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ORAL MUCOSITIS AND PERIODONTAL DISEASE IN PATIENTS WITH PRIMARY OR ACQUIRED IMMUNODEFICIENCIES

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to the patients

ABSTRACT

The infection and inflammation in the oral cavity are commonly related to the skewed interaction between host immunity and inhabiting microorganisms. Both primary and acquired immunodeficiency conditions are frequently associated with oral symptoms. In this thesis two oral manifestations, chronic periodontitis and oral mucositis, were investigated in patients with severe congenital neutropenia and in patients with malignant diseases respectively.

In Paper I, a genotype-phenotype correlation between *ELANE* mutations and chronic periodontal disease was described in a group of patients with severe congenital neutropenia. Patients that harbor mutations in the gene *ELANE*, encoding neutrophil elastase, presented with poorer periodontal health as compared to those with *HAX1* mutations or unknown mutations. The periodontal pockets of the patients with *ELANE* mutations displayed a skewed microflora and elevated levels of proinflammatory cytokine IL-1β.

In Paper II and III we studied the oral mucositis, an acute side effect frequently encountered during cytostatic chemotherapy, in a group of pediatric patients diagnosed with various types of malignancies. In Paper II, we showed that at the time of malignancy diagnosis, patients with acute leukemia had highest risk of oral mucositis, presenting with high concentrations of inflammatory cytokines IL-6, IL-8, IL-10, and TNF-α and low levels of pro-LL-37 (hCAP-18) in the blood plasma. In Paper III we conducted high-throughput sequencing of the oral mucosal bacterial community in an attempt to investigate the role of bacteria in the pathogenesis of oral mucositis development. At the time of malignancy diagnosis, patients who later developed oral mucositis were found to have higher oral mucosal microbial diversity and were more heterogeneous among one another compared to those that did not develop mucositis. A more pronounced modification of the bacterial community by chemotherapy was detected in patients that later developed oral mucositis, indicating a beneficial role of stable oral microbiota.

In the last paper of this thesis, we investigated the variation of blood pro-LL-37 levels in a cohort of patients presenting with chronic neutropenia. The lower values of pro-LL-37 were found in cases with severe congenital neutropenia, as compared to autoimmune, idiopathic, and ethnic neutropenia. The findings support the view that benign forms of neutropenia could be distinguished from the severe forms at an early stage. Plasma pro-LL-37 levels may potentially work as a diagnostic parameter and this analysis could be developed for clinical use.

Providing adequate and personalized oral care to the patients with immunodeficiencies has necessitated more knowledge regarding the predisposition and

pathobiology of their oral manifestations. The findings in this thesis may benefit the clinical management and subsequently improves the patient's quality of life.

LIST OF PUBLICATIONS

I. YING YE, Göran Carlsson, Biniyam Wondimu, Annika Fahlén, Jenny Karlsson-Sjöberg, Mats Andersson, Lars Engstrand, Tülay Yucel-Lindberg, Thomas Modéer, Katrin Pütsep. Mutations in the *ELANE* gene are associated with development of periodontitis in patients with severe congenital neutropenia. *Journal of Clinical Immunology* 2011; 31(6): 936-45.

II. YING YE, Göran Carlsson, Monica Barr Agholme, Jenny Karlsson-Sjöberg, Tülay Yucel-Lindberg, Katrin Pütsep, Thomas Modéer. Pretherapeutic plasma pro- and anti- inflammatory mediators are related to high risk of oral mucositis in pediatric patients with acute leukemia: a prospective cohort study. *PLoS ONE* 2013; 8(5): e64918.

III. YING YE, Göran Carlsson, Monica Barr Agholme, John A.L. Wilson, Annika Roos, Birgitta Henriques-Normark, Lars Engstrand, Thomas Modéer, Katrin Pütsep. Oral bacterial community dynamics in paediatric patients with malignancies in relation to chemotherapy-related oral mucositis: a prospective study. *Clinical Microbiology and Infection* 2013; doi: 10.1111/1469-0691.12287.

IV. YING YE, Göran Carlsson, Jenny Karlsson-Sjöberg, Niels Borregaard, Thomas Modéer, Mats Andersson, Katrin Pütsep. Plasma pro-LL-37 level as a diagnostic tool in the evaluation of chronic neutropenia: a prospective study. *Manuscript*

LIST OF ABBREVIATIONS

AMP	antimicrobial protein/peptide
ANC	absolute neutrophil count
hCAP-18	human cationic antimicrobial protein-18 kDa
CMV	cytomegalovirus
DMFT/dmft	decayed, missing and filled teeth in deciduous/permanent teeth
EBV	Epstein-Barr virus
ELANE	elastase, neutrophil expressed
ELISA	enzyme-linked immunosorbent assay
GBI	gingival bleeding index
GCF	gingival crevicular fluid
G-CSF	granulocyte colony-stimulating factor
HAX1	HS1-associating protein X-1
HBD	human β-defensin
HNP	human neutrophil peptide
HSCT	hematopoietic stem cell transplantation
HSV	herpes simplex virus
IL	interleukin
OTU	operational taxonomic unit
PCoA	principle coordinate analysis
PMN	polymorphonuclear neutrophil
RDP	Ribosomal Database Project
rRNA	ribosomal RNA
SCN	severe congenital neutropenia
TLR	Toll-like receptor
TNF	tumor necrosis factor
WHO	World Health Organization

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1 INTRODUCTION

The oral cavity harbors one of the most complex mixtures of microorganisms in human body, which is estimated to have over thirty thousand phylotypes in healthy individuals (Dewhirst *et al*, 2010). Host immunity is critical in maintaining oral health. The microbes are largely commensal and cause no harm under normal circumstance. However in the cases of diseases that affect the host immune system, oral tissues are frequently involved in infection and inflammation. These oral diseases negatively influence the patients' quality of life and may further promote invasion of endogenous bacteria causing systemic infections.

This thesis will focus on manifestations of periodontal and oral mucosal tissues in relation to the immunodeficient diseases or conditions, in which two patient groups will be highlighted, patients with severe congenital neutropenia and pediatric patients with malignancies and undergoing chemotherapy. This thesis is aimed to provide knowledge in order to understand the pathobiology of their oral diseases, and thus benefit the development of clinical proactive management.

1.1 BACTERIA AND HOST IMMUNITY IN ORAL HEALTH

1.1.1 Oral bacterial flora

Human microbes constitute around 90% of the total number of cells associated with our bodies (Luckey, 1972). These microbes heavily populate the essential surfaces of the human body, such as oral cavity, intestinal tract, skin, upper respiratory tract, and vagina. Despite the extensive use of antimicrobials in this age, the microbes inhabiting our body are largely commensal and provide us with genetic variation and functions.

Microbial communities vary widely among these human habitats with strong niche specialization (Figure 1). In comparison with other habitats, the oral bacterial community is especially diverse in terms of species richness. It has been estimated that the oral microbiota has over thirty thousands phylotypes and over 600 prevalent taxa at the species level in healthy individuals (Keijser *et al*, 2008; Dewhirst*, et al*, 2010). However, more than half of the oral bacterial taxa have not yet been cultivated or validly named (Dewhirst*, et al*, 2010). Furthermore, only some species are common to different oral sites, whereas the majority of species are selective for a particular oral habitat, such as teeth, gingival sulcus, tongue, lip, cheek, hard and soft palates, and oropharynx (Aas *et al*, 2005).

The complex oral microbiota is associated with the common infectious diseases in the oral cavity, including periodontitis, caries, and endodontic infections. In addition, accumulating evidence has linked oral bacteria to a number of systemic diseases, such

as cardiovascular disease (Seymour *et al*, 2007; Koren *et al*, 2011), stroke (Joshipura *et al*, 2003), preterm birth (Offenbacher *et al*, 1998), diabetes (Zeigler *et al*, 2011), and pneumonia (Bahrani-Mougeot *et al*, 2007). These associations might be as results of the microbiota priming of the immune system, or the long-term effects on inflammation.

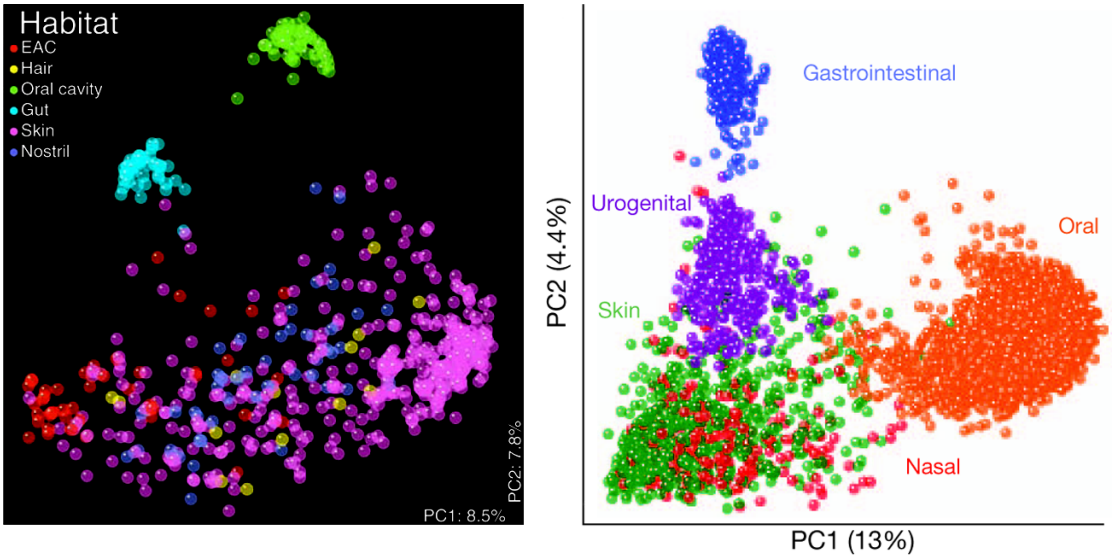

Figure 1. The oral microbial communities in relation to microbiota from other human habitats. From Costello *et al*, 2009 (left) and Human Microbiome Projects Consortium, 2012 (right).

