The microbiome in primary Sjögren's syndrome
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CHAPTER 2

The microbiome–systemic diseases connection

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Chapter 2

ABSTRACT

The human microbiome consists of all microorganisms occupying the skin, mucous membranes and intestinal tract of the human body. The contact of the mucosal immune system with the human microbiome is a balanced interplay between defense mechanisms of the immune system and symbiotic or pathogenic microbial factors, such as microbial antigens and metabolites. In systemic autoimmune diseases (SADs) like rheumatoid arthritis, systemic lupus erythematosus and Sjögren’s syndrome, the immune system is deranged to a chronic inflammatory state and autoantibodies are an important hallmark. Specific bacteria and/or a dysbiosis in the human microbiome can lead to local mucosal inflammation and increased intestinal permeability. Proinflammatory lymphocytes and cytokines can spread to the systemic circulation and increase the risk of inflammation at distant anatomical sites, such as the joints or salivary glands. Increased intestinal permeability increases antigen exposure and the risk of autoantibody production. If the human microbiome indeed plays such a critical role in SADs, this finding holds a great promise for new therapeutic strategies, such as diet interventions and pro- and prebiotics. This review provides a background on the human microbiome and mucosal immunity in the gut and oral cavity, and gives a summary of the current knowledge on the microbiome SADs connection.
INTRODUCTION

Despite the enormous effort from investigators over the world, the etiopathogenesis of systemic autoimmune diseases (SADs) like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren’s syndrome (SS), systemic sclerosis and vasculitis is only partially understood. These SADs have a multifactorial etiopathogenesis, meaning that a genetic background, environmental factors, hormones and a deranged immune system are all involved to a more or lesser extent.

The genetic contribution to RA and SLE has been well studied, but is yet understudied in SS (Lessard et al, 2012). Concordance rates for RA and SLE in monozygotic twins are 15% and 24%, respectively (Deapen et al, 1992; Silman et al, 1993). Thus, siblings with identical genomes often do not share a systemic disease phenotype. However, the heritability - which estimates the extent to which variation in liability to disease in a population can be explained by genetic variation - is estimated to be 60% and 44% for RA and SLE, respectively (Kuo et al, 2015; MacGregor et al, 2000). No data on twin concordance or heritability in SS is yet available (Bogdanos et al, 2012; Lessard et al, 2012).

Genome-wide association studies (GWAS) have revealed that single nucleotide polymorphisms (SNPs) in the human leukocyte antigen (HLA) gene are the major genetic risk factor to develop a SAD. SNPs in the HLA gene locus account for maximum odds ratios (ORs) of 3.7 in RA, 2.9 in SLE and 3.5 in SS (Castaño-Rodríguez et al, 2008; Lessard et al, 2013; Raychaudhuri et al, 2012), but these ORs are low compared to other autoimmune diseases like ankylosing spondylitis and type 1 diabetes (T1D) with HLA-related ORs of 41 and 11, respectively (Lin et al, 2011; Noble and Erlich 2012). Many non-HLA encoding genes related to suspected pathogenic pathways are associated with RA, SLE and SS, but these ORs are seldom higher than 1.5 (Harley et al, 2008; Lessard et al, 2013; Okada et al, 2014). To summarize, although variations in the human genome explain only a small part of the aetiology of SADs, a relatively strong heritability and familial aggregation of SADs is noted (Cárdenas-Roldán et al, 2013). This apparent discrepancy in the role of genetics in the aetiology of SADs might be explained by the fact that not only the human genome is inherited, but also microorganisms that colonize the human body (Frankenfeld et al, 2004; Goodrich et al, 2014; Li et al, 2007).

Environmental factors are considered a key factor in the development of systemic diseases (Wahren-Herlenius and Dörner, 2013). Especially (infections with) microorganisms are thought to play a causative role in the initiation of RA, SLE and SS, although mechanisms are
poorly understood. Examples of microorganisms that are associated with systemic diseases are the periodontal pathogen *Porphyromonas gingivalis* with RA and Epstein-Barr virus (EBV) with SLE and SS (Croia *et al*, 2014; Hanlon *et al*, 2014; Quirke *et al*, 2014).

An important argument of viral involvement in the etiopathogenesis is the demonstration of an increased production of type 1 interferon (type 1 IFN) in patients with RA, SLE, SS and other SADs (Gottenberg *et al*, 2006; Higgs *et al*, 2011). Type 1 IFN is upregulated upon cellular encounter with a virus and induces a cell-intrinsic antimicrobial state of infected and neighboring cells, limiting the spread of viral pathogens. Furthermore type 1 IFN promotes antigen exposure, natural-killer cell function and activates adaptive immunity to develop high-affinity antigen-specific T- and B-cells (Ivashkiv and Donlin 2014). Chronic type 1 IFN production is also associated with the development of autoreactivity, ultimately leading to autoimmunity (Ivashkiv and Donlin, 2014). Although microorganisms are suspected to play an initiating role in SADs, most auto-antibodies present in the serum of SS, SLE and RA are yet not directly related to associated microbial infections. An exception is the anti-citrullinated protein antibody (ACPA) in RA patients with periodontal disease and *P. gingivalis* colonization (see section on ‘The microbiome RA connection’) (Montgomery *et al*, 2015).

The knowledge of the role of microorganism in the development of SADs is still inconclusive. We propose three possible explanations for this gap in understanding the role of microorganisms in systemic disease development. First, investigators might have overlooked possible pathogenic bacteria or viruses because they were limited to culture-dependent and targeted DNA detection of microorganisms, revealing only one or several microorganisms. Currently less than half of the bacteria present in the oral cavity and only 20% of bacteria in the gut are cultivated, but techniques aimed to cultivate ‘unculturable’ bacteria are still developing (Dewhirst *et al*, 2010; Eckburg *et al*, 2005; Vartoukian *et al*, 2016). Secondly, investigators have possibly searched in the wrong anatomic location for pathogenic microorganisms causing an autoimmune response. Although the human gut harbors the majority of microorganisms present in our body, investigators have mainly focused on detecting microorganisms and their associated antibodies in the blood and the affected organ or tissue (Croia *et al*, 2014; Gill *et al*, 2006; Hanlon *et al*, 2014; Martinez-Martinez *et al*, 2009). Finally, the microorganisms found to be associated with systemic diseases may very well be the ‘second hit’ in patients already predisposed to autoimmunity because of a chronic pro-inflammatory state of the immune system. For example, Epstein-Barr virus (EBV) infection is the microorganism strongest related to SS and SLE (Croia *et al*, 2014; Hanlon *et al*, 2014). However, 95% of the general adult population has been infected with EBV during life (Luzuriaga and Sullivan, 2011). An underlying - not clinically evident - chronic pro-inflammatory state of the immune system (‘first hit’) might be present in future
SLE or SS patients before they are infected with EBV (‘second hit’). This pro-inflammatory state causes an exaggerated and self-perpetuating immune response to a common viral infection such as EBV.

