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## Novel innate immune functions of saliva

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# Novel innate immune functions of saliva

Tirthankar Mohanty

DOCTORAL DISSERTATION

by due permission of the Faculty Medicine, Lund University, Sweden.

To be defended on the ***November 19<sup>th</sup> at 9.00 A.M*** in Belfragesalen  
Biomedical center, Lund, Sweden

*Faculty opponent*

Professor Dr. Maren von Köckritz-Blickwede

Department of Physiological Chemistry

University of Veterinary Medicine

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Faculty of Medicine

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Title and subtitle Novel innate immune functions of saliva		
<p><b>Abstract</b></p> <p>The innate immune system in humans has succeeded in developing numerous mechanisms against the injurious effects of bacteria, along with their toxins as a result of several thousand years of co-evolution. The oral mucosal surface represents such a unique environment, where despite being constantly exposed to microbes and their products, overt infection and damage is a rarity. An important component of the human oral cavity is saliva that is known to aid a wide variety of functions, which not only includes basic physiological activities like swallowing, but also preservation of the overall health of the oral cavity. Saliva flow that is diminished in either quantity or quality is often linked to development of numerous oral maladies. The oral cavity harbors a diverse and abundant microflora interacting with saliva. Saliva has innate immune functions and many direct interactions between saliva and bacteria have been described previously. We therefore chose to study the interaction between saliva and other components of the innate immune system in the oral cavity. Salivary lipids were found to improve antimicrobial peptide (AMP) synthesis and promote clearance of intracellular bacteria in keratinocytes. We also observed how saliva modulates the functions of the prime guardian leucocyte, the neutrophil. Saliva triggers a response via salivary mucins, which stimulates neutrophils to undergo a rapid mode of cell death called NETosis, wherein the neutrophils extrude a web to catch microbes in the form of a DNA framework decorated with AMPs known as neutrophil extracellular traps (NETs). These saliva-induced NETs had great capacity for entrapment and killing of bacteria. Lastly, we explored the effect of saliva on plasma, in presence of pathogenic streptococci. Streptococcal pharyngitis is defined by plasma exudation into a saliva rich environment. We found that upon intermixing with plasma, saliva triggers several proteolytic cascades within plasma that include the complement system and both arms of the coagulation systems. This results in clot formation that entraps bacteria of the oral flora. Using a well-characterized virulence factor, streptokinase, the important human pathogen <i>Streptococcus pyogenes</i> subsequently sequester and activate host plasminogen, enabling them to escape the clots. This thesis describes how saliva boost the function of other important components of the innate immune system in the oral cavity, namely keratinocytes, neutrophils and plasma and thereby shed light on some of the molecular mechanisms involved in health and disease.</p>		
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# Novel innate immune functions of saliva

Tirthankar Mohanty



**LUND UNIVERSITY**  
Faculty of Medicine



Tirthankar Mohanty

Department of Clinical Sciences  
Division of Infectious Diseases  
Faculty of Medicine  
Lund University

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Cover: Immunofluorescence image of saliva collected immediately after waking up containing bacteria, leucocytes, neutrophil elastase and integrin  $\beta$ -3.

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*To Bapa, Baba, Mama, Bhai and Bhauja.*

*All knowledge is good.*

*It is impossible to say any fragment of knowledge,  
however insignificant or remote from one's ordinary pursuits,  
may not some day be turned to account.*

- Thomas Huxley, *Science and Education: Essays*



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# *Preface*

I began my work at the Sørensen lab as a part of my master's thesis in the beginning of 2010. The unifying element of this thesis is saliva, an often ignored and challenging body fluid to work with. My initial reaction towards the idea of working with saliva was in fact a mixture of astonishment and anguish. But after five years (time sure flies by!), my limited efforts have culminated in this thesis.

The broad range of subjects dealt with in the thesis makes it difficult to address each individual topic in detail. Therefore, the structure of the thesis includes a brief introduction to some key concepts. These, I assume will help the reader before the study of the manuscripts included in the thesis.