1.1.2 Immune components in keeping oral health

The complex oral microflora is a constant challenge to the host immune system. The primary function of the immune system in the oral cavity is to protect teeth, periodontal tissues, and oral mucosa from infection. The host immunity against invading microorganisms is generally characterized by immediate recognition followed by innate and adaptive immune responses. However, the immune defense can be specialized in different oral microenvironments, represented by periodontal sulcus, oral mucosa, and saliva.

In the periodontal sulcus, there is a close contact of epithelium and dental plaque biofilm, which is the initiator and essential for the progression of tooth-supporting tissues destruction, periodontitis. The bottom of the gingival crevice is formed by a highly porous epithelium, junctional epithelium, which attaches soft tissue to the tooth surface. It only contains a few desmosomes and occasional gap junction between cells, resulting in large intracellular spaces. Thus, even in healthy gingiva there is a continuous traffic of neutrophils from gingival capillaries into the periodontal sulcus, and these granulocytes constitute an important line of defense against microbes from the oral biofilm. It has been calculated that approximately 30,000 leukocytes transit

through periodontal tissue every minute and it is the major source of leukocytes found in the oral cavity (Schiött & Löe, 1970). It is also estimated that there are around 2,000 to 400,000 polymorphonuclear neutrophils (PMNs) per µl in the gingival crevicular fluid (GCF) (McKay *et al*, 1999), which might be associated with transepithelial gradient of IL-8 and intercellular adhesion molecule-1 (ICAM-1) (Tonetti *et al*, 1998). Both quantitative deficiencies and qualitative defects of neutrophils by either innate or induced abnormalities can result in aggressive forms of periodontitis.

The oral mucosa exploits various mechanisms to protect the host against invading microbes. Contrary to the junctional epithelium in periodontal sulcus, intact stratified squamous epithelium on oral mucosa forms a mechanical barrier. In addition, the elaborate mucosal immune system, including intra-epithelial dendritic cells that patrol the tissue microenvironment and antibodies that transudate through the epithelium, maintains the homeostasis of the oral mucosa.

Saliva secretion is important in keeping mucosa moist and limiting excessive bacterial accumulation. There are three paired extrinsic salivary glands in human: parotid, submandibular, and sublingual glands. Saliva is made up of secretions from a number of sources, predominantly the extrinsic glands, but also fluids from the small intrinsic glands, epithelial cell secretions, and the GCF. It is very rich in the soluble antibody secretory IgA and antimicrobial proteins and peptides, including lysozyme, lactoferrin, histatins, defensins, and cathelicidins, which prevent bacterial expansion and protect tooth and mucosa (Farnaud *et al*, 2010).

The bacterial flora is initially controlled by the innate immune system of oral epithelia, saliva, and GCF, which are all rich in antimicrobial proteins and peptides (AMPs). To date over 45 AMPs are identified in oral cavity and these diverse classes of molecules offer broad protection against invading microbes (Gorr & Abdolhosseini, 2011). A great number of the AMPs come from neutrophils, which can release an assortment of proteins from three types of granules as the result of degranulation or apoptosis. The contents of these granules have antimicrobial properties and help to combat infection. For instance, it has been shown that some patients with chronic periodontitis or aggressive periodontitis are lacking a bactericidal peptide, LL-37, in the GCF (Puklo *et al*, 2008), which suggests an important role in protecting periodontal health. LL-37 is the C-terminal fragment derived from its precursor pro-LL-37, which is stored in the specific granule of neutrophils. In addition to the main origin from neutrophils, LL-37 can be produced by the epithelial cells in the oral cavity (Hosokawa *et al*, 2006).

Further, gingival tissue and oral epithelium expresses numerous other innate host mediators, such as human β-defensins (HBDs) (Diamond *et al*, 2001; Lu *et al*, 2004; Lu *et al*, 2005), CD14 (Jin & Darveau, 2001), and lipopolysaccharide-binding protein (LBP) (Ren *et al*, 2004), which all have been shown to be associated to oral health. To

provide a complete barrier to microbial colonization, both neutrophil and epithelial antimicrobial peptides complement each other in the periodontal sulcus and oral mucosa.

1.1.3 Oral manifestation in patients with immunodeficiency

Under normal circumstances, the interaction of host immune system and microbes are in a dynamic state of homeostasis, which however could be interrupted if the host defense is inadequate or skewed. Both primary and acquired immunodeficiency diseases or conditions may result in profound effect on oral tissues (Leggott *et al*, 1987). As summarized in Table 1, phagocytic cell defects in either number or transit are invariably associated with progressive forms of periodontal disease, often accompanied with mucosal lesion. Moreover, recurrent aphthous ulcer, candidiasis, and herpes simplex infection are reported frequently in patients with T cell or combined immunodeficiency disorders.

Table 1. Immunodeficient diseases or conditions and their oral manifestations.

Immunodeficiencies	Mainly affected components	Oral manifestation
Primary		
Congenital neutropenia	Neutrophils	Periodontitis; Ulcers
Leukocyte adhesion deficiency	Neutrophils	Periodontitis; Ulcers
Severe combined immunodeficiency	T and B cells	Candidiasis and ulcers
Common variable immunodeficiency	B cells	Ulcers
Wiskott-Aldrich syndrome	Hematopoietic stem cell derivatives	Candidiasis, herpes and ulcers Gingival bleeding
Down's syndrome	Innate and adaptive immunity	Gingivitis and periodontitis
Secondary		
Cytostatic treatment	Hematopoietic stem cells	Ulcers, candidiasis, and viral infection
Diabetes	Phagocytes	Periodontitis; Candidiasis
Acquired immunodeficiency syndrome (AIDS)	$CD4^+$ T cells	Candidiasis; Necrotizing gingivitis and periodontitis

In this thesis, patients with a primary immunodeficiency disease named severe congenital neutropenia and patients with primary malignancies will be highlighted and investigated regarding their oral manifestations.

1.2 SEVERE CONGENITAL NEUTROPENIA

Severe congenital neutropenia (SCN, also known as Kostmann disease) is a primary immunodeficiency disease that includes a heterogeneous group of disorders, characterized by low absolute neutrophil count (ANC) (< 0.5×10^9/l) in the peripheral blood, early onset of bacterial infections, and a maturation arrest of the myelopoiesis in the bone marrow at the level of promyelocyte/myelocyte stage (Kostmann, 1956; Kostman, 1975; Carlsson *et al*, 2006a; Zeidler *et al*, 2009). It was first reported with the name "infantile genetic agranulocytosis" by the Swedish pediatrician Rolf Kostmann in 1956, based on 14 affected children in a district of Norrbotten, the northernmost province of Sweden (Kostmann, 1956). It is estimated that the incidence of SCN is 1 in 100,000 live births in Sweden (Carlsson *et al*, 2012). The international registries of patients with SCN were developed in the 1990s.

1.2.1 Pathogenesis genetics

The inheritance patterns of SCN is mainly autosomal dominant or recessive, and in some cases sporadic. There are several gene mutations involved in the etiology of SCN (Klein, 2011), out of which mutations in the *ELANE* (formerly *ELA2*) gene encoding neutrophil elastase, a serine protease, are most commonly identified for most dominant or sporadic SCN (Dale *et al*, 2000; Xia *et al*, 2009). On the other hand, the recessive form of SCN is mostly associated with homozygous mutations in the *HAX1* gene (Klein *et al*, 2007), which encodes the mitochondrial antiapoptotic protein HS1-associating protein X-1. The other less frequently mutated genes include *G6PC3* in recessive SCN, *GFI1* in autosomal dominant SCN, and *WAS* in Wiskott Aldrich syndrome, an X-linked SCN (Klein, 2011). Recently, mutations of endosomal trafficking gene *VPS45* have been identified in a recessive form of congenital neutropenia (Vilboux *et al*, 2013). Approximately one third of the SCN cases present with no known disease-causing mutation (Boztug & Klein, 2009).

The prevalence of the different gene defects in SCN varies between different ethnic backgrounds of the patient population. For instance, *ELANE* mutations are variable, ranging from 30% in a British population (Smith *et al*, 2009), 35% in French registry (Bellanne-Chantelot *et al*, 2004), 56% in North America (Xia*, et al*, 2009), to 57% in European registry (Zeidler*, et al*, 2009). The *HAX1* mutations also vary in prevalence, for instance no case in a North America study (Xia*, et al*, 2009), 12% in the

European registry (Zeidler*, et al*, 2009), and up to 40% in an Iranian population with SCN (Alizadeh *et al*, 2013).

Today close to two hundred distinct *ELANE* mutations have been recognized in association with SCN or a less severe inherited disorder, cyclic neutropenia (CyN) (Horwitz *et al*, 2007; Germeshausen *et al*, 2013). CyN is characterized by a periodic decrease in circulating neutrophils that occurs approximately every 21 days and lasts for 3-6 days (Reimann & De, 1949; Horwitz *et al*, 1999). *ELANE* gene encodes the neutrophil elastase, which is stored as an active protease in primary granules and released upon exposure of the neutrophil to inflammatory stimuli. However, it is so far not completely understood why *ELANE* mutations cause neutropenia. Furthermore, it remains unclear why certain *ELANE* mutant alleles may cause either of the two different phenotypes, congenital or cyclic neutropenia (Newburger *et al*, 2010). There are many theories on the pathogenesis of SCN, including mislocalization of elastase, altered biochemical properties of elastase, and unfolded protein response (Kollner *et al*, 2006; Horwitz*, et al*, 2007), however lack of animal models in which the mutations give the human phenotype has hampered the testing of these different ideas. It was also pointed out that type or localization of mutation only partially determines the clinical phenotype, despite differences in the spectrum of mutations.

1.2.2 Clinical characteristics

SCN is usually diagnosed in infancy. Children with SCN, if untreated, usually present with invasive bacterial infections such as septicemia, omphalitis, skin abscesses, cellulitis, or pneumonia. Unlike healthy individuals, there is typically a decreased formation of pus at sites of infection. The infection sites do not distinguish between patients harboring different gene mutations. In addition to the infection, some patients with *HAX1* mutations present with neurological symptoms during childhood (Carlsson *et al*, 2008). Further, decreased bone mineral density leading to osteopenia or osteoporosis has been reported in some patients (Yakisan *et al*, 1997).