Next-generation sequencing (NGS) is a great tool to explore the role of microorganisms in the development of SADs, because with NGS the complete composition and functions of a microbial community can be defined. First, NGS can detect previously unknown and uncultured microorganisms. Second, NGS can be applied to many human microbial habitats including the oral cavity or gut. Third, NGS has revealed that the gut microbial composition affects local and systemic immunity (Hooper et al., 2012). In this review we summarize the current general knowledge of the human microbiome in relation to the development and pathogenesis of three SADs: RA, SLE and SS.

Defining the human microbiome

In scientific literature the term microbiome is approached from two directions. From a biologist point of view the human microbiome is defined as “a characteristic microbial community occupying a reasonably well defined habitat which has distinct physio-chemical properties”. This definition not only refers to the microorganisms involved, but also encompasses their “theatre of activity” (Whipps et al., 1988), and emphasizes on –biome (as in community). As research on microorganisms has partly moved from biology towards genetics, the term microbiome is also defined from a genetic point of view with the emphasis on –ome (as in ‘–omics’ research, i.e. genomics). Herewith the human microbiome is defined as “the collective of genomes of the microbes that live with us” or as a “second genome of the host”. The microorganisms themselves are defined as ‘microbiota’ (Turnbaugh et al., 2007). In this review we will use the latter mentioned definitions for microbiome and microbiota. The term metagenome will be used to refer to the collective set of all genomes of a microbial community (Petrosino et al., 2009). The human microbiome includes the collective of genomes from viruses, bacteria, archaea and fungi, but the focus of this review is on the bacterial composition in the gut and oral cavity, referred to as the gut and oral microbiome. The role of viruses in systemic diseases is extensively reviewed by others (Ball et al., 2015; Hanlon et al., 2014; Triantafyllopoulou and Moutsopoulos, 2007) and will only briefly be discussed here.

Next-generation sequencing and ‘meta-omics’

Sampling a complete microbial community has become possible with the culture independent method NGS. NGS is a DNA-sequencing technique which allows massively parallel sequencing, during which millions of fragments of DNA from a single sample are sequenced in unison (Grada and Weinbrecht, 2013). NGS facilitates high-throughput
sequencing, which allows an entire microbial community to be sequenced, herewith providing much more information than culture or targeted DNA studies. With this greatly increased amount of microbial data, the range of possible statistical calculations in microbial research has also increased. This has led to new associations between the microbial composition and the health status of the human host (Scher et al, 2013; Turnbaugh et al, 2009).

In microbiome research the two major NGS approaches are 16S ribosomal RNA (rRNA) gene sequencing and metagenomic shotgun sequencing. Both methodologies have been thoroughly reviewed by Zarco et al, (Zarco et al, 2012) and will be summarized here briefly. The 16S rRNA gene approach is used to identify and classify bacteria that are present in a community based on sequence reads of variable regions within the 16S rRNA gene (Yarza et al, 2014). With metagenomics shotgun sequencing the functional characteristics ('what can the community do?') is studied (Gill et al, 2006; Zhang et al, 2015). Metagenomic shotgun sequencing is more complex, time-consuming and requires more computational power than 16S rRNA sequencing especially in processing the massive amount of reads to useful information about putative functional pathways (Bikel et al, 2015; Thomas et al, 2012). Metagenomics gives an answer to the potential function of a microbial population, but has a limited role in revealing the microbial activity measured by gene expression (Bikel et al, 2015). The whole process from DNA, to messenger RNA (mRNA), to proteins and finally metabolites can be studied with a variety of ‘omics’ techniques. Metatranscriptomic shotgun sequencing, or in short RNAseq, answers the question ‘which metabolic pathways are currently active in a certain microbial population? With RNAseq all RNA present in a sample is sequenced and the mRNA reads are analysed. For example, comparing the relative abundance of mRNA reads of a certain gene or pathway with the relative abundance of the equivalent DNA gives insight in the relation of the functional activity to the functional potential (Franzosa et al, 2015a).

Sequencing microbial DNA gives insight in the functional potential of a microbial community, but measuring protein abundance (metaproteomics) provides a more direct measure of the functional activity of a microbial community. Proteomic methods rely on mass spectrometry-based shotgun quantification of peptide mass and abundance (Franzosa et al, 2015a). Finally, the study of metabolomics aims to identify and quantify all the small-molecule, microbial-produced metabolites in order to unravel the dynamic nature of the metabolic function of a microbial community and understand how it influences its human host (Cénit et al, 2014).

In the future, multiple ‘omics’ approaches (multi-omics) will be integrated to take the next step forward in understanding the biology of microbial communities and a better
understanding of the complex mechanisms of host-microbiome interactions (Franzosa et al, 2015a). The multi-omic approach has a great potential in unravelling the microbiome-systemic disease connection, but considerable work needs to be done, especially in the investigative tools and integrate the massive amount of data in bioinformatic pipelines.

The human superorganism

Ever since Antony van Leeuwenhoek discovered his “animalcules” in the 17th century, we know that the human body has always been inhabited by many different microorganisms. In one of his letters van Leeuwenhoek wrote: “…the people living in our United Netherlands are not as many as the living animals that I carry in my own mouth…”. Taking a step of 400 years to the 21st century, we now know that each human being is colonized with trillions of bacteria and that the human body can therefore be addressed as a ‘superorganism’ (Gill et al, 2006). A superorganism is an organism made out of many smaller organisms, acting in concert to produce phenomena governed by the collective.

Each human being is colonized with roughly the same amount of bacteria \((4 \times 10^{13})\) as human cells \((3 \times 10^{13})\) (Sender et al, 2016). The collective of genes in these microorganisms outnumbers the human genome by a factor of more than 100 (Qin et al, 2010). The potential metabolic functions of all these bacterial genes also exceed that of the human body (Maccaferri et al, 2011). Thus, humans can be described as superorganisms whose metabolism is a concert of microbial and human instruments.

Each human being is born almost sterile, but directly after birth the human body becomes colonized with microorganisms from its environment. The complex and critical assembly of the host with its microorganisms starts at birth and takes several years to form a stable composition (Koenig et al, 2011; Yatsunenko et al, 2012). Delivery mode (vaginal birth versus Caesarean section), breast feeding, diet, use of antibiotics, use of proton pump inhibitors, home country and the host genome are all known to influence the composition of the bacterial community in the gut (David et al, 2014; Dominguez-Bello et al, 2010; Goodrich et al, 2014; Imhann et al, 2015; Koenig et al, 2011; Yatsunenko et al, 2012; Zaura et al, 2015). Thus, each adult ‘human superorganism’ has evolved through a series of events and the individual composition of this superorganism appears to be so unique that individuals can be distinguished based on human-associated microbial communities (Franzosa et al, 2015b).

