can be compared to 16S databases such as SILVA (18), Green Genes (19) and the Human Oral Microbiome Database (HOMD) (20), to identify them as precisely as possible. The community can be described in terms of which OTUs are present, their relative abundance, and/or their phylogenetic relationships. This method was used in the studies described in chapters 3-6. An alternate method of identifying community taxa is to directly metagenomically sequence community DNA (i.e., whole genome sequencing) and compare it to reference genomes or gene catalogs (see shaded area in the figure). This method is more expensive and computationally demanding, but provides improved taxonomic resolution and allows observation of single nucleotide polymorphisms (SNPs) and other variant sequences. This method was not used in this thesis. Figure and text reproduced with permission from: X.C. Morgan and C. Huttenhower, Chapter 12: Human Microbiome Analysis, PLoS computational biology, 2012. 8: e1002808 (21).

The oral microbiome

The oral microbiome has a highly diverse bacterial composition, with over 700 microbial species included in the 16S rRNA gene Human Oral Microbiome reference Database (HOMD) (20). In one individual person, the microbiota composition differs between the buccal mucosa, tongue, dental surfaces, the periodontal sulcus and saliva (25,26). Therefore, it is important to consider where a microbial sample is taken from, when one refers to ‘the oral microbiome’.

Despite the high load and large variety of bacteria, viruses and fungi, the oral mucosa remains in a relative state of health, due to its pro-tolerogenic nature and the antimicrobial defense mechanisms (27). Interaction between oral microbiota and immune cells in the oral mucosa mainly take place under the influence of dendritic cells in the mucosa and lymphoid structures (tonsils) in the oropharyngeal region (28).

Thus far, three relatively small studies (≤10 pSS patients) have assessed the oral microbiome in patients with pSS, using different oral sampling methods (saliva, buccal mucosa and tongue) (29–31). These preliminary studies showed that oral microbiota compositions differ between pSS patients and healthy controls (Table 2), but were unable to elucidate whether the differences in oral microbiota composition are specific for pSS and whether oral dryness itself already leads to these differences.
### TABLE 2: Previous studies on the oral and gut microbiome in pSS patients versus healthy controls (HCs)*

<table>
<thead>
<tr>
<th>Study</th>
<th>Study population</th>
<th>Sample and 16S region</th>
<th>Alpha-diversity</th>
<th>Beta-diversity</th>
<th>higher in (p)SS compared to HCs</th>
<th>lower in (p) SS compared to HCs</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>De Paiva et al. 2016 (30)</td>
<td>10 SS patients 11 HCs</td>
<td>Tongue swab \ V1-V3</td>
<td>Lower Shannon diversity in SS</td>
<td>PCoA: significant difference between pSS and HCs</td>
<td>Streptococcus</td>
<td>Leptotrichia, Fusobacterium, Bergeyella, Peptostreptococcus, Butyrivibrio</td>
<td>No correction for multiple testing.</td>
</tr>
<tr>
<td>Li et al. 2016 (31)</td>
<td>10 pSS patients 10 HCs</td>
<td>Buccal mucosa \ V1-V3</td>
<td>Lower number of genera per sample in SS</td>
<td>Redundancy analysis: Haemophilus, Gemella, Neisseria more closely related to HCs. Streptococcus, Lactobacillus more closely related to SS.</td>
<td>Leucobacter, Delfia, Pseudochrobactrum, Ralstonia, Mitsuaria</td>
<td>Proteobacteria Haemophilus, Neisseria, Comamonas, Granulicatella, Limnobias</td>
<td>No correction for multiple testing.</td>
</tr>
<tr>
<td>Siddiqui et al. 2016 (29)</td>
<td>9 pSS patients 9 HCs</td>
<td>Whole saliva \ V1-V2</td>
<td>Lower observed OTUs and lower Shannon diversity in pSS</td>
<td>PCoA shows 2 separate groups based on disease status using PC2 and PC3.</td>
<td>Firmicutes, Streptococcus, Veillonella</td>
<td>Synergistetes, Spirochaetes, Bacteroidetes (NS), Proteobacteria (NS), Treponema, Morvella, Fretibacterium, Porphyromonas, Tannerella, Catonella, Haemophilus (NS) and Neisseria (NS)</td>
<td>All patients and HCs had a normal salivation rate of &gt;0.1mL/min. FDR correction was applied.</td>
</tr>
<tr>
<td>De Paiva et al. 2016 (30)</td>
<td>10 pSS patients Unknown number of HCs from HMP</td>
<td>Fecal \ V4</td>
<td>Not assessed</td>
<td>Not assessed</td>
<td>Pseudobutyrivibrio, Escherichia/Shigella, Blautia, Streptococcus</td>
<td>Bacteroides, Parabacteroidetes, Faecalibacterium, Prevotella</td>
<td>Comparison of sequence data between different cohorts can induce false-positive results based on technological differences</td>
</tr>
<tr>
<td>Mandl et al. 2017 (34)</td>
<td>44 pSS patients 35 age+sex matched controls</td>
<td>Fecal 16S rRNA gene based dysbiosis test (54 16S gene probes)</td>
<td>Not assessed</td>
<td>More pSS patients had a high Dysbiosis score than controls (21% vs 3%)</td>
<td>none</td>
<td>Bifidobacterium, Alistipes, Faecalibacterium (NS)</td>
<td>Only 15 individual taxa were compared between pSS and HCs</td>
</tr>
</tbody>
</table>