It is clinically important to maintain the ANC above 1.0×10^9/l to avoid severe infections. The majority of the patients with SCN died of infection at young age before introduction of the maintenance therapy with granulocyte colony-stimulating factor (G-CSF), which was introduced in late 1980s and since then has a major impact on the management of SCN (Bonilla *et al*, 1989; Dale *et al*, 1993). It is now the standard of care to prevent life-threatening bacterial sepsis (Carlsson & Fasth, 2001). The majority of the patients respond to G-CSF with increased neutrophils, reduced infections, and improved survival. However, the only curative treatment for SCN is hematopoietic stem cell transplantation (HSCT), which is indicated when patients are refractory to G-

CSF therapy, develop G-CSF receptor mutations, or develop myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) (Carlsson *et al*, 2011).

SCN is recognized as a premalignant disorder. It was reported that around 9-25% patients with SCN developed MDS/AML after long-term G-CSF therapy (Freedman *et al*, 2000; Rosenberg *et al*, 2010; Carlsson*, et al*, 2012), which is similar to the risk of AML in some other inherited hematologic disorders, such as Fanconi anemia (Rosenberg *et al*, 2003) and dyskeratosis congenita (Alter *et al*, 2009). It has been shown that sequential gain of mutations in SCN at different phases progresses to AML (Germeshausen *et al*, 2008; Beekman *et al*, 2012).

It has been demonstrated that *ELANE* mutations correlate with more severe expression of neutropenia, *i.e.* younger median age at diagnosis, lower ANC levels and higher prevalence of bacterial infections in periods without G-CSF, higher frequency and median dose of G-CSF therapy (Bellanne-Chantelot*, et al*, 2004; Zeidler*, et al*, 2009). In a recent study based on nearly four hundred patients with SCN, the frequency of acquired mutations on *CSF3R* gene encoding colony-stimulating factor 3 receptor (OR = 1.86) and the risk of malignant transformation, MDS/leukemia (OR = 2.00), are significantly higher in individuals with *ELANE* mutations compared to those with wild-type *ELANE* (Germeshausen*, et al*, 2013). Furthermore, the need for HSCT was also significantly higher in patients with *ELANE* mutations (OR = 3.32) (Germeshausen*, et al*, 2013). In an earlier study, discrimination of G-SCF dosages by genotype showed that patients with *HAX1* mutations require a lower median dose as compared to patients with *ELANE* mutations, suggestive for the differentiation of severity in the underlying defects (Zeidler*, et al*, 2009). However, the variations of neutropenia expression and malignant transformation have not been clearly explained by the location or nature of the mutations.

1.2.3 Periodontal infections

As discussed earlier in the 1.1.3 section, patients with SCN are predisposed to a major oral disease, periodontitis (Figure 2).

Periodontitis is a bacteria-induced chronic inflammatory disease of the periodontium, which consists of the epithelial, connective and bone tissues that surround and support the teeth. It is the most common cause of tooth loss worldwide. It has been well accepted that tissue destruction in periodontal disease is a result of an imbalance between the host inflammatory process and pathogenic bacteria residing in the periodontal crevicular space. Periodontal destruction in subjects at puberty or younger is usually associated with immunological disorders.

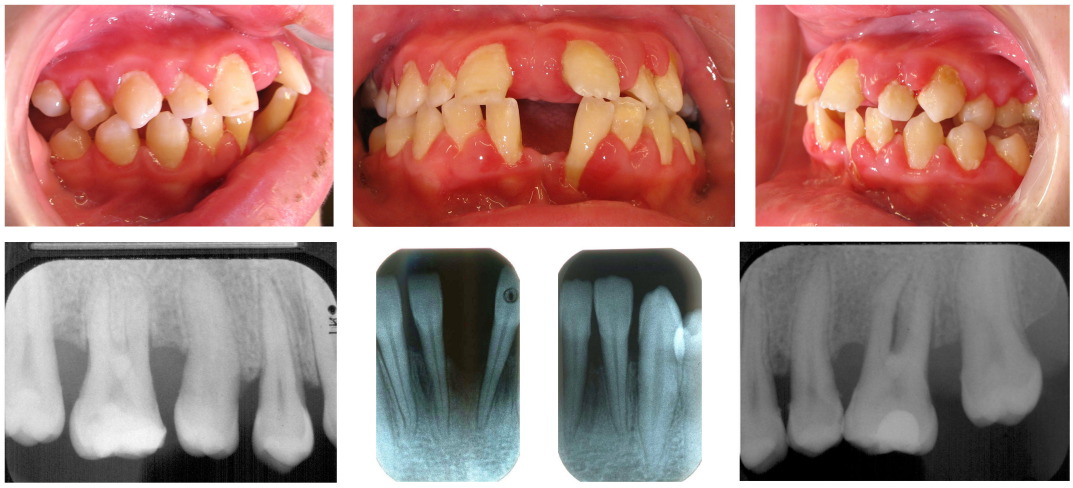

Figure 2. Periodontitis in a 17-year-old patient with SCN, harboring *ELANE* mutation. Photos courtesy of Dr. Biniyam Wondimu.

Neutrophils are the predominant phagocytes that provide protection against bacterial and fungal infections. Genetically determined neutrophil depletion confers a predisposition to severe infections. Neutropenia may also impair the function of other immune component, like NK cell maturation and function (Jaeger *et al*, 2012). Thus, a profound state of immunodeficiency caused by neutrophil deficiency not surprisingly leads to periodontal infection. The destructive periodontal disease affecting both deciduous and permanent dentitions is often found in patients with SCN (Mishkin *et al*, 1976; Defraia & Marinelli, 2001; Carlsson *et al*, 2006b) or cyclic neutropenia (Scully *et al*, 1982). Early severe periodontal disease is characterized by gingival inflammation, excessive tooth mobility, and extensive loss of alveolar bone. Although in some cases the oral hygiene was poor, the destruction usually far exceeded that seen in otherwise healthy individuals of the same age. Further, it is usually present as generalized periodontitis rather than localized periodontitis that is seen in patients at puberty without systemic disorders. Because of the low prevalence of SCN, the microbiology associated with this disorder is not well described, with only some evidence of the presence of *A. actinomycetemcomitans* and *P. gingivalis* in periodontal pockets (van Winkelhoff *et al*, 2000).

The periodontal disease is not only a consequence of neutropenia but may also in return increase the severity of neutrophil deficiency in circulation. In chronic periodontal diseases, neutrophil migrate in large numbers into the sulcular area in response to chemokines and cytokines, which is an unceasing consumption of peripheral neutrophils. It was observed that the peripheral levels of leukocytes and neutrophils were partly restored after improvement of periodontal disease status, which means gained attachment levels and decreased probing depth in patients with SCN, both with G-CSF treatment (Goultschin *et al*, 2000) and without (Tozum *et al*, 2003).

The oral hygiene instructions and routine dental check-up should be strictly followed in patients with SCN, to control disease and reduce tragic evolution. It was reported that in patients receiving local antibiotic prophylaxis and supervised oral hygiene, the gingival conditions was improved with elimination of periodontal pathogens (Okada *et al*, 2001).

1.2.4 Pro-LL-37 deficiency in SCN

The G-CSF therapy drastically changed the clinical outcome by increasing neutrophil level and diminishing vulnerability to bacterial infections. However, it was noticed that some patients with SCN might still suffer from periodontitis, despite G-CSF-elevated ANC and professional dental care. To date it is well recognized that aside from the quantitative defect of neutrophils, patients with SCN also present qualitative neutrophil aberrations. It has been found that neutrophils from patients with SCN are deficient of the antimicrobial protein pro-LL-37 and display reduced level of the defensins human neutrophil peptide 1-3 (HNP1-3) (Pütsep *et al*, 2002; Andersson *et al*, 2007), indicating that G-CSF treatment reverses neutropenia but is not correcting the granule proteins.

Neutrophils are short lived, non-mitotic cells generated from pluripotential stem cells residing in the bone marrow. There are approximately 10^{11} neutrophils produced daily. The differentiation and maturation of neutrophils involves the progression through five stages before they are released into circulation (Figure 3) (Badolato *et al*, 2004). During this process of myelopoiesis, the neutrophils acquire necessary capabilities to detect infection, migrate to the site of infection, ingest, and kill microorganisms.

Neutrophils contain several types of granules that are important for their microbicidal activity and migration. The primary (azurophilic) granules store enzymes including myeloperoxidase, elastase, and proteinase 3 but also the antimicrobial defensins. In the secondary (specific) granules a number of antimicrobial components are stored, including pro-LL-37, lactoferrin, and lysozyme. The secondary granules are more easily mobilized for exocytosis than the primary granules and thus essential in extracellular killing of microbes. The tertiary (gelatinase) granules and secretory vesicles of neutrophils are the most readily mobilized. The secretory vesicles contain intergrins that attach to the endothelium while the gelatinase granules contain metalloproteases for tissue modulation during neutrophil migration.

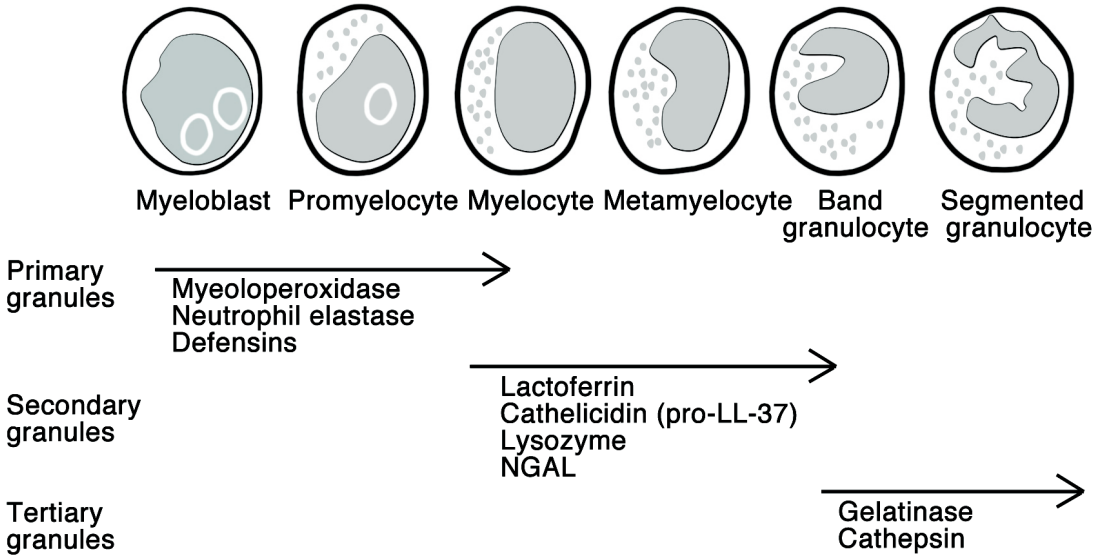

Figure 3. Myeloid maturation and neutrophil granule-associated proteins. Adapted from Badolato *et al*, 2004.