The human microbiome in healthy people – is there a ‘core’ human microbiome?

In 2007, the Human Microbiome Project (HMP) was announced as a logical conceptual and experimental extension of the Human Genome Project (Turnbaugh et al, 2007). One of the
main goals of the project was to investigate the concept of a ‘core’ human microbiome, which is defined as the set of microbial genes present in a given habitat in all or the vast majority of humans (Turnbaugh et al, 2007). Shortly after the start of the HMP in 2008 this concept was largely discarded, because the variability of the human microbiome between individuals appears to be very high (Hamady and Knight, 2009; Huttenhower et al, 2012). As a consequence of the enormous variability in the human microbiome, it is very difficult (or even impossible) to use the presence or abundance of specific microorganisms as biomarkers for disease. When looking at higher-order taxonomic levels (i.e., the genus or phylum level) human microbiome communities begin to resemble one another more, although variation in the relative abundance in the shared genera or phyla is still large (Hamady and Knight, 2009; Zaura et al, 2009; Zhang et al, 2015a). Although the concept of a core human microbiome, defined by a set of abundant microorganisms, is largely discarded, it appears that a core microbiome does exist in the gastrointestinal tract (gut) microbiome at the level of shared genes, especially those involved in metabolic functions (Turnbaugh et al, 2009; Zhang et al, 2015a). Thus, although the bacterial composition shows a large variability between individuals, the metabolic functions executed by these microorganisms are more similar in a healthy population (Lozupone et al, 2012).

**Observational and experimental study designs in microbiome research**

Several studies have demonstrated associations of the microbiome with RA, SLE, SS and systemic sclerosis in humans and in mice (Arron et al, 2014; Hevia et al, 2014; Scher et al, 2013; Szymula et al, 2014). Up to now, human studies investigating the microbiome-systemic disease connection are observational case-control studies in which the human microbiome (or metagenome or metabolome) of mainly the gut, oral cavity and/or skin is compared between patients with systemic diseases and controls (Arron et al, 2014; Hevia et al, 2014; Scher et al, 2013; Zhang et al, 2015b). Because of the observational design with single time point measurements, these studies are unable to answer the question whether changes in the microbiome are a cause or effect of the disease. Furthermore, because of the exploratory nature and relatively low number of subjects in human microbiome studies (usually dozen to a hundred) compared to human genome studies (usually thousands), there is a considerable risk of finding false positive associations.

In GWAS studies, a genetic difference found between two populations needs to exceed the threshold of $P = 5 \times 10^{-8}$ to be considered significant (Lessard et al, 2013). Data in microbiome studies differs greatly from genome studies and specific biostatistic pipelines (multiple statistical analyses performed successively) have been developed to find associations between microbiome data and meta-data, such as disease parameters (Morgan et al, 2012). These biostatistic pipelines often include a calculation to correct for the false discovery rate.
(Benjamini and Hochberg method) or for multiple testing (Bonferroni correction) (Morgan et al, 2012; Scher et al, 2013). Applying these methods aids in finding significant associations in the immense amount of microbial sequence data and diseases or clinical metadata.

To investigate the effect of microorganisms on the initiation of systemic diseases animal studies are very helpful. In these studies germ-free (GF), specific pathogen free (SPF) and gnotobiotic animals (usually mice) are compared with mice grown under conventional conditions. GF mice are born and raised in sterile conditions and SPF mice are mice that are free of specific pathogens (and commensals) through the administration of antibiotics. Gnotobiotic (Greek gnōstos = ‘known’) mice are GF mice that are exposed to one or several known microorganisms at a certain point in life. For example, autoimmune arthritis was strongly attenuated in a K/BxN mouse model under GF conditions and the introduction of segmented filamentous bacteria (SFB) into GF animals re-established arthritis rapidly (Wu et al, 2010).

**Mucosal immunity in the gut**

The microbiome-host immunity connection involves the bidirectional relationship between microorganisms and the host innate and adaptive immune system. The microbiome-host immunity connection is mainly investigated in the gut, both in humans and mice (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Vossenkämper et al, 2013; Wen et al, 2008). The high density of bacteria (≥10^{11}/cm^{3} intestinal content) and the large surface area of the gut (30-40m^{2}) emphasize the major exposure of the gut epithelium to bacteria and the potential effect of the gut microbiome on host immunity (Helander and Fändriks, 2014).

The mucus layer residing on the epithelial lining of the intestinal tract forms a physical barrier which minimizes direct contact between bacteria and epithelial cells (van der Waaij et al, 2005). A firmly adherent (inner) mucus layer is attached to the intestinal mucosa and a more loose (outer) mucus layer covers the adherent layer and has direct contact with the luminal contents of the gut (Atuma et al, 2001). The mucus layer contains a large amount of secretory immunoglobulin A (SIgA) which blocks pathogens from binding to epithelial cells and traps bacteria in the mucus layer (Mantis et al, 2011). Physical clearance of entrapped bacteria is facilitated by the peristaltic movement of the bowel. Bacterial contact with the intestinal epithelium is also restricted by the antibacterial lectin RegIIIγ, which limits bacterial penetration of the mucus layer in the small intestine (Vaishnava et al, 2011). Antimicrobial peptides (AMPs), such as α-defensin and the human cathelicidin LL37, are produced by Paneth cells in the small intestine (Mukherjee and Hooper, 2014). Via granule exocytosis AMPs are brought into the intestinal lumen, where they can kill bacteria through membrane disruption (Mukherjee and Hooper, 2014; Schauber et al, 2003). The epithelium itself is also a central component of the intestinal immune system. It serves not only as a physical barrier,
but also shares immunological functions, by expressing pattern recognition receptors (PRRs) that recognize microbial-associated molecular patterns (MAMPs, also named pathogen-associated molecular patterns (PAMPs)). When a MAMP binds to a PRR (such as a Toll-like receptor, TLR) an intracellular signaling cascade in the epithelial cell activates the cell to stimulate the transcription of antibacterial proteins, pro-inflammatory cytokines and chemokines (Cerf-Bensussan and Gaboriau-Routhiau, 2010). Microfold cells (M cells) are specialized cells located between enterocytes and in close proximity of mucosa-associated lymphoid tissue (MALT, in the gut also called, gut-associated lymphoid tissue, GALT) beneath the epithelial layer. M cells are highly specialized for the phagocytosis, receptor mediated endocytosis and transcytosis of gut lumen antigens and microorganisms across the intestinal epithelium (Mabbott et al, 2013). Herewith luminal antigens are transported to the organized lymphoid tissue of the MALT located directly beneath the gut epithelium. Therefore, M cells fulfill an important immunosurveillance post in the intestinal epithelium (Mabbott et al, 2013). Intraepithelial lymphocytes (IELs) are another immune component of the intestinal epithelium. IELs are a heterogeneous group of antigen-experienced T-cells that have selectively migrated to the intestinal epithelium (Cheroutre et al, 2011). They comprise both thymus-induced, as well as peripheral-induced T-cells, many of them expressing the γδ T-cell receptor (up to 60%). IELs are located in the epithelial layer, in direct contact with enterocytes, which is mediated by CD103 on the surface of IELs. IELs are thus in close proximity of intestinal microorganisms, thereby fulfilling their role in the front line of immune defense against invading pathogens. IELs protect and restore the integrity of the epithelium and maintain local immune quiescence by secreting a wide range of cytokines. For example, transforming growth factor beta (TGFβ) has a role in protecting the integrity of the epithelium and tumor necrosis factor (TNF) and IFNγ function as protective cytokines (Cheroutre et al, 2011).