The data presented in the thesis is indicative towards the beneficial role of saliva in maintaining a healthy oral cavity. There have been a fair amount of challenges and exciting results and they have provided me with a fascinating and creative experience over the years. I sincerely hope that the reader enjoys reading the thesis and learns something new in the process.

Tirthankar Mohanty

Lund, 2015



# *Abstract*

The innate immune system in humans has succeeded in developing numerous mechanisms against the injurious effects of bacteria, along with their toxins as a result of several thousand years of co-evolution. The oral mucosal surface represents such a unique environment, where despite being constantly exposed to microbes and their products, overt infection and damage is a rarity. An important component of the human oral cavity is saliva that is known to aid a wide variety of functions, which not only includes basic physiological activities like swallowing, but also preservation of the overall health of the oral cavity. Saliva flow that is diminished in either quantity or quality is often linked to development of numerous oral maladies.

The oral cavity harbors a diverse and abundant microflora interacting with saliva. Saliva has innate immune functions and many direct interactions between saliva and bacteria have been described previously. We therefore chose to study the interaction between saliva and other components of the innate immune system in the oral cavity. Salivary lipids were found to improve antimicrobial peptide (AMP) synthesis and promote clearance of intracellular bacteria in keratinocytes. We also observed how saliva modulates the functions of the prime guardian leucocyte, the neutrophil. Saliva triggers a response via salivary mucins, which stimulates neutrophils to undergo a rapid mode of cell death called NETosis, wherein the neutrophils extrude a web to catch microbes in the form of a DNA framework decorated with AMPs known as neutrophil extracellular traps (NETs). These saliva-induced NETs had great capacity for entrapment and killing of bacteria. Lastly, we explored the effect of saliva on plasma, in presence of pathogenic streptococci. Streptococcal pharyngitis is defined by plasma exudation into a saliva



rich environment. We found that upon intermixing with plasma, saliva triggers several proteolytic cascades within plasma that include the complement system and both arms of the coagulation systems. This results in clot formation that entraps bacteria of the oral flora. Using a well-characterized virulence factor, streptokinase, the important human pathogen *Streptococcus pyogenes* subsequently sequester and activate host plasminogen, enabling them to escape the clots.

This thesis describes how saliva boost the function of other important components of the innate immune system in the oral cavity, namely keratinocytes, neutrophils and plasma and thereby shed light on some of the molecular mechanisms involved in health and disease.

# *List of papers*

## **Paper I**

Mohanty T., Alberius P., Schmidtchen A., Reiß K., Schröder J. M., Sørensen O.E. Saliva induces expression of antimicrobial peptides in human keratinocytes and accelerates antimicrobial peptide expression in injured human skin. (Manuscript).

## **Paper II**

Mohanty T., Sjögren J., Kahn F., Abu-Humaidan A.H.A, Fisker N., Assing K., Mörgelin M., Bengtsson A. A., Borregaard N., Sørensen O. E. A novel mechanism for NETosis provides antimicrobial defense at the oral mucosa. Blood. Prepublished August 4, 2015: DOI 10.1182 blood-2015-04-641142. [Epub ahead of print]

## **Paper III**

Wollein Waldetoft K., Mohanty T., Karlsson C., Ruttardottir S., Mörgelin M, Frick I.M., Malmström J., Björck L. Saliva Activates the Intrinsic Pathway of Coagulation to Capture Streptococci but the Bacteria Escape by Inducing Fibrinolysis. (Manuscript).



# *List of papers not included*

## **Paper I**

Jena P., Mohanty S., Mohanty T., Lindstrøm T., Borregaard N., Sonawane A., Sørensen O. E. (2012) Azurophil granule proteins constitute the major mycobactericidal proteins in human neutrophils and enhance the killing of mycobacteria in macrophages. PLoS One. 2012;7(12):e50345.

## **Paper II**

Murphy E.C., Mohanty T., Frick I.M. FAF and SufA: proteins of *Finleishia magna* that modulate the antibacterial activity of histones. Journal of innate immunity. 2014;6(3):394-404.