Pro-LL-37, also named human cationic antimicrobial protein-18 kDa (hCAP-18), is synthesized during myeloid cell differentiation in the bone marrow and stored in the secondary granules of neutrophils (Sørensen *et al*, 1997a). It can also be detected in the peripheral blood where it is stabilized by binding to apolipoproteins (Sørensen *et al*, 1999). In patients with SCN, the bone marrow usually shows a maturation arrest phenomenon of neutrophil precursors at the promyelocyte/myelocyte stage, thus causing neutropenia. The maturation arrest is a phenomenon of elevated myeloid cell apoptosis, consequently leading to severely depressed pro-LL-37. The G-CSF treatment overcomes the apoptosis, but pro-LL-37 deficiency is not corrected, independent of the inheritance subtypes. However, it should be noted that other granule protein including lysozyme, myeloperoxidase, and lactoferrin displayed similar level compared to control individuals (Andersson*, et al*, 2007). The mechanism behind is so far unclear.

Pro-LL-37 is the precursor of the antimicrobial peptide LL-37, the only cathelicidin found in human (Gudmundsson *et al*, 1996; Sørensen *et al*, 2001). Apart from the leukocyte, it is also expressed in epithelial cells of the testis, skin, and the gastrointestinal, urinary, and respiratory tracts. LL-37 exhibits a broad-spectrum of antimicrobial activity. It could show synergistic effect with defensins or lysozyme in killing microorganisms. Below bactericidal concentrations, it has also been found to have additional defensive roles such as regulating the inflammatory response and chemo-attracting cells of the adaptive immune system to infection sites, binding and neutralizing lipopolysaccharide, and promoting wound healing (Yang *et al*, 2001; Yu *et al*, 2007; Alalwani *et al*, 2010).

1.3 CHEMOTHERAPY-RELATED ORAL MUCOSITIS

In this section we will discuss pediatric patients with malignant diseases and a major oral complication, oral mucositis, related to anticancer treatment.

Anticancer treatment containing cytostatic drugs virtually affects all the organ systems of the body. The common acute toxicities related to classic chemotherapy include myelosuppression, mucositis, nausea, vomiting, and alopecia, while the long-term side effects may include toxicities affecting puberty and fertility, growth, intellectual development, and damages on other organs like heart, lung, and kidney (Figure 4). Among these complications, oral mucositis is the most common side effect found in the oral cavity.

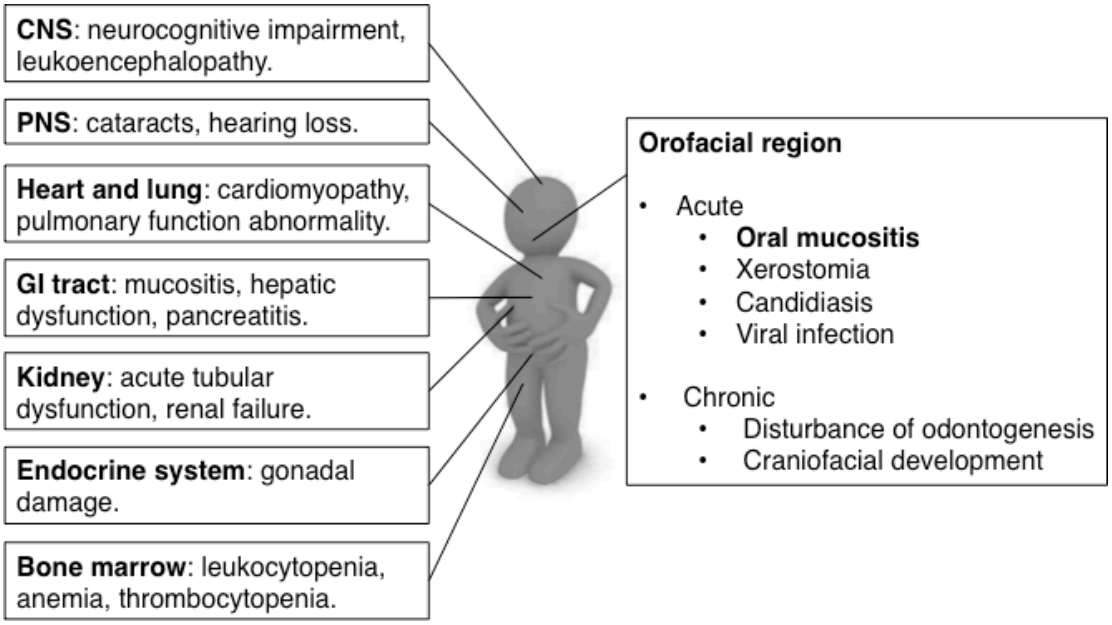

Figure 4. Toxicities of chemotherapy in children. CNS, central nervous system; PNS, peripheral nervous system; GI, gastrointestinal.

1.3.1 Clinical characteristics

Oral mucositis is one of the major complications seen during cytostatic treatment (Scully *et al*, 2006; Sonis, 2009). It is the damage that occurs in the mucosal lining of the oral cavity and oropharynx. It has variable clinical symptoms ranging from erythematous patches to infection-prone ulcerations with lesions mostly seen on the nonkeratinized mucosa. Oral mucositis usually cause severe pain, bleeding, and in severe cases, dysphagia, which subsequently restrain the ability to maintain adequate oral intake and sufficient body weight, and ultimately reduce the patient's quality of life (Cheng *et al*, 2013). In addition to the pain it causes, oral mucositis has also been recognized as a major toxicity that promotes infection because the disruption of

mucosal barrier leads to a greater penetration of microorganism. Unanticipated mucositis may thus further delay or complicate the anticancer treatment.

Oral mucositis occurs in around 40-75% of the pediatric cancer population with higher frequency in patients receiving HSCT (Sonis *et al*, 2004; Fadda *et al*, 2006; Figliolia *et al*, 2008). The severity of oral mucositis is commonly graded on two scales in clinics, World Health Organization (WHO) and National Cancer Institute-Common Toxicity Criteria (NCI-CTC). Both grading scales are based on the clinical manifestation and function related to eating and swallowing. The more commonly used scale, WHO scoring system (WHO, 1979), rates the oral toxic effect into five levels: grade 0, no change; grade 1, soreness/erythema; grade 2, erythema, ulcers, can eat solids; grade 3, ulcers, requires liquid diet only; grade 4, alimentation not possible. In grade 3 and 4, patients either are limited to predominately liquid diet or completely lose the possibility of oral alimentation, both of which require the parenteral nutrition.

The individual risk of oral mucositis during chemotherapy has been investigated in several studies on pediatric patients, in which body weight prior to chemotherapy (Cheng *et al*, 2008), blood type (Otmani *et al*, 2008), underlying malignant disease (Otmani *et al*, 2011), specific chemotherapy regimens or protocols (Fadda*, et al*, 2006; Figliolia*, et al*, 2008), serum creatinine level (Cheng*, et al*, 2008), blood methotrexate concentration (Cheng, 2008), and neutropenia (Arya *et al*, 2008; Cheng*, et al*, 2008; Cheng *et al*, 2011) have been suggested as risk factors for developing oral mucositis. However, the relative contribution of these risk factors in relation to mucositis is not clear, and little conclusive result has been achieved. It was recently reported that the G-CSF responsiveness appeared as a predictor of oral mucositis, febrile neutropenia, and infection after high-dose chemotherapy in adult patients with myeloma or lymphoma (Straka *et al*, 2011). It was also revealed that the ability of monocytes to synthesize IL-10 before chemotherapy was inversely related to gastrointestinal mucositis (Schauer *et al*, 2010). These findings indicated that the individual differences in immune response could attribute to the oral mucositis development.

Although a great number of studies have investigated the possible preventive or therapeutic management of oral mucositis, there is scarce evidence that could confirm the demonstrable anti-mucositis efficiency (Stokman *et al*, 2006; Worthington *et al*, 2007; Clarkson *et al*, 2010). So far the only agent that show robust activity in preventing oral mucositis is palifermin, a recombinant human keratinocyte growth factor (Spielberger *et al*, 2004). The tissue-protective capacity of palifermin was primarily attributed to its mitogenic effect that stimulates mucosal epithelial cell proliferation. In addition palifermin up-regulates genes that encode reactive oxygen species-scavenging enzymes (Braun *et al*, 2002) and stimulates the generation of the anti-inflammatory cytokine IL-13 (Panoskaltsis-Mortari *et al*, 2000), which collectively reduce inflammation and prevent mucositis. Several practical therapies for mucositis

are also available but have less evidence-based data to prove their effectiveness, such as cryotherapy, low level laser therapy, L-glutamine, non-steroidal anti-inflammatory mouth rinse. Although some of these therapies have been incorporated into some practice guidelines as recommended preventive measures of oral mucositis, the results from previous studies investigating the outcome of these preventive managements have been controversial. While limited medication has been proven to successfully eliminate mucositis, management of painful symptoms can help to alleviate oral discomfort, encourage eating, and improve patient's quality of life. High-grade mucositis pain is commonly relieved with potent analgesic medications, for instance morphine (Keefe *et al*, 2007).

1.3.2 Pathobiology of oral mucositis

The oral epithelium maintains its structural integrity by a process of continuous cell renewal and migration from the deepest layers to the surface in order to replace those cells that were shed. Thus, the etiology of mucositis was historically connected to epithelial injury that was caused by the preferential effect of chemotherapeutic agents on proliferating basal cells, which resulted in eventual loss and ulceration of the epithelium.