Along the intestinal tract, dendritic cells (DCs) reside in the lamina propria (LP), in MALT and in the mesenteric lymph nodes (MLNs) (Coombes and Powrie 2008). DCs in the LP and MALT have cellular extensions passing the epithelial cells into the lumen of the gut, where they can phagocytose bacteria, viruses and food peptides. MALT associated DCs present antigen to T-cells in the MALT and in MLNs and DCs from the LP migrate directly to the MLNs (Iwasaki, 2007; Liu and MacPherson, 1995). B-cells that are activated in the MALT or MLNs enter the circulation and home to the LP where they become IgA secreting plasma cells (Hooper et al, 2012). The human gut contains almost 80% of all plasma cells and produces the largest amount of IgA in the body (Brandtzæg and Johansen 2005). The vast majority (80-90%) of the immunoglobulins produced by plasma cells in the LP is IgA (Brandtzæg et al, 1999). The polymeric immunoglobulin receptor located on the basolateral surface of epithelial cells mediates transcytosis of locally produced IgA into the gut lumen where it is secreted as SlgA (Kaetzel, 2014). Besides the entrapment of bacteria in the mucus, SlgA
reduces bacterial virulence factors (Forbes et al., 2008 and 2011), prevents toxin attachment to epithelial receptors (Dallas and Rolfe, 1998) and aids in the phagocytosis of antigen by M cells to Peyer’s patches (Rey et al., 2004). Vice versa, gut microbiota and butyrate can modulate SlgA transport by influencing the expression of the polymeric immunoglobulin receptor (Bruno et al., 2010; Kvale and Brandtzaeg, 1995). How the gut microbiome affects local and systemic immunity is currently a major topic of research and is discussed in more detail in the section on the microbiome systemic immunity connection below.

Mucosal immunity in the oral cavity

The oral mucosa is also exposed to a very high diversity of microorganisms (=700 different bacterial species) and food peptides (Dewhirst et al., 2010). Despite this high antigen load, the oral mucosa remains in a relative state of health due to the pro-tolerogenic nature of the mucosal immune system and its antimicrobial defense mechanisms (Dewhirst et al., 2010, Hovav, 2014).

The epithelium of the oral cavity is a stratified layer of non-keratinized (except for the keratinized gingiva) squamous cells and has a much less absorptive and permeable nature than the gut epithelium which is composed of a single cell layer. Cell shedding from the surface layer of the oral epithelium minimizes colonization of bacteria (Hovav, 2014).

Saliva is one of the major factors responsible for microbial homeostasis in the oral cavity which is illustrated by the fact that reduced salivary secretion in patients with SS leads to an increase in microbial related diseases, such as dental caries and oral Candidiasis (Arendorf and Walker, 1980; Christensen et al., 2001). Homeostasis of the oral microbiome is maintained by saliva through modulation of bacterial attachment, modulation of bacterial growth and inhibition of bacterial growth (van ’t Hof et al., 2014). Salivary Agglutinin and SlgA both bind to bacteria and prevent bacterial attachment to the oral mucosa. Bacterial modulation involves mucin 5B (MUC5B), the largest molecule in saliva. MUC5B is a peptide that is heavily glycosylated with an extremely heterogeneous set of oligosaccharides. MUC5B is the major carbohydrate source for microorganisms when external supply is absent (van ’t Hof et al., 2014). Inhibition of bacterial growth is controlled by salivary antimicrobial components, such as lysozyme, histatins, β-defensins and the human cathelicidin LL37. These antimicrobial components exert their antibacterial function by affecting cell wall integrity and pore formation and indirectly by immune signaling (van ’t Hof et al., 2014). Considering the many functions of saliva in the homeostasis of the oral microbiome, it is not surprising that hyposalivation may disturb this homeostasis.

All of the above mentioned antimicrobial components of saliva are also present in the gingival crevicular fluid (GCF) (Fábián et al., 2012). The composition of GCF is very similar to
serum transudate and therefore contains proteins that are also present in blood such as the complement system and IgG (Fábián et al, 2012). Leukocytes are also present in the GCF, with the majority being neutrophils (>90%) and the remaining fraction lymphocytes and monocytes (Delima and Van Dyke, 2003).

Dendritic cells present in the oral mucosa are mainly Langerhans cells and are capable of antigen capture, migration to lymph nodes (LNs) and antigen presentation to T-cells (Hovav, 2014). However, because of the non-adsorptive nature of the oral epithelium and because the extensions of these DCs do not protrude into the oral cavity, the mucosa must be penetrated or damaged first, before antigen capture by DCs can take place at these sites. The sublingual mucosa is an exception to this, because antigen is easily adsorbed and captured by DCs (Kweon, 2011). After capture and antigen presentation in the peripheral LNs, the DCs of the sublingual mucosa are capable of initiating broad systemic and antigen specific protective immune responses (Kweon, 2011). Currently, this route is used in sublingual immunotherapy for type 1 respiratory allergies and may be used as a route for vaccinations in the future (Kweon, 2011; Moingeon, 2013). Furthermore, immunisation with an orally administered cholera vaccine has been shown to induce strong intestinal antibody responses with local immunological memory (Quiding, 1991).

The palatine tonsils are important lymphoepithelial organs in the oral cavity, located strategically at the surface of the digestive and respiratory tract (Nave et al, 2001). The adenoids, palatine tonsils and other smaller lymphoid structures of Waldeyer’s pharyngeal ring are collectively called the nasopharynx-associated lymphoid tissue (NALT) (Brandtzaeg et al, 2008). M cells are also present in the epithelium of the palatine tonsils (as in GALT) (Nave et al, 2001). T-cells recognize presented antigen in the palatine tonsils (and other lymphoid tissues of NALT) and can activate B-cells to become plasma cells. Plasma cells originating from both the NALT and the GALT can home to the salivary glands to become SIgA secreting plasma cells (Brandtzaeg 2013).