## **Paper III**

Abu-Humaidan, A.H.A., Ananthoju, N., Mohanty T., Sonesson, A., Alberius, P., Schmidtchen, A., Garred, P., Sørensen, O. E. The epidermal growth factor receptor is a regulator of epidermal complement component expression and complement activation. Journal of immunology 2014 Apr 1;192(7):3355-64.



# *Introduction*

## **Saliva**

### ***Function and composition***

Saliva functions to facilitate taste, maintain enamel integrity, buffering activity, mastication and lubrication of the food bolus to aid in swallowing, speech and cleaning of the teeth. Apart from this, saliva as a mucosal fluid plays a major role in keeping the oral cavity moist, prevention of cellular desiccation and protects the mucosa from harmful foreign agents.

Saliva is an exocrine mucoserous secretion, with a pH range of about 6.2-7.4. The contribution of saliva as a valuable oral fluid is often overlooked and does not receive much attention till the quality or quantity of secretion is affected adversely. About 1-1.5 liters of saliva is secreted daily in a healthy individual, most of which is swallowed and reabsorbed in the gut. The major salivary glands contributing to saliva flow are the paired parotid glands (20% of total secretion), submandibular glands (65-70% of total secretion) and the sublingual glands (7-8% of total secretion). Minor glands present on the lower lip, tongue, palate, cheeks and pharynx also add to the total secreted saliva (10%). About 98% of saliva is water. Saliva contains electrolytes like bicarbonate, sodium, magnesium, potassium, calcium, phosphate and chlorine. The buffering capacity of saliva is important in prevention of colonization with microbes and maintenance of the integrity of the dental enamel (Dodds *et al.*, 2005).

It is noteworthy that in comparison to plasma or extracellular fluid, saliva is hypotonic. Its organic constituents include the large glycoprotein molecules known as mucins that give saliva its characteristic viscosity, enzymes for digestion, antimicrobial peptides and immunoglobulins. Saliva also contains a natural analgesic called opiorphin, which has been shown to have a greater effect than morphine in rats (Wisner *et al.*, 2006). Apart from proteins, saliva is also known to contain lipids that may be neutral (e.g.- cholesterol, cholesteryl esters, free fatty acids), polar (e.g.- phospholipids) and glycolipids (e.g.- cerebrosides) (Larsson *et al.*, 1996). Some phospholipids, which are bioactive like lysophosphatidic acid (LPA) (Moolenaar *et al.*, 1997), platelet activating factor (PAF) (Cox *et al.*, 1981) and sphingosine-1-phosphate (S1P), are also found. Peptide growth factors like epidermal growth factor (EGF) and nerve growth factor (NGF) are only present in minute quantities in human saliva (Bodner, 1991); (Li *et al.*, 1980). Unlike its intravascular counterpart plasma, resting saliva is nutrient poor (low in glucose) and hypotonic, possibly to prevent overgrowth of bacteria and facilitate taste.

Saliva also contains antimicrobial substances derived from neutrophils, oral keratinocytes or salivary glands. Peptides like  $\alpha$ -defensins (McKay *et al.*, 1999) and LL-37 (Murakami *et al.*, 2002) are neutrophil-derived;  $\beta$ -defensins are derived from oral keratinocytes (Abiko *et al.*, 2003) and histatins are secreted by salivary glands (Ahmad *et al.*, 2004). Sir Alexander Fleming discovered the antibacterial role of lysozyme when he famously demonstrated that nasal mucus was able to reduce bacteria grown on a plate. It was subsequently demonstrated to be present in other body fluids including saliva (Fleming, 1932).