Nevertheless, studies in the past decade have indicated a more complex pathomechanism. Considerable progress has been made in defining a cascade of destructive progress in the aspect of molecular and cellular pathobiology, which was characterized as initiation, signaling, amplification, ulceration and healing (Sonis, 2004). In this multifactorial event, the upregulation of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, was found to be critical in the initial phase (Sonis, 2004; Logan *et al*, 2008a; Logan *et al*, 2008b). Early release of inflammatory cytokines and reactive oxygen species in mucosal tissue activates transcription factors such as NF-κB and further apoptosis that leads to epithelial ulceration.

Other factors leading to ulceration may include submucosal damage and apoptosis of epithelial cells. It was suggested that damage to components of the submucosa is apparent before injury to the overlying epithelium can be detected (Sonis, 2010). The submucosal damage may include injury and eventual apoptosis of fibroblasts and vascular endothelial cells. Mechanistically, injury to endothelial cells results in loss of secreted epithelial growth factors such as keratinocyte growth factor, which may explain disturbance of the normal growth patterns of the mucosal epithelium (Wearing & Sherratt, 2000). As it appears that much tissue damage in mucositis could be a consequence of apoptosis, some studies have explored whether cytotoxicity to epithelial cells from anticancer therapy could be achieved by suppressing apoptosis through toll-like receptors mediated cell proliferation (Fukata *et al*, 2006; Burdelya *et*

al, 2008). However, the mechanism is unclear and optimal prevention is thus subject to further investigation.

1.3.3 Oral microbiota in oral mucositis

The oral cavity harbors a diverse, abundant and complex microbial community and is one of the most contaminated sites of the human body. In parallel to the studies of inflammatory mediators, an important aspect to be considered has emerged: the contribution of human microbiota to mucositis.

In the five-phase model of mucositis development that was proposed in the last decade (Sonis, 2004), the microbiota does not play a significant role in the pathophysiology of mucositis. It is commonly believed that bacterial colonization and/or secondary infection increase the duration of ulcers or prevent healing, rather than promote the occurrence of mucositis. However, the immunodeficiency that generally featured by patients with malignancies and undergoing anticancer treatment predisposes to a disturbed microbial equilibrium in the oral cavity, which may cause the development of exogenous and opportunistic infections and subsequently promote the development of oral mucositis. Recently some evidence has appeared indicating a change of bacterial community after chemotherapy that may contribute to the development of mucositis (van Vliet *et al*, 2009; Napenas *et al*, 2010; van Vliet *et al*, 2010).

In addition to the immunodeficiencies in the host, other potential contributors to the oral microbial shift could be the use of prophylactic antibiotics before placing central venous catheter, the antibacterial effect of anticancer treatment, and/or altered salivary output (Napenas *et al*, 2007). Nevertheless, so far no clear pattern regarding qualitative and quantitative oral flora changes emerged among studies, and little conclusive evidence has been published considering the role of microorganisms in the pathogenesis of mucositis.

1.3.3.1 Antibacterial prophylactic therapies for oral mucositis

Various antimicrobial agents have been evaluated for their prophylactic effect on oral mucosits. However no agreement has been reached regarding the effectiveness of these agents (Stokman*, et al*, 2006; Saunders *et al*, 2013). This is one essential reason why it is so far unclear if mucositis is of infectious etiology, and further, if secondary microbial colonization of oral lesions has definite relation with clinically relevant local or systemic infection.

Iseganan, a microbicidal agent against a broad spectrum of endogenous oral flora, including both Gram-positive and Gram-negative bacteria as well as yeast, was initially

considered as a promising candidate that possesses the attributes essential for preventing oral mucositis. However, in the phase III clinical trial it failed to show clinical benefit in preventing oral mcuositis occurrence (Giles *et al*, 2004; Saunders*, et al*, 2013).

A combination of antimicrobials named PTA, namely polymyxin, tobramycin, and amphotericin B, targeting aerobic Gram-negative bacteria and yeast, was also studied in terms of reduction of mucositis. Polymyxin is selectively toxic for Gram-negative bacteria due to their specificity for the lipopolysaccharide molecule; tobramycin is an aminoglycoside antibiotic derived from *Streptomyces tenebrarius* and especially effective against species of *Pseudomonas*; amphotericin B is a polyene antifungal drug. However, the results are controversial with some studies showing effectiveness in preventing oral ulceration in radiotherapy treated patients, whereas other studies that did not support the clinical benefit (Wijers *et al*, 2001; El-Sayed *et al*, 2002; Stokman *et al*, 2003).

Other topical antimicrobial agents, including clarithromycin and triclosan, have also been studied, however these agents cannot be recommended due to insufficient or conflicting result (Saunders*, et al*, 2013). Some antiseptic agents were also studied, including chlorhexidine, benzydamine, and povidone-iodine, which are all effective against some oral bacteria. However, these agents applied in the form of mouth rinse did not show enough clinical evidence to be effective (Clarkson*, et al*, 2010; Saunders*, et al*, 2013).

The non-conclusive results with respect to the efficacy of local antimicrobial treatment indicate that the role of microbiota played in mucositis development is not only related to increased bacterial load. In addition, many of the traditional "pathogens" were first identified because they grow readily in culture, but they may not be neither the predominant species nor essentially virulent to oral mucosa. Thus, antimicrobials targeting these microbes may not significantly change the clinical outcome. It is therefore of importance to draw a better picture of bacterial composition and dynamics in patients receiving chemotherapy. If specific microbial colonization intensifies the inflammatory process, then interventions targeting these pathogenic organisms might alter the cascade of events that lead to oral mucosits.

1.3.3.2 Antibacterial activities of cytostatic drugs

Many commonly used cytostatic drugs have been found to have antimicrobial, especially antibacterial activities. Drugs including actinomycin, doxorubicin, and cisplatin were initially known or isolated as antibiotics and then applied in anticancer treatment. Cytostatic chemotherapy may thus affect the oropharyngeal commensal flora (Renard *et al*, 1986).

Among the cytostatic drugs commonly used in the treatment of childhood malignancies, methotrexate, doxorubicin, cyclophosphamide, etoposide, and vincristine show antibacterial activities against Gram-positive *S. epidermidis* and *S. aureus*, however do not effectively kill Gram-negative *E. coli*, *P. aeruginosa*, or *K. pneumoniae* (Gumpert *et al*, 1982; Gieringer *et al*, 1986; Renard*, et al*, 1986; Calame *et al*, 1988; Peiris & Oppenheim, 1993; Kruszewska *et al*, 2000). The actinomycin, similarly, has marked antimicrobial properties against Gram-positive bacteria, for instance *Micrococcus* spp., *Bacillus* spp., and *Staphylococcus* spp., and to a lesser extent is active on Gram-negative bacteria (Katz *et al*, 1956; Abbott & Sudo, 1977). The Gram-negative bacteria like *F. nucleatum*, *Bacteroides* spp., *E. coli*, and *P. aeruginosa* are not sensitive to actinomycin. On the contrary, cisplatin is active against *E. coli*, *P. aeruginosa*, *A. faecalis*, and *K. pneumoniae*. The cisplatin shows variable effect on Gram-positive species, with inhibitory effect on some *Bacillus* spps. but not on *S. aureus, S. faecalis*, *S. lutea*, or *N. catarrhalis* (Rosenberg *et al*, 1967; Joyce *et al*, 2010).

1.3.3.3 Oral bacteria and systemic infection

Oral mucositis has a major impact on the systemic status, which is largely attributed to the invasion of oral microorganisms after increased epithelial permeability. Oral mucositis has been shown to predispose patients with cancer to systemic infections with *Staphylococcus*, alpha-hemolytic *Streptococcus* (Bochud *et al*, 1994; Rossetti *et al*, 1995), *Capnocytophaga* (Parenti & Snydman, 1985; Baquero *et al*, 1990), or, although uncommonly, Gram-negative anaerobic bacteria *Leptotrichia*, *Fusobacterium*, *Pseudomonas*, and *Klebsiella* (Baquero*, et al*, 1990; Landsaat *et al*, 1995; Schwartz *et al*, 1995; Kersun *et al*, 2005). A shift of spectrum of pathogens from gram-negative to antimicrobial-resistant gram-positive organisms has been noticed in the bloodstream (Zinner, 1999; Madani, 2000). However, most of these studies were hampered by the restrictions of culturing techniques, because a large fraction of bacteria has never been cultured and thus escapes detection.

In the work of this thesis, we tested the following hypotheses: (1) in patients with SCN, the periodontal health might be associated to the underlying genetic defect to SCN; the patients with SCN featuring pro-LL-37 deficiency might be utilized in determining clinical diagnosis in chronic neutropenia; (2) in pediatric patients with malignancies and undergoing chemotherapy, the circulating inflammatory mediators and dynamics of oral bacterial community may be related to the risk of oral mucositis development.

2 AIMS

The general aim of this thesis is to increase our understanding of the oral infection and inflammation in patients with primary or acquired immunodeficiencies, and to find connections that may shed light on the driving mechanisms behind these oral diseases and potential factors in maintaining tissue intact and homeostasis.

Specific aims:

- To study the periodontal diseases in patients with SCN harboring different gene mutations, together with underlying immune modulators including pro-LL-37, pro-/anti- inflammatory cytokines, and the composition of periodontal bacterial flora.
- To investigate the clinical diagnostic value of plasma pro-LL-37 level in indicating the myelopoietic activity in patients with chronic neutropenia.
- To study the potential clinical risk indicators and pro-/anti- inflammatory modulators of chemotherapy-related oral mucositis in children with malignancies.
- To study the composition and dynamics of oral mucosal bacterial community in children with malignancies in relation to the development of oral mucositis during chemotherapy.

3 METHODOLOGICAL CONSIDERATIONS

In this section, an overview of the materials and methods applied in the work of this thesis will be described. Most of the methodologies have been established and published previously. Detailed descriptions of these methods can be found in the papers as referred below. In addition, information regarding processing 454 pyrosequencing data of bacterial 16S rRNA gene will be described and discussed in this section.