Sublingual antigen exposure has been shown to induce tolerance to antigens in respiratory allergies and strong intestinal antibody responses (Moingeon, 2013; Quiding, 1991). In conclusion, microbial homeostasis in the oral cavity is an effect of the combined function of the oral epithelium, saliva, GCF and adaptive immune responses that take place in lymphoid organs of the NALT and GALT.

The microbiome–systemic immunity connection

The ability to investigate the whole bacterial composition and function in the gut and oral cavity with NGS has resulted in a major increase of understanding the bidirectional relationship of the immune system with the gut and oral microbiome (Hooper et al, 2012;
Scher et al, 2014; Zarco et al, 2012). Several excellent reviews have been published about how the microbiome affects local and systemic immunity and the relation between the microbiome and autoimmunity (Belkaid and Hand, 2014; Belkaid and Naik, 2013; McLean et al, 2014; Wu and Wu, 2012). In this section we explain the current knowledge of how bacteria and their metabolites in the gut can influence mucosal and systemic immunity. The connection of the gut and oral microbiome with RA, SLE and SS is discussed after this section.

The effect of microorganisms on shaping the immune system has been investigated for over 50 years. The first studies were done with germ-free mice and collectively they showed that lymph nodes, spleen and Peyer’s patches from these mice were underdeveloped, small and relatively inactive compared to conventional raised mice (Bauer et al, 1963; Pollard and Sharon, 1970). When these mice were challenged with antigen from Salmonella paratyphi A by swabbing the oral cavity or intraperitoneal injection, the lymph nodes, spleen and Peyer’s patches increased in size and showed distinct germinal zones (Pollard and Sharon, 1970). This and other observations indicate that the immune system is dependent on contact with microorganisms to develop to a well-functioning state (Talham et al, 1999). In 1989 Strachan found that presence of hay fever (or allergic rhinitis, which is a type 1 allergic reaction) was inversely correlated with the number of children in a household, which he linked to a reduced opportunity for cross infection in young families (Strachan, 1989). Furthermore, children living on farms are exposed to a wider range of microbes than children not living on a farm and this exposure explained a substantial fraction of the inverse relation between allergic asthma and growing up on a farm (Ege et al, 2011). Recently the mechanism behind this relation was clarified in mice as it was shown that exposure to endotoxin (also called lipopolysaccharide, a cell wall component of Gram-negative bacteria) was capable of suppressing a type 2 immune reaction to house dust mite, by modifying the communication between barrier epithelial cells and DCs (Schuijs et al, 2015). Finally, early life consumption of raw cow’s milk reduces the risk of manifest respiratory infections and fever by about 30% in infants, implicating that bacteria or peptides present in untreated milk positively affect the immune system (Loss et al, 2015). Thus, it seems that the presence of certain microorganisms early in life is necessary to boost an immune system that prevents infections and will not overreact to antigens generally present in the environment (Olszak et al, 2012; Riedler et al, 2001).

Early life (0 – 3 years) dynamics of the gut microbiome also affect the development and progression towards type 1 diabetes (T1D). In the study by Kostic et al, (Kostic et al, 2015) the gut microbiome was investigated in infants genetically predisposed to T1D. A marked drop in alpha-diversity (diversity within one faecal sample) was seen in T1D progressors in the time window between seroconversion and T1D diagnosis, compared to infants who
did not progress to T1D (Kostic et al., 2015). A decreased diversity of microorganisms in the gut bacterial composition is a hallmark of intestinal dysbiosis. Besides reduced diversity, dysbiosis can be defined by (a combination of) loss of beneficial microorganisms (such as Bacteroides strains and butyrate producing bacteria) and expansion of pathobionts (such as Proteobacteria including Escherichia coli) (Petersen and Round, 2014). Intestinal dysbiosis has been observed in patients with obesity (Turnbaugh et al., 2009), intestinal bowel diseases (Manichanh et al., 2012) and SLE (Hevia et al., 2014).

One of the factors leading to intestinal dysbiosis is diet. A high-fat/high-sugar diet in C57BL/6 mice induces a dysbiosis in the gut microbiome, illustrated by a decreased total bacterial abundance and an increased absolute abundance of Escherichia coli and Bacteroides-Prevotella spp (Martinez-Medina et al., 2014). Furthermore, release of the proinflammatory cytokines TNFα and IL-1β into the colonic mucosa was seen in mice treated with the high-fat/high-sugar diet. Overexpression of claudin-2 was shown by immunohistochemical staining of intestinal biopsies (Martinez-Medina et al., 2014). Claudin-2 is an integral membrane protein in the epithelium and plays an important role in the regulation of epithelial permeability by creating paracellular pores. Overexpression of claudin-2 is associated with increased permeability in the gut and is expressed as a response to several inflammatory cytokines (IFNγ, TNFα, IL-1β, IL-4, IL-6, IL-13) (Suzuki, 2013). Thus, dietary changes can lead to an increased intestinal permeability (Martinez-Medina et al., 2014). Increased intestinal permeability can lead to translocation of bacteria between enterocytes into the LP of the intestine (Lewis et al., 2010). However, cause and effect of gut dysbiosis and mucosal inflammation are difficult to distinguish and therefore the relation between the gut microbiome and inflammatory bowel diseases is an important research field (Huttenhower et al., 2014).

Depending on which bacterial or food antigens are presented by APCs in the immunologic compartments in the LP, the local immunologic response is directed towards inflammation or regulation (tolerance) (Belkaid and Hand, 2014). For example, segmented filamentous bacteria (SFB) in mice promote the accumulation of T helper (Th)17 and Th1 cells in the small intestine, both involved in the production of pro-inflammatory cytokines (Gaboriau-Routhiau et al., 2009, Ivanov et al., 2009). An anti-inflammatory response is facilitated by the induction of regulatory T-cells (TREG cells) as a result of the presence short chain fatty acids (SCFA) in the intestine (Arpaia et al., 2013). SCFA are produced by bacteria as a result of the breakdown of indigestible dietary components such as fibre. Also vitamin A (Mucida et al., 2007), Clostridium spp. (Atarashi et al., 2013), Bacteroides fragilis (Round and Mazmanian, 2010) and Faecalibacterium prausnitzi (Qiu et al., 2013) have been identified to promote the development of TREG cells. Thus, metabolites and bacteria in the gut lumen are important in maintaining immunologic balance in the mucosal immune system between
inflammatory and regulatory functions. It is therefore suspected that changes in the gut microbiome concomitantly lead to changes in this balance, initially affecting the gut mucosa, but also leading to systemic immune effects (figure 1). In mouse models it has indeed been demonstrated that changes in the gut microbiome play a significant role in the development of SADs (Johnson et al, 2015; Praet et al, 2015; Scher and Abramson, 2011).