## ***Salival mucins***

Mucins are one of the major components of saliva that render saliva viscous. Salival mucins are heavily glycosylated glycoproteins, consisting of about 80% carbohydrates that are secreted by both major and minor saliva glands of the oral cavity. The sublingual and submandibular salivary glands are the major mucin-producing glands. The chief mucin components found in saliva are the gel-forming MUC5B (also known as high-molecular-mass salivary mucin MG1) and the soluble monomeric MUC7 (also known as low-molecular-weight salivary mucin MG2). Both mucins possess a net negative charge and consist of a protein core (about 20%) and are made up of large number of tandem repeats of serine, threonine and proline. The proteinaceous backbone is densely surrounded by moderately branched carbohydrate structures consisting primarily of *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, galactose and sialic acid (*N*-acetylneuraminic acid), and trace amounts of sulfates of mannose. *O*-glycosidic bonds constitute the majority of the bonds and connect the hydroxyl groups of serines and threonines on the protein core to the carbohydrate side chains. *N*-linked glycosidic bonds are lesser in number and are centered on the –N and –C termini of the protein core, which has lower *O*-glycosidic bonds. The final structure of the mucins possesses a characteristic ‘bottle-brush’ or ‘Christmas tree’ appearance. Due to their massive and complex glycoprotein branches, both MUC5B and MUC7 are able to interact with an extensive array of salivary molecules as well as cells like neutrophils and keratinocytes. Mucins form viscoelastic hydrophilic gels that not only lubricate and moisten the oral cavity, but also contribute to the protection of the oral mucosa from irritants, maintenance of tooth enamel and aggregation and bacterial clearance from the oral cavity (Karlsson and Thomsson, 2009; Thomsson *et al.*, 2002).

Interestingly, sialidase producing bacteria seem to take advantage of terminal host sialic acid residues, by harvesting them and metabolizing them, or displaying them on their surface to facilitate immune evasion



(Carlin *et al.*, 2009b; Corfield, 1992). Removal of terminal sialic residues from mucins may also result in reduced clearance of bacteria, thereby promoting survival of sialidase producing bacteria (Corfield, 1992).

Analysis of individual salival components for study is excruciatingly difficult. It is largely due to the mucins due to their property to aggregate host molecules as well as the commensal microflora. Removal of mucins during the processing, can often lead to loss of certain desirable molecules. Therefore, we did not use any chemical agent like DTT as a thinning agent to retain function of salivary components.

### ***Role of saliva in maintaining oral homeostasis and role in innate immunity***

In humans, a disturbance in both the quality and/or quantity of saliva results in delayed wound healing and leads to a variety of conditions. Xerostomia, which is also commonly known as ‘dry mouth’, is such a condition that is noticed in patients who suffer from acquired immunodeficiency syndrome (AIDS), autoimmune diseases (e.g. Sjögren’s syndrome) and patients undergoing radiotherapy. As a consequence of reduced saliva flow associated with this disorder, fungal infections of the oral cavity with *Candida albicans* and inflammation of salivary glands and lips are commonly observed maladies. Diseases associated with reduced saliva flow thus emphasize the importance of saliva in maintaining a proper and healthy oral cavity (Dodds *et al.*, 2005; Napenas *et al.*, 2009).

It is indeed tempting to speculate that diminished saliva flow may hamper the defense pathway in which bacteria attached to shed-off

epithelial layers are swallowed and neutralized by stomach acids. This may also result in aiding to the bacterial overgrowth.

### ***Woundlicking and growth promoting effects of saliva***

An interesting role attributed to saliva is the phenomenon of wound licking, which is an instinctive response exhibited by higher animals towards external injury. A variety of animals like cats, dogs, rodents, and primates, including humans, are known to demonstrate this behavior. This not only keeps the wound physically clean but it also may deliver antimicrobial substances and growth promoting factors in and around the wound area. Inhibition of wound licking in rats either by removal of salivary glands or preventing the act of licking each other's wounds in a community (also referred to as 'community licking') leads to delayed wound healing. Licking of mammary and anogenital areas prior to birth by rodents is a noticeable phenomenon. This act of self-licking strengthens the nipples and prevents the new born pups from acquiring infections post parturition (Bodner, 1991; Bodner *et al.*, 1991a; Bodner *et al.*, 1991b). Saliva from mice also contains high amounts of epidermal growth factor (EGF) and nerve growth factor (NGF) that aids rapid healing of wounds (Li *et al.*, 1980). However, human saliva only contains minute quantities of these growth factors. In humans, histatins are found in saliva, which have been demonstrated to accelerate wound closure and have been hypothesized to be an important salivary component required for protection of tissues (Oudhoff *et al.*, 2009).