3.1 CLINICAL EXAMINATIONS

The clinical examination work conducted includes periodontal examinations (Paper I), oral mucositis evaluation (Paper II and III), and clinical diagnosis of chronic neutropenia with various etiologies (Paper IV). In Paper I, the periodontal status was evaluated based on bleeding on probing (BOP, %), probing depth (mm), and alveolar bone loss shown on radiographs. In Paper II and III, the oral mucositis was evaluated using WHO scoring system (WHO, 1979), as introduced in the section 1.3.1. In Paper IV, the referring physicians reported the final clinical diagnose which was made according to disease definition and diagnostic criteria.

3.2 ORAL SAMPLE

The oral samples analyzed include GCF samples from periodontal sulcus (Paper I) and bacterial samples from either periodontal sulcus (Paper I) or oral mucosal surface, which includes central lower lip and bucca (Paper III). Sample retrieval was performed using a prefabricated paper strip (PerioPaper, Oralflow, US, Figure 5), which is around 2×6 mm^2 of the absorbent part (white). The paper strip is either inserted into the periodontal pocket or placed on the surface of oral mucosa.

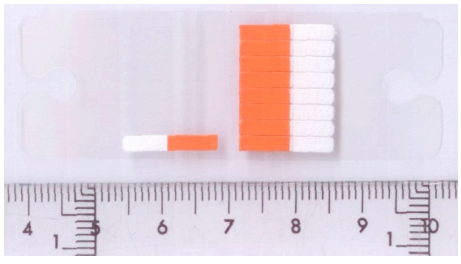

Figure 5 Paper strips used to retrieve GCF and microflora samples from oral cavity.

3.3 PLASMA SAMPLE

In Paper I, II, and IV, peripheral blood samples were retrieved for analysis of pro-LL-37 or cytokines. For all samples, EDTA-anticoagulated blood was centrifuged at 200 g

for 8 minutes at room temperature. The plasma was collected from the top layer for further analysis.

3.4 PROTEIN DETECTION

The detection of pro-LL-37 and its cleaved peptide LL-37 was performed using immunoblot. To determine the relative level of pro-LL-37 we use a commercially available human serum as the standard (Sigma-Aldrich, Sweden). This analysis is included in Paper I, II, and IV.

In Paper IV, plasma pro-LL-37 was further analyzed for the absolute concentration by a pro-LL-37 (hCAP-18) ELISA established by Borregaard and coworkers, in which the pro-LL-37 standard is of recombinant source (Sørensen *et al*, 1997b). In addition, a commercially available human LL-37 ELISA (Hycult Biotech, US) was assessed for the quantitative analysis of pro-LL-37 in plasma and the result was compared with that from pro-LL-37 ELISA.

The concentration of the cytokines, including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17, IFN-γ, and TNF-α, was determined using a fluorescent bead-based immunoassay with human cytokine LINCOplex kit (Millipore, Sweden) and Bio-Plex 200 system (Bio-Rad, Sweden), which was included in Paper I and II. In this method, the reader combines two lasers, fluidics, and real-time digital signal processing to distinguish up to 100 different sets of color-coded polystyrene beads, each bearing a different assay. Thus, the advantage of this method is to enable the detection and quantification of multiple analytes in a single sample volume.

3.5 DETERMINATION OF BACTERIAL FLORA

The microbial ecosystems in human have traditionally been characterized using culture-based methods. However, in the complex ecosystems like oral cavity or gastrointestinal tract, only a small proportion of the bacteria species could be cultivated and there has been a growing need to explore the full diversity of bacteria communities, which could be achieved using genomic identification.

3.5.1 16S ribosomal RNA gene

The elucidation of bacterial phylogeny is based on the 16S ribosomal RNA (rRNA), which is a component of the 30S small subunit of prokaryotic ribosomes. It is evolutional conserved and thus suitable as a phylogenetic target (Olsen *et al*, 1986). The 16S rRNA gene is around 1500 nucleotides in length and contains 9 species-specific hypervariable regions with interspersed conserved regions (Baker *et al*, 2003).

By sequencing the variable regions, 16S rRNA gene can provide a taxonomic identification down to species level, which is commonly defined as operational taxonomic unit (OTU) with 97% identity in a group of reads.

The sequence length used in high-throughput sequencing does not usually cover the whole 16S rRNA gene. Analyzing a longer region will provide a higher taxonomic precision, which however should be balanced with sampling depth (Hamady & Knight, 2009). In addition, the taxonomic assignment is also influenced by the variable region sequenced and assignment tool used. Many different primer pairs have been designed and evaluated, and there is so far no consensus regarding the length and variable region to use. In the work of this thesis, we have used the primer pair consisting of the forward primer 341f (CCTACGGGNGGCWGCAG) and the reverse primer 805r (GACTACHVGGGTATCTAATCC), both of which match to more than 90% bacterial sequences of *E. coli* in the Ribosomal Database Project (RDP) (Cole *et al*, 2009; Herlemann *et al*, 2011). The sequencing length with this primer pair is around 450 base pair (bp), which is generally accepted for the diversity estimation and community profiling. The primers were incorporated with an additional sequence tag (5 nucleotide long in Paper I and 7 nucleotide long in Paper III), which is unique for each sample and therefore allows multiple samples in one run.

3.5.2 454 pyrosequencing

The exploration of microbial genomes using next generation sequencing (NGS) technologies has rapidly increased our knowledge of the structure and diversity of microbial communities both from human and in the environment. A higher throughput is achieved by sequencing a large number of DNA molecules in parallel. The 454 Genome Sequencer (Roche, Switzerland), as the pioneer of NGS platforms, was introduced in 2005 for bacterial whole genome sequencing (Margulies *et al*, 2005) and it was soon applied in bacterial community profiling (Sogin, 2006), including gut (Andersson *et al*, 2008) and oral (Keijser*, et al*, 2008) microbiome. As technologies advance, the platform is continuously upgraded with a higher capacity of throughput and read length. In Paper III, the 454 GS FLX Titanium XLR70 kit was able to sequence around 700,000 amplicon per run with 450 bp average read lengths.

3.5.3 Bioinformatics

The sample-specific barcodes are introduced into each sample during the PCR step. After sequencing, individual sequences can then be traced back to samples using the barcodes they contain. The sequences from each sample are then separated, aligned, and then either used directly for taxa-based analyses or used to build trees for phylogenetic analyses.

The bioinformatics analysis proceeds through several steps as described in a workflow (Figure 6). The sequences were firstly denoised for errors introduced in PCR amplification and pyrosequencing using AmpliconNoise (Quince *et al*, 2011), as well as for chimeric sequences using PerseusD. Denoised sequences were aligned and sorted into OTUs in the RDP. Thereafter the sequence aligner SINA was performed against the SILVA SSU reference database (Quast *et al*, 2013) in order to identify the taxonomical belonging of each OTU cluster.

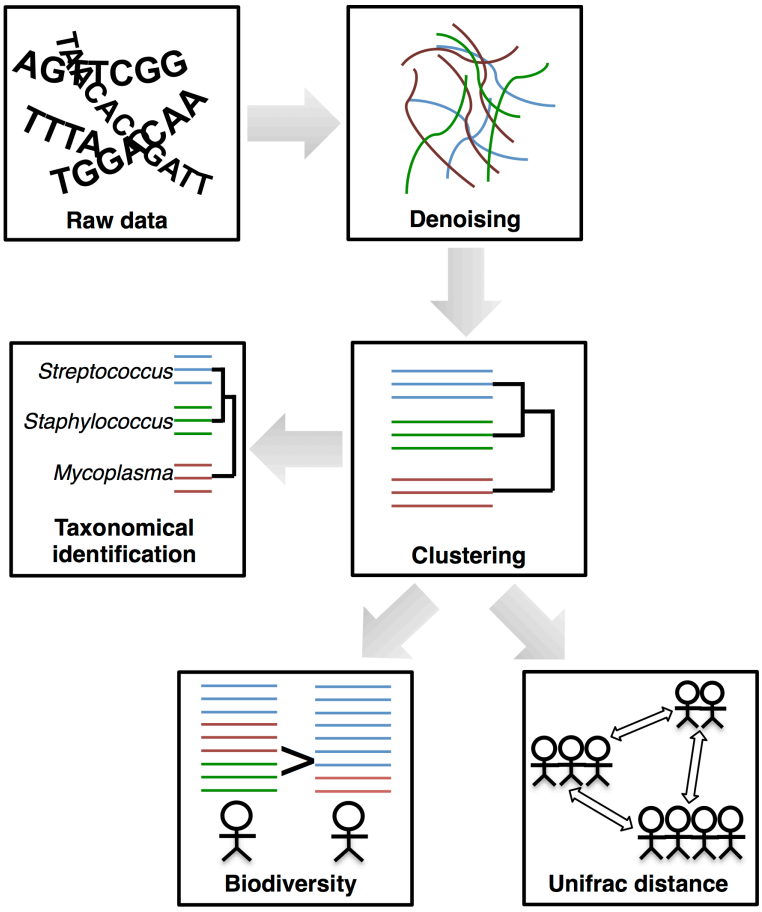

Figure 6. Workflow of the bioinformatic analysis on bacterial sequence data derived from 454 pyrosequencing. 454 pyrosequencing raw data –> 454 and PCR denoising and chimera removal (running AmpliconNoise) –> Clustering (running RDP cluster tool) –> Taxonomically identifying clusters (running SINA anligner) –> Statistic analysis: alpha diversity (Shannon index); beta diversity (Unifrac distance); visualization (PCoA); taxa comparison (edgeR).

The statistic analyses have included the following tests:

(1) The α-diversity in terms of Shannon diversity index, which evaluates the number (richness) and abundance distribution (evenness) of distinct types of microorganisms within a bacterial community, *i.e.* a specimen.

(2) The β-diversity in terms of Unifrac distance (Hamady *et al*, 2010), which measures the inter-sample variability and determines differences between communities. The β-diversity can be visualized using Principal Coordinate Analysis (PCoA) in the Unifrac platform.

(3) The comparison of the relative abundance of individual taxon between samples was performed using the R package empirical analysis of digital gene expression in R (edgeR) (Robinson *et al*, 2010).

3.5.4 Strengths and weaknesses of amplicon-based analysis

The quality and interpretation of the NGS data could be undermined at numerous steps, from sample collection, storage, and DNA extraction to PCR bias, sequencing errors, choice of algorithms for data processing, and statistical analyses. There have been and will be challenges in dealing with these data.