The microbiome RA connection

RA is a systemic autoimmune inflammatory disease characterized by joint swelling, joint tenderness, destruction of synovial joints and the presence of autoantibodies such as rheumatoid factor (RF) and, more specifically, anti-citrullinated protein antibody (ACPA). The prevalence of RA in North America and Northern Europe is 500-1000 per 100,000 (Shapira et al, 2010). The 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for RA include the presence of synovitis and the number of joints involved, serology (presence of RF or ACPA), acute phase reactants for inflammation (C-reactive protein and erythrocyte sedimentation rate) and a duration of symptoms of at least 6 weeks (Aletaha et al, 2010).

Periodontal disease is more prevalent in RA patients than in controls and patients with periodontitis are at an increased risk of developing RA (de Smit et al, 2012; Koziel et al, 2014; Nesse et al, 2010). Therefore, the role of Porphyromonas gingivalis, a key pathogen in periodontitis, has been studied in RA patients. P. gingivalis has the unique ability to convert the amino acid arginine in a protein to the amino acid citrulline, a process called citrullination (Gabarrini et al, 2015). However, protein citrullination is also a physiological process in the human body and therefore citrullinated proteins are also normally present. ACPAs are found in about 50% of early RA patients and serum levels of ACPAs correlate with disease severity (Koziel et al, 2014). Furthermore, ACPAs are highly specific for RA (more specific than rheumatoid factor) and are included in the 2010 classification criteria for RA (Aletaha et al, 2010). Although the exact link between ACPAs and synovial inflammation in RA patients is not fully understood, ACPA producing B-cells are enriched in the synovial fluid, indicating that ACPAs and/or ACPA producing B-cells play a role in synovial inflammation (Amara et al, 2013).

Fifty years ago, it was shown that the amount of Clostridium perfringens in faecal samples of RA patients was increased compared to controls (Mansson and Colldahl, 1965; Olhagen and Månsson, 1968). Ten years later it was hypothesized that not just one bacteria is involved in the pathogenesis of RA, but probably many bacterial species (Gullberg, 1978). Recently, Scher et al, (Scher et al, 2013) investigated the complete gut microbial composition in RA patients with NGS and demonstrated that the gut microbiome of new-onset RA patients is characterized by an increase of Prevotellae, a decrease of Bacteroides and a loss of reportedly
UV

M cell

Microbial antigen

Bacteroides

SCFA

TH17 T cell

TH17

Treg

B cell

Proteobacteria

Bacteroides

SCFA

B cell

Autoantibody

Rheumatoid arthritis

Systemic lupus erythematosus

Sjögren's syndrome

Plasma cell

LUMEN

EPITHELIUM MUCUS LAYER

LAMINA PROPRIA

MESENTERIC LYMPH NODES

CIRCULATION

TARGET ORGAN / TISSUE

bacteria

Rheumatoid arthritis

virus

Sjögren's syndrome

UV
beneficial bacteria. Interestingly, the higher abundance of *Prevotella copri* in new-onset RA patients was inversely correlated with genetic predisposition for RA. They explain this inverse correlation by stating that a certain threshold for *P. copri* abundance is necessary to develop RA (Scher *et al.*, 2013). This *P. copri* threshold is possibly higher in patients who do not carry risk-alleles for RA. Thus, whether an individual develops RA might be the result of the sum of risk-alleles and *P. copri* abundance (Scher *et al.*, 2013).

A recent study using metagenomic shotgun sequencing and a metagenome-wide association study of faecal, dental and salivary samples from treatment-naïve RA patients, RA patients treated with disease modifying antirheumatic drugs (DMARDs) and healthy controls demonstrated an altered gut, dental and salivary microbiome in RA patients compared to controls (Zheng *et al.*, 2015). With complex bioinformatical genetic analysis the investigators also showed that the altered microbiome could be used to identify RA patients, correlated with clinical measures and could be used to stratify individuals on the basis of their response to therapy. Although this sounds very promising, it must be noted that the statistical methods were so complex that an accompanying paper was needed to explain the results (Rogers, 2015).
The ‘gut-joint axis’ has been postulated for RA as a mechanism for disease development. An increased intestinal permeability, caused by a dysbiosis in the gut microbiome, leads to immune stimulation with increased citrullination by bacteria, leading to new immunogenic epitopes in the intestine which induce autoantibody production and activate pathogenic pathways (Lerner and Matthias, 2015). Many assumptions in this hypothesis have not been clarified yet, but the first steps in unraveling the role of the oral and gut microbiome in RA pathogenesis have been taken. Furthermore, it has been shown that microbiome analysis in RA patients can function as a biomarker for response to therapy.

The microbiome SLE connection

SLE is a multisystemic autoimmune disease characterized by the production of numerous auto-antibodies and the involvement of skin, joints, kidneys, brain, serosal surfaces, blood vessels, blood cells, lungs and heart (Goldblatt and O'Neill, 2013; Lipsky, 2001). SLE is a very heterogeneous disease, affecting individuals with a wide range of symptoms and disease courses, of which a butterfly rash in the face, photosensitivity, arthritis or arthralgia and renal symptoms are the most common (Goldblatt and O'Neill, 2013). SLE is ten times more prevalent in women than in men and the incidence of the disease is highest in women of childbearing age (Danchenko et al, 2006). Autoreactive B-cells produce auto-antibodies against nuclear peptides, nucleosome, double-stranded DNA and Sjögren’s syndrome related antigen A (SSA, also called ‘Ro’) and are present in 50-95% of SLE patients (Goldblatt and O'Neill, 2013; Olsen and Karp, 2013).

Recently, it has been shown that the GALT is an important checkpoint for the removal of autoreactive B-cells (Vossenkämper et al, 2013). In SLE patients immature B-cells were poorly equipped to access the GALT due to a reduced expression of the gut homing receptor α4β7. Thus, failure of immature B-cells to access the GALT efficiently in SLE patients may contribute to the increased autoreactive B-cells and autoantibody production in SLE (Vossenkämper et al, 2013; Yurasov et al, 2005). Furthermore, examples of GALT were not found in the biopsies of SLE patients, whereas multiple examples of GALT were observed in healthy controls. A lower density of IgA secreting plasma cells was present in intestinal histological biopsies of SLE patients compared to healthy controls (Vossenkämper et al, 2013). The absence of GALT and reduced amount of IgA secreting plasma cells may contribute to a disturbed mucosal immunity in the gut, leading to a dysbiosis in the bacterial composition (Benckert et al, 2011).