*Bacterial taxa higher or lower in pSS compared to HCs were all significant, according to the statistics used in the study, except when indicated as NS (not significant). FDR = false discovery rate. HMP = Human Microbiome Project. Genera are indicated in italic.
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The gut microbiome

The gut is home to most bacteria in the human microbiome and the composition is more diverse than in any other location in the body (23). The concentration of bacteria in feces is, together with the bacterial concentration in dental plaque, the highest in any microbial sample from the human body ($10^{11}$ bacteria/gram) (32). Fecal sampling is the most widely used method to determine the gut microbiota composition and provides a valuable method to assess the connection between microbiota and gut mucosal immunity. The mucosal immune system in the gut contains 80% of all human immunoglobulin (Ig) producing cells (i.e., plasma cells and plasmablasts) (33). Furthermore, many different types of T cells are also abundantly present in the gut mucosa, which play a fundamental role in regulating immunologic responses to gut microbiota (10). Therefore, the gut microbiome is highly relevant to study in patients with chronic inflammatory autoimmune diseases, especially those in which B cells, T cells and autoantibodies play a fundamental role in the pathogenesis, as is seen in pSS.

Until now, two relatively small studies have assessed fecal samples from pSS patients, suggesting an association between gut microbiota dysbiosis and pSS (Table 2) (30,34). However, several questions remain poorly answered or unanswered. 1) Does the gut microbiome from pSS patients differ from that of the general population? 2) If so, what are the characteristics of the gut microbiome in pSS patients? 3) Is the gut microbiota composition in pSS patients specific for the disease?

The vaginal microbiome

The vaginal microbiome is unique compared to the oral and gut microbiome, because *Lactobacillus* species largely dominate (often >90% of the relative abundance) the healthy vaginal microbiome (35,36). Consequently, a healthy vaginal microbiome is mostly associated with low microbial diversity, while high diversity is frequently observed in women diagnosed with bacterial vaginosis (35,36). Vaginal dryness is frequently reported by women with pSS (37,38), but it is unknown whether this affects the vaginal microbiome. If the vaginal microbiome in women with pSS is altered more towards dysbiosis, then this may result in increased cervicovaginal inflammation and mucosal barrier changes (39). Inflammatory infiltrates have already been observed in vulvar biopsies from women with pSS (40), but, whether the vaginal microbiome is involved in the lymphocyte infiltration in the vaginal epithelium is unknown (40). To date, there are no studies available that have investigated the vaginal microbiome in women with pSS.
AIM AND OUTLINE OF THIS THESIS

Aim

The overall aim of the research described in this PhD thesis was to assess whether pSS is associated with a disease-specific dysbiosis of the microbiota composition in the oral cavity, gut and vagina. Therefore, the microbiota composition in oral, fecal and vaginal samples from pSS patients was studied in comparison with that of healthy and/or population controls and disease controls using 16S rRNA gene sequencing. This technique allows to compare the diversity, the overall bacterial composition and the relative abundance of individual bacterial taxa between pSS patients and controls.

Outline

In chapter 2, the currently available evidence of a connection between the human microbiome and three of the most common systemic inflammatory autoimmune diseases (i.e., rheumatoid arthritis, systemic lupus erythematosus (SLE) and pSS) is reviewed. Furthermore, we explain how the human microbiome is assessed with next-generation sequencing and describe the interaction of mucosal and systemic immunity with microbiota.

In the studies described in chapters 3 and 4, we aimed to find an answer to the question whether patients with pSS have a disease-specific composition of the oral microbiome. Therefore, we did not only include pSS patients and healthy controls in the analysis, but also disease-controls, comprising of individuals with oral dryness complaints not fulfilling the classification criteria for pSS (i.e., non-SS sicca patients). In the study described in chapter 3, the overall oral microbiota composition – measured in oral washings – of pSS patients was analyzed in pSS patients, healthy controls and non-SS sicca patients. In chapter 4, a study is described in which the microbiota composition of the buccal mucosa is compared between pSS patients, non-SS sicca patients, healthy controls and population controls who participated in the LifeLines DEEP study (41,42). In both studies, richness, diversity and overall composition of the microbiome, as well as the relative abundance of individual bacterial taxa were used as outcome measures. The effect of salivary secretion rate was tested on the complete microbiota composition in a sample and on the relative abundance of individual bacterial taxa.

The main goal of study described in chapter 5 was to assess whether pSS patients have a specific dysbiosis of the gut microbiome in comparison with a large group of general population controls who participated in LifeLines DEEP (42,43) and with disease controls (i.e., SLE patients). We used fecal samples to compare the richness, diversity, overall microbiota composition and the relative abundance of individual bacterial taxa in the
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gut microbiome between the three study groups. Furthermore, we compared the oral microbiome, determined in oral washings and buccal swabs, from the same pSS and SLE patients. Finally, we assessed the connection between the oral and gut microbiome in these diseased patient groups.

The study described in chapter 6 was performed in order to assess whether premenopausal women with pSS-associated vaginal dryness show dysbiosis of the vaginal microbiome, in comparison with premenopausal control women. Endocervical swabs and cervicovaginal lavage samples were obtained from women and analyzed with 16S rRNA sequencing. Clustering analyses were performed and vaginal bacterial community state types were compared between the two groups.

In chapter 7, the results of our studies are discussed in further detail. We provide future perspectives on how future studies can further elucidate the cause and effect relationship between oral/gut microbiota and pSS. Furthermore, we discuss possible interventions that may positively influence the oral and gut microbiome in pSS patients.
REFERENCES

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