The earliest microbial surveys were based on fingerprinting techniques, which relied upon physical separation of 16S rRNA gene by denaturing gradient gel electrophoresis and terminal-restriction fragment length polymorphism (T-RFLP) analyses. Greater specificity was gained with Sanger sequencing. The long read lengths produced by this method are still a benefit in many studies.

The advent and commercialization of highly parallel sequencing techniques have revolutionized microbial community analyses by the greater sequencing depth and much lower cost. In comparison with another cornerstone of the NGS technologies Illumina, 454 has substantial longer read length, comparable sequencing error, but higher cost as of today due to the lower read density (Luo *et al*, 2012).

As all NGS technologies are PCR-based, the amplified DNA can only reflect the relative quantitative abundance. In addition, a cloning bias could be introduced in the amplification (von Wintzingerode *et al*, 1997). The variations in the 16S rRNA gene, for instance secondary structures like GC content and gene copy number, could contribute to the uneven amplification of the template and subsequently give the quantitative skewing in the PCR product. Therefore, a given taxon might finally be over- or under- estimated when such techniques are used for community screening. Further, as read extends during sequencing, a certain population of molecules might lose synchronicity, called dephrasing, which causes an increase in noise and errors. In addition, the process of amplification increases the complexity and time associated with sample preparation.

To data, a third generation also known as single-molecule sequencing technologies is emerging. Unlike major NGS that rely on PCR to grow clusters of a given DNA template, the new generation technologies interrogate single molecules of DNA with no

synchronization required, thereby overcoming issues related to the biases introduced by PCR amplification and dephasing. In addition, the new generation technologies have the potential to substantially increase throughput and accuracy, reduce sequencing time and cost, prolong read length up to tens of thousands bp per read, and require small amounts of starting material (Schadt *et al*, 2010).

4 RESULTS AND DISCUSSION

4.1 SEVERE CONGENITAL NEUTROPENIA

Two studies (Paper I and IV) were performed with patients with severe congenital neutropenia (SCN) to investigate the oral manifestation and the clinical indication of pro-LL-37 deficiency.

4.1.1 Periodontitis in patients with severe congenital neutropenia

The aim was to investigate if there is a genotype-phenotype relation regarding the periodontal health in patients with SCN. Previous studies have shown that patients with *ELANE* mutations correlate with more severe expression of neutropenia. We therefore addressed a hypothesis about a possible link between *ELANE* mutations and enhanced inflammatory reactions in periodontal tissue leading to periodontal break down.

Periodontitis is a bacterial infection affecting the tooth-supporting tissues and neutropenia is a putative risk factor for this disease. In Paper I, the periodontal condition of the patients with SCN varied from healthy to severe periodontitis and two patients were edentulous due to periodontitis, indicating great variation of the chronic inflammatory condition in the periodontium among patients with SCN.

We showed that patients with *ELANE* mutations presented with a poorer periodontal health compared to patients with *HAX1* or unknown mutations. This result is in line with the clinical findings that patients with *ELANE* mutations are associated with more severe phenotypes and need a more intensive G-CSF treatment (Bellanne-Chantelot*, et al*, 2004; Zeidler*, et al*, 2009). In total 13 patients with SCN were recruited, representing a majority of the patients diagnosed in Sweden. In this cohort, 5 out of 6 patients with *ELANE* mutations were found to have periodontitis or edentulism (being toothless) due to chronic periodontal disease despite of the different mutation sites harbored. In GCF, we tested the local concentration of the neutrophil granule-associated proteins pro-LL-37 and HNP1-3, which are important in maintaining the periodontal health due to their broad spectrum of antimicrobial activity. However, they did not show different levels between patients with *ELANE* mutations and other mutations. In addition, the results showed that the epithelial contribution to LL-37 levels was noticeably low. These findings indicated that other neutrophil related immunity might be different between patient groups.

As expected in the inflamed periodontium, the GCF samples from subjects with mutant *ELANE* had significantly higher levels of IL-1β and higher mean and median levels of IL-17, IL-6, and TNF-α than in those with *HAX1* or unknown mutations. Furthermore, along with the clinical manifestation, the periodontal bacterial flora from

patients with *ELANE* mutation was also different from patients with *HAX1* or unknown mutations, but similar to the flora in periodontitis from individuals without systemic disorder. Although it has been speculated that patients with a compromised host response may harbour selected and specific microorganisms with unique virulent potentials not found in normal patients (Baehni *et al*, 1983), we did not see a distinctive bacterial profile in patients with SCN compare to otherwise systemically healthy individuals. The periodontal pathogens of the genera *Fusobacterium*, *Prevotella*, *Treponema*, and TM7 domain are more likely to grow in the crevices of inflamed periodontium.

The neutrophils with *ELANE* mutations carry mutated elastase proteins that may be aberrant in their localization and functions such as proteolytic processing of other proenzymes or cytokines, which in turn may affect the local periodontal immune response (Meyer-Hoffert & Wiedow, 2011). Although it has been shown that neutrophil elastase activity was reduced in patients with SCN irrespective of the mutation status, the enzymatic activity was significantly lower in patients with *ELANE* mutations compared with those with wild-type *ELANE* (Germeshausen*, et al*, 2013). It has been shown that neutrophil serine proteases act as alternative processing enzymes of proinflammatory cytokines IL-1β and IL-18 *in vivo* and modulate other inflammation control mechanisms, such as progranulin inactivation, matrix metalloproteinase-9 activation, and IL-6 inactivation. Thus in addition to the antimicrobial peptide deficiency that is shared by all patients with SCN, it is possible that the *ELANE* mutations confer an additional neutrophil dysfunctionality, leading to more severe periodontal disease.

We also included one patient with cyclic neutropenia. This patient presented with healthy periodontal tissue and higher circulating pro-LL-37 at the time of sampling.

These findings indicate that the breakdown of periodontal tissue in patients with SCN might be the result of skewed balance of host immune defense and bacterial virulence, which is similar to the periodontitis in individuals without systemic disorder. However, the number of patients in our study is limited since SCN is a rare disease. Further investigation involving a larger cohort will be needed to confirm our findings.

4.1.2 Plasma pro-LL-37 in evaluation of chronic neutropenia

Chronic neutropenia is the reduction in the absolute number of neutrophils (segmented cells and bands) in the blood circulation and lasts two to three months or longer. It is a relatively frequent finding in clinics.

Chronic neutropenia encompasses many disease entities. It may arise secondarily to causes extrinsic to bone marrow myeloid cells, which is common, including autoimmune neutropenia or ethnic neutropenia; it may be as an acquired disorder of

myeloid progenitor cells, which is less frequent, for instance drug or virus induced myelopoiesis depression; or it is as an intrinsic defect affecting the proliferation and maturation of myeloid progenitor cells, which is rare, like SCN. In other occasions, when there is no clear reason for the neutropenia, it is categorized as idiopathic neutropenia. While differing in clinical severity, early diagnosis and treatment is of considerable importance for all forms of chronic neutropenia to prevent life-threatening infections. Plasma sampling is a simple method with limited invasiveness for determining a diagnosis. We assessed the possibility of using a plasma protein, pro-LL-37 as an additional tool for differentiating benign cases of chronic neutropenia from the severe forms with poor prognosis.

In this study the primary focus was on entities that lead to neutropenia as an isolated finding, but cases with neutropenia in the context of other immune defects are also reported. We showed that the group of patients with the disease SCN (n = 24) display the lowest levels of plasma pro-LL-37, as compared to the diagnostic groups of autoimmune neutropenia (AIN), idiopathic neutropenia (IN), and ethnic neutropenia (EN). We previously demonstrated that neutrophils of patients with SCN lack the proprotein pro-LL-37 and its active peptide LL-37, and this deficiency is common for all patients with SCN irrespective of disease-related mutations (Pütsep*, et al*, 2002; Andersson*, et al*, 2007). Plasma pro-LL-37 stems mainly from the neutrophil precursors in the bone marrow during myelopoiesis (Sørensen*, et al*, 1997a). The therapy by G-CSF, which leads to increased myelopoiesis and elevated ANC values, does not affect the plasma levels of pro-LL-37 (Karlsson *et al*, 2007). Therefore, using plasma pro-LL-37 in facilitating clinical diagnosis is valid irrespective of the use of G-CSF treatment.

In patients with neutropenia not as the sole clinical finding, we showed that the pro-LL-37 levels of patients with Shwachman-Diamond syndrome, Barth syndrome, aplastic anaemia, and AML were reduced and similar to those of patients with SCN. These diseases are characterized with either accelerated apoptosis of myeloid precursors or impaired myeloid development (Makaryan *et al*, 2012; Tulpule *et al*, 2013), which are similar features as in SCN. In addition, the patient with human herpesvirus 6 (HHV6) infection and the patients receiving treatment with thiamazole, presented with severely reduced plasma pro-LL-37 levels as well. Both HHV6 infection and thiamazole treatment are known to cause neutropenia possibly through affecting myelopoiesis. On the contrary, the plasma pro-LL-37 levels from patients with neutropenia secondary to other diseases, including ataxia telangiectasia, glucose-6-phosphate dehydrogenase deficiency, Graves' disease, Hyper IgE syndrome, or Papillon-Lefèvre syndrome were in the range higher than that from patients with SCN.

We hypothesize that the reduction of plasma pro-LL-37 is as a reflection of impaired myelopoiesis. Interestingly, the plasma pro-LL-37 levels did not correlate

with ANC levels in many cases. The levels of the neutrophils in the circulation may be affected by peripheral destruction as in immune neutropenia, exhausted as during post-bacterial infection, or low as a consequence of drug-induced redistribution of neutrophils. Therefore the ANC levels may in a number of clinical situations fail be an indicator of myelopoietic activity.

In the Swedish Paediatric Haematology Care Program issued in 2010, the analysis of pro-LL-37 was endorsed for diagnosis of chronic neutropenia although still as part of a translational research project. Our experience from using this analysis is thus positive in the sense that benign forms of neutropenia could be distinguished from the severe forms at an early stage. It is shown in this paper that plasma pro-LL-37 levels determined by a pro-LL-37 ELISA presented high specificity, sensitivity, and predictive values in distinguishing SCN from AIN, IN and EN, hence we suggest that the use of this diagnostic parameter could be developed for clinical use.