The first study to investigate the gut microbial composition in SLE patients demonstrated that active SLE patients have more different biotypes of Enterobacteriaceae than healthy controls or inactive SLE patients (Apperloo-Renkema et al, 1994). The hypothesis behind this study was that a dysbiosis between indigenous and foreign bacteria can lead to translocation
of bacteria from the lumen to the immunologic compartments of the gut, ultimately leading to cross reactivity and antibody production. This hypothesis has gained support from a recent microbiome study confirming that intestinal dysbiosis is associated with SLE (Hevia et al, 2014). In this study intestinal dysbiosis was defined as a reduced Firmicutes/Bacteroidetes ratio. The Firmicutes/Bacteroidetes ratio in SLE patients was significantly lower than that of healthy subjects (median ratio 1.97 vs median ratio 4.86; \( P < 0.002 \)), although the bacterial diversity was not significantly different (Hevia et al, 2014). Another study from the same group performed a metabolome-wide scan of gut microbiota in SLE patients and healthy controls (Rojo et al, 2015). A difference in the gut metabolome (but not in the bacterial composition) was found between SLE patients and controls, suggesting that SLE affects the functionality of the gut microbiome (Rojo et al, 2015). However, only 0.72% of the metabolic mass features were found to significantly differ between the SLE and healthy controls, which just might be a variation within a common range and raises the question whether this subtle difference has physiological implications (Rojo et al, 2015).

The above mentioned human studies have demonstrated that there is a connection between the gut microbiome, gut mucosal immunity and SLE, but it remains unclear whether an altered gut microbiome causes SLE or that the altered gut microbiome is an effect of the disease. Two recent mouse studies have demonstrated, however, that an altered gut microbiome indeed might be a causal factor in the development of SLE. Comparing the gut microbiome of healthy mice (MRL/Mp) and lupus-prone mice (MRL/lpr) demonstrated that the relative abundance of Lactobacillaceae was significantly decreased and the relative abundance of Lachnospiraceae was significantly increased in the lupus-prone mice (Zhang et al, 2014). Vitamin A (retinoic acid) treatment restored the relative abundance of Lactobacillaceae to the level of healthy MRL/Mp mice. Furthermore, strong correlations were found between the relative abundance of Lactobacillaceae and Lachnospiraceae, and lupus disease indexes (spleen weight, MLN weight and renal function) (Zhang et al, 2014). Another study showed that a change of pH of drinking water affected the gut microbiome, gut inflammation and disease progression in a different spontaneous mouse model of SLE (SWR x NZB F1, (SNF1) mice) (Johnson et al, 2015). Mice receiving acidic water (pH 3.0-3.2) had a slower disease progression, less autoantibody (anti-dsDNA and anti-nucleohistone) production and lower immune cell infiltrates in the kidney compared to mice receiving neutral water (pH 7.0-7.2). Because the investigators expected to find the primary effect of a dietary intervention in the gut mucosa, they investigated the immune phenotype of small intestinal tissue of the SNF1 mice. Mice receiving normal water showed higher expression levels of proinflammatory cytokines, such as IL17, IL21-23 and IFNα. Gut microbiome analysis showed that the relative abundance of many bacteria significantly differed between the acidic water recipient and normal water recipient mice, with an overall trend of promoted growth of Firmicutes in the acid water recipient mice. Finally, by
transferring the gut microbiota of acidic water recipient mice into normal water recipient mice by oral gavage, the investigators were able to suppress lupus progression in the normal water recipient mice. This finding suggests that disease progression in lupus-prone SNF1 mice is dependent on gut microbiota (Johnson et al, 2015).

In conclusion, studies on the human microbiome SLE connection suggest a connection between dysbiosis in the gut microbiome and SLE, although cause and effect remain unclear. Mouse models of SLE (lupus-prone) have demonstrated that an altered gut microbiome not only affect local intestinal inflammation, but also systemic autoimmunity illustrated by autoantibody production and lymphocyte infiltration in the kidneys. To investigate whether the gut microbiome in SLE patients plays a role in disease activity, the experimental approach could be the transfer of human gut bacteria of healthy and SLE patients to germ-free lupus-prone mice (Faith et al, 2014).

The microbiome SS connection

SS is a systemic auto-immune disease characterized by lymphocyte infiltration in the salivary and lacrimal glands, causing reduced saliva and tear production which subsequently leads to the two most prominent symptoms of SS: a sensation of a dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca) (Cornec et al, 2015; Kalk et al, 2001). Fatigue is another pronounced symptom of the disease. SS is 10 times more prevalent in women than in men (Qin et al, 2014). Depending on whether SS presents as a single disease or concurrently with other autoimmune disorders, such as SLE or RA, SS is divided into primary SS (pSS) and secondary SS. Systemic features of SS appear in extraglandular sites like joints, skin, peripheral and central nerves, lungs, kidneys, liver and thyroid gland (Moreira et al, 2014; Tincani et al, 2013). pSS patients have a 13-fold increased risk for developing a non-Hodgkin lymphoma, occurring in 5–10% of all pSS patients (Liang et al, 2014). Auto-antibodies against the ribonuclear proteins SSA/Ro and SSB/La and rheumatoid factor (RF) are frequently present in SS patients; these autoantibodies can be detected many years before disease onset (Jonsson et al, 2013; Kyriakidis et al, 2013). Because the presence of anti-SSA/SSB antibodies is fairly specific for SS, it is used in the classification criteria for SS (Shiboski and Shiboski, 2015; Shiboski et al, 2012; Vitali et al, 2002).

The majority of studies on the role of the microbiome on SS, focused on the effect of hyposalivation on the oral microorganisms applying culture techniques or targeted DNA methods to determine the presence of bacterial species. Hyposalivation in patients with SS has been associated with a higher number of Candida species on mucosal surfaces and an increase in acidogenic (acid producing) and aciduric (thriving well in relatively acidic environment) microorganisms such as Lactobacillus spp. on tooth surfaces (Almståhl et al, 2003; Leung et al, 2007 and 2008; MacFarlane and Manson, 1974; Shinozaki et al, 2012). This
The microbiome-systemic diseases connection

is clinically relevant, because patients suffering from hyposalivation are at increased risk of (typically cervical) caries and oral candidiasis (González et al, 2014; Mathews et al, 2008). A Taiwanese population based study revealed that the frequency of dental visits already increased years prior to the diagnosis of pSS because of dental caries, gingivitis and stomatitis (Lu et al, 2014). In all these manifestations bacteria are involved in the pathogenesis, thus a shift in the oral microbiome might be present before the more characteristic oral symptom of pSS, which is xerostomia. No data was available on oral candidiasis and cervical caries. In contrast to RA patients, the prevalence of periodontitis is not increased in pSS patients (Lugonja et al, 2016). The authors conclude that microbiome analysis is needed to identify a possible triggering bacterial pathogen for pSS. Since periodontitis was not increased in pSS patients, it is unlikely that the presumed bacterial trigger for SS is a pathogen currently associated with periodontitis.

The only study available on the role of bacteria in SS development has demonstrated that T-cells from mice immunized with the Ro60 peptide (derived from the SSA autoantigen) could be activated in vitro by peptides from oral, skin and gut bacteria (Szymula et al, 2014). The most potent activator of the mouse Ro60 reactive T-cells was a peptide from the von Willebrand factor type A domain protein (vWFA). Basic Local Alignment Search Tool (BLAST) analysis revealed that vWFA is present in (amongst others) the oral microorganism Capnocytophaga ochracea and the gut microorganism Bacteroides intestinalis. This study supports the hypothesis that T-cells with a receptor for Ro60 (SSA) can be activated by DCs who present this specific peptide of vWFA as a result of interaction with for example B. intestinalis. SSA autoantibodies might be produced when the activated Ro60 reactive T-cells in turn activate B-cells to become plasma cells. If NGS of the oral and gut microbiome in patients with SS reveals an increased relative abundance of C. ochracea in the oral cavity or B. intestinalis in the gut, then, the microbiome SS connection might be explained by the molecular mimicry theory.

Evidence of a direct connection between the presence of SS and the human microbiome is currently absent in human and scarce in animal studies. However, pathogenic features of SS, such as the presence of autoreactive B-cells and the involvement of T_{\gamma,17}-cells (Barrera et al, 2013; Kroese et al, 2014; Lin et al, 2014) have been associated directly or indirectly with the human microbiome. The mechanism behind the survival of autoreactive B-cells in SLE patients, might also be present in SS since SLE and SS have a comparable pathogenesis (Vossenkämper et al, 2013). T_{\gamma,17} cells have been demonstrated in the salivary glands of SS patients (Sakai et al, 2008) and in our own cohort of Sjögren’s patients we find elevated numbers of T_{\gamma,17} cells in patients’ peripheral blood (unpublished data). As discussed above, specific bacteria and possibly also dysbiosis in the gut microbiome can both lead to an increase in T_{\gamma,17} cells in the LP of the gut. If these proinflammatory T_{\gamma,17} cells enter the
circulation, they may migrate to the exocrine glands in Sjögren’s syndrome. Furthermore, in an experimental SS mouse model (C57BL/6 mice immunized with submandibular autoantigens) it was shown that TH17 cells were able to drive the development of experimental SS as featured by reduced salivary secretion and tissue destruction in the salivary gland (Lin et al, 2014, Sakai et al, 2008). The question whether the TH17 cells, found in the salivary glands of SS patients, have migrated from the LP would be an interesting topic for future research.

Because we hypothesized that bacteria may play a more direct role in the recruitment of lymphocytes into the salivary glands of pSS patients, we have investigated bacterial presence in parotid gland biopsies. Biopsies of five different pSS patients were investigated with fluorescent in situ hybridization. However, by using a bacterial kingdom-specific oligonucleotide probe (Bact338), we were unable to find bacterial DNA (and thus bacteria) in these parotid gland biopsies (unpublished data). Although one case report states that *H. pylori* is present in a parotid gland MALT lymphoma in a SS patient (Nishimura et al, 2000), we were again unable to find bacterial DNA in a similar SS related MALT lymphoma of the parotid gland (unpublished data).

To conclude, indirect evidence and evidence from related systemic diseases, suggests a role for the gut and/or oral microbiome in the development of SS. Future studies will first need to find associations between the oral and/or gut microbiome and the presence of SS in patients and investigate the effect of raising a SS mouse model in a germ-free environment.

**CONCLUDING REMARKS**

From birth on, the human body is inhabited with an enormous amount and diversity of microorganisms. The human microbiome acts in close concert with the mucosal surfaces of the body and is capable of inducing inflammatory and regulatory immunologic effects locally and at distant sites. NGS, combined with other ‘omics’ methods and established methods such as flow cytometry, has increased the knowledge concerning the mechanism behind the microbiome immunity connection, but there are still many gaps. NGS of the human microbiome has, however, not yet been widely used to study the role of microbiota in the development and pathogenesis of SADs.

In SLE and RA patients an altered gut microbiome has been found, but the number of patients in these studies are too small for definite conclusions. Thus it remains unknown whether the observed differences in the oral and gut microbiome of SLE and RA patients are a cause or effect of the disease. Animal studies have provided evidence for a causal role
The development of systemic autoimmune diseases in time. A genetic predisposition for SAD (mostly HLA related) is present before birth.

The first three years of life are crucial for the assembly of a healthy human microbiome. Dysbiosis in the gut microbiome can ensue as a result of a Caesarean section (instead of vaginal birth), lack of breastfeeding, use of antibiotics and lack of exposure to environmental microbes. Dysbiosis in the gut microbiome leads to a systemic proinflammatory state (see Figure 1), which predisposes to an excessive and self-perpetuating immune response after a second hit in a joint, the skin or salivary glands. The excessive immune response becomes chronic and causes tissue damage, leading to respectively arthritis, dermal inflammatory infiltrates or sialadenitis. After a certain time, the inflammatory damage is irreversible and the function of the inflamed organ starts to fail, which is experienced as, e.g., arthralgia (RA), malar rash (SLE) or xerostomia (SS). After symptoms occur it can still take several years before the patient presents to a medical professional for the first time or the underlying disease is recognized by a physician and the diagnosis of a systemic autoimmune disease is drawn.
of the microbiome in the development of SADs, but this is still to be proven in humans. Based on the available evidence we postulate the following hypothesis on the role for the gut microbiome in the etiopathogenesis of SADs (figures 1 and 2).

A variety of environmental factors during life – from delivery mode to antibiotic use and diet – affects the human microbiome. Unfavorable environmental factors, such as birth by C-section (because the baby is not colonized with bacteria from the urogenital and anal area of the mother), frequent antibiotic use (disturbing gut homeostasis) and a high-fat/low-fiber diet (reducing SCFA producing bacteria), all may lead to dysbiosis in the gut microbiome. Long persisting dysbiosis in the gut leads to a (low-grade) inflammatory state of the mucosal immune system with increased permeability of the gut epithelium as a consequence. The increased permeability increases the translocation of bacteria and other antigens from the lumen to the LP, exacerbating the inflammatory response in the mucosal immune system of the gut. The chronic inflammatory state of the gut mucosa will ultimately affect the systemic immune system, because proinflammatory lymphocytes and cytokines will be released in the circulation. The increased exposure of bacterial and food antigens increases the risk of exposure to mimicry autoantigens and/or bystander activation of autoreactive cells, and herewith the break of tolerance for self-antigens. This process may take effect without specific symptoms. Local injury in a joint or the skin or a local (viral) infection in the salivary gland may function as the second hit and arouse the chronic proinflammatory immune system. Because the proinflammatory status rules over the regulatory functions of the immune system, the second hit causes a local chronic and self-perpetuating immune response directed against autoantigens. After a certain period of time, clinical symptoms begin to appear, such as joint inflammation in RA, skin involvement in SLE and reduced saliva production in SS.
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