4.2 CHEMOTHERAPY-RELATED ORAL MUCOSITIS

In Paper II and III we studied the epidemiologic characteristics, predispositions, and microbial dynamics of oral mucositis, a frequently encountered adverse effect in patients undergoing cytostatic treatment. In these two papers, pro- and anti-inflammatory modulators in peripheral blood and oral bacterial flora from mucosal surface, which might underlie the development of oral mucositis, were studied.

4.2.1 Clinical risk factor and inflammatory mediators

In Paper II we aimed to investigate the clinical parameters in early identification of the patients with high risk of oral mucositis and also to increase our understanding regarding predispositions in the mucositis development. We showed that at the time of malignancy diagnosis, patients with acute leukemia, who had highest risk of oral mucositis, presented high concentrations of inflammatory cytokines and low levels of pro-LL-37 in the plasma.

The first episode of oral mucositis did not in all cases appear after the first course of the entire chemotherapy protocol. In many cases mucositis was detected after several courses of chemotherapies. The median time to onset of mucositis is six weeks in acute leukemia cases, one week in lymphoma, and three weeks in solid tumors. This finding could be related to the accumulative toxicity of chemotherapy reagents after continuous administration.

We included variables present before chemotherapy and those arising during chemotherapy in the univariable logistic regression model to evaluate their relation to the mucositis development. Parameters that showed significances were then further

included in the multivariable model in order to identify the most essential clinical indicator(s). In the final model based on all the patients, the malignancy diagnosis exclusively showed significance after the adjustment of the potential confounder methotrexate. To better evaluate the direct association between cytostatic regimens and the oral mucositis, we included individual cytostatic drugs as covariates instead of different chemotherapy protocols, a design which differs from some studies (Cheng*, et al*, 2008). The adjustment of the potential confounder methotrexate did not affect the significance of malignancy diagnosis in the multivariable model, which indicates that the association of acute leukemia and oral mucositis might be far more complex than the direct cytostatic effect of the intensive chemotherapy protocol applied.

To understand the high incidence of oral mucositis in the group of patients with acute leukemia, we compared the pretherapeutic levels of plasma cytokines across malignancy type. The plasma from patients with acute leukemia displayed significantly higher concentrations of both pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) and the anti-inflammatory cytokine (IL-10). This finding suggests that in patients with acute leukemia, a significantly elevated inflammatory status before the start of chemotherapy might contribute to the development of oral mucositis after the initiation of chemotherapy treatment. This finding is line with the current view that inflammatory cytokines is a key player in the pathogenesis of oral mucositis (Sonis, 2004; Logan*, et al*, 2008b; Meirovitz *et al*, 2010; Morales-Rojas *et al*, 2011).

We also showed that the pro-LL-37 level at the time of malignancy diagnosis was lower in patients with acute leukemia compared to those with lymphoma or solid tumors. Pro-LL-37, the proform of antimicrobial peptide LL-37 (Agerberth *et al*, 1995; Sørensen*, et al*, 2001), plays a protective role in the oral health. Therefore, pretherapeutic low levels of pro-LL-37 may render oral mucosa more vulnerable to the destruction caused by commensal or pathological oral bacteria, leading to the breakdown of epithelium of oral mucosa.

The neutropenia has been previously suggested as a risk indicator of oral mucositis in patients receiving chemotherapy (Cheng*, et al*, 2008), however in our study, we did not find neutropenia or neutrophil counts at the time of malignancy diagnosis to be associated with oral mucositis. The occurrence of neutropenia was evaluated strictly only before the time point of oral mucositis appearance, which suggested that neutropenia might only be a concomitant side effect rather than a risk indicator of oral mucositis. Cytostatic treatments generally reduce the number of leukocytes and it has been controversial regarding the role of neutropenia in the development of oral mucositis (Locatelli *et al*, 1996; Gandemer *et al*, 2007; Cheng*, et al*, 2011). Future research based on single type of malignancy is needed to confirm our finding.

Due to the limited number of patients included in this paper, it is difficult to compare the plasma parameters between mucositis and no mucositis cases within the

same malignant type. This could be studied in the future to see if the relation between pretherapeutic inflammatory mediators and mucositis risk could be generalized in the context of a single malignant type. This kind of study will further elucidate the role of pretherapeutic plasma cytokines in the development of oral mucositis.

In conclusion, in Paper II we mainly show that at the time of malignancy diagnosis, patients with acute leukemia, who had the highest risk of oral mucositis, presented high concentrations of inflammatory cytokines and low levels of pro-LL-37 in the plasma. We demonstrate the pretherapeutic elevated inflammatory state might predispose the patients to oral mucositis, which is frequently overlooked when current research focus has been mainly put on the pathogenic events after the initiation of chemotherapy.

4.2.2 Bacterial flora in oral mucositis

The pathogenesis of oral microorganisms in mucositis remains largely unknown, yet determination of the potential role of oral entrogeneous bacterial species in the development of oral mucositis, is important. In Paper III, we looked into the oral mucosal bacterial dynamics in relation to chemotherapy-related oral mucositis. We showed that at the time of malignancy diagnosis, a more heterogeneous bacterial community with higher microbial diversity was found in the patients that later developed oral mucositis compared to no mucositis group. In addition, we found a more pronounced shift of the bacterial composition after the initiation of chemotherapy in patients that later developed oral mucositis compared to those that did not.

We firstly showed that the entire group of patients with malignancies exhibited a less diverse bacterial community and presented more dissimilarity among one another compared to the reference children. This difference potentially may be attributed to the compromised host immunity and systemically altered inflammatory response caused by the malignancies (Ascierto *et al*, 2011), and can be a consequence of the single-dose prophylactic cefotaxime.

At the time of malignancy diagnosis, a higher level of diversity was detected in patients that later developed mucositis compared to the group that did not. In addition, the bacterial community composition of the mucositis group was found to be more heterogeneous than the no mucositis group. The comparison of inter-group and intra-group Unifrac distance did not reveal a distinct profile between mucositis and no mucositis group, which is likely due to the fact revealed by recent evidence showing that inter-subject diversity occupies a smaller dynamic range than the diversity within communities, *i.e.* the entire bacterial profile from the same habitat is similar among subjects (Human_Microbiome_Project_Consortium, 2012).

In both patient groups, the mean and median values of microbial diversity were decreased after the start of chemotherapy, although no significant difference was found. This finding is contrary to the result from a previous study showing a more complex microbial community after chemotherapy using Sanger sequencing (Napenas*, et al*, 2010). The discrepancy may be partly attributed to the method difference. After receiving chemotherapy, a more pronounced shift of bacterial community profile was found in the patients that later developed mucositis compared to the ones that did not, which might indicate a beneficial effect of a higher microbial stability. Notably, a significant decrease of the phylum Proteobacteria was detected in the mucositis group during chemotherapy, while no such change was found in the group without mucositis. This difference between patient groups might not be simply explained by the antibacterial agents or cytostatic treatment, as these parameters did not show significant difference between groups.

Microbial colonization of the mucositis lesion might intensify the ulceration severity and increase the risk of systemic infection. The breakdown of mucosal barrier provides a port for the endogenous bacteria invasion. In this study, we identified a distinctive bacterial composition from the mucositis lesions compared to all the mucosal samples from lip and bucca. Although the bacterial profile from the mucositis lesions may not help to understand the pathomechanisms of mucositis development, it is of clinical importance to investigate the potential oral pathogens that can cause systemic infection. The ulcerations represent a different niche in which virulent pathogens can compete successfully with the resident bacteria, in addition to the effect of the empirical antimicrobial therapy administered.

The contribution of the oral microflora to mucositis remains to be clarified. So far a large number of studies investigating preventive management failed to achieve any conclusive findings (Jensen *et al*, 2013; Saunders*, et al*, 2013), which is mostly due to our limited knowledge of the pathobiology of oral mucositis. This study is the first attempt to use next generation sequencing method to map the microbial profile in relation to the occurrence of oral mucositis in patients receiving chemotherapy. The limitations of this study include, (1) the heterogeneity of patients in terms of malignancies, which makes the control of clinical confounders difficult; (2) due to the different treatment protocol involved in treating malignancies, the sampling time interval was not the same in all patients, although we controlled for the cytostatic agents and antibacterial treatment.

In conclusion, at the time of malignancy diagnosis, patients who later developed oral mucositis showed higher oral mucosal microbial diversity and were more heterogeneous among one another compared to those who did not develop mucositis. A more pronounced modification of the bacterial community by chemotherapy was detected in patients that later developed oral mucositis, indicating that oral microbial

stability might be beneficial. These findings might contribute to the development of better prophylactic treatments and improved intervention protocols against oral mucositis, tailored to individual patient.

5 CONCLUDING REMARKS

Oral health appears to depend on the nature of host immunity defect. Immunity defects, whether caused by primary immunodeficiency, by hematologic malignancies, or by intensive cytostatic therapy, have profound effects on the oral tissues.

We showed that the patients with SCN harboring *ELANE* mutations presented with a poorer periodontal health, which is in line with other medical findings, *i.e.* more severe neutropenia and higher dose of G-SCF treatment. This finding may be of clinical importance in the early proactive management of oral care in these patients. Future studies are needed to elucidate the pathobiology of the *ELANE* mutation in disturbing neutrophil maturation, and affecting functions of neutrophils and possibly other immune defense components. Moreover, the potential pathogenic differences of periodontal destruction in patients with SCN and those without systemic disorders are of interest to study.

Oral mucositis is by far a poorly managed clinical complication during cytostatic chemotherapy with mainly palliative approaches available. In pediatric patients with malignancies and undergoing chemotherapy, we have studied the inflammatory state and dynamics of the oral bacterial community, which all may be of significance in the development of oral mucositis. The work of this thesis may be clinically important in developing better prophylactic and therapeutic intervention for oral mucositis. Future studies illustrating the relation between oral microbial composition and local capacity of antimicrobial defense along the chemotherapy are needed to understand if mucositis is of infectious etiology.

The now emerging third generation also known as single-molecule sequencing technologies overcomes issues related to the biases introduced by PCR amplification and require small amount of starting material. These techniques may further help to decipher the details of the pathogenetic influence of oral microbiota in the process of periodontitis and oral mucositis.

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