### ***Influence of saliva on complement and coagulation cascades***

Activation of the complement system in response to intruders forms an integral part of the innate immune system. Complement deposition products like C3a and C5a are powerful chemoattractants, priming

agents and act as danger signals for neutrophils as well as peripheral blood monocytes. Another important function of complement deposition on bacteria as well as dying cells is marking them for clearance by phagocytes. Saliva contains very little effector complement components, but rather contains complement regulatory elements. However, complement rich body fluids like gingival crevicular fluid and tissue transudates come in contact with saliva during inflammation of the gums (periodontitis) or of the throat (pharyngitis). Saliva contains aggregating substances like mucins, immunoglobulins and non-immunoglobulin agglutinins that collaborate in initiating the complement cascade. Interestingly, saliva owing to its low osmolarity enhances C1 binding and can lead to notable C4b deposition (Boackle, 1991).

The coagulation cascade is an important part of the innate immune system. Following mechanical or infection caused injury both the extrinsic and intrinsic pathways of coagulation are activated. The deposition of the end products of coagulation, particularly the fibrin/platelet web and antimicrobial substances generated during the process promote bacterial capture as well as killing (Frick *et al.*, 2006; Wollein Waldetoft *et al.*, 2012). Saliva has also been demonstrated to efficiently trigger coagulation even though it lacks coagulation components or fibrinogen (Berckmans *et al.*, 2011). In fact, the capacity of saliva in accelerating clotting (prothrombin time) is comparable to the snake venom reptilase from *Bothrops atrox*, which acts on the final step of coagulation. Tissue factor, which initiates the extrinsic pathway of coagulation, is known to be present on salivary microparticles and aid in clotting. Salivary tissue factor has been alleged to provide an extravascular source of tissue factor that could play a role in reducing blood loss and prevent dissemination of bacteria into the blood stream after wound licking (Berckmans *et al.*, 2011)

# **Innate immune system**

The total number of commensal bacteria on human skin and mucosal surfaces is estimated to be  $10^{14}$ , which is roughly ten times the number of cells comprising the entire human body. These microbes are involved in highly complex interactions with the host immune system. The exchange between the host and the commensal flora develops the immune system, while simultaneously the specialized commensals outcompete potential exogenous pathogenic bacteria.

Although the commensals are beneficial to survival of the host, these may invade host tissues, resulting in pathogenesis. Therefore, a proper immunological barrier is important in preventing invasion by commensals. In fact, opportunistic infections in immunocompromised individuals are an example of how easily a harmless commensal might turn into a pathogen due to the lack of intervention of the immune system. The immune system that provides security to the host's internal environs can be broadly classified into adaptive and innate immune systems. The innate immune system is sufficient enough to prevent the human body from being invaded periodically by its overwhelming commensal flora. Once, microorganisms have bypassed the innate immune system and garnered a foothold in a more internal niche, only then the adaptive immune system is activated. Both arms of the immune system in concert thwart off the invading microbe. Again, microorganisms that have the ability to bypass the immune system and invade host tissues, resulting in symptomatic disease can be labeled as pathogens (Potempa and Pike, 2009).

The innate immunity is active against microbes regardless of prior exposure. In a wider sense, the innate immune system includes physical cellular barriers, along with their effector molecules. Unlike the adaptive immunity, this ancient immune system is found in both invertebrates and vertebrates. Pattern recognition is key in defining the innate immune

response. Conserved bacterial motifs (or patterns) are recognized by pattern recognition receptors (PRRs) present on various effector cells. The patterns that are recognized can either be of bacterial origin (Pathogen associated molecular patterns, PAMPs) or that may arise due to damage to host tissues (Danger associated molecular patterns, DAMPs) (Matzinger, 1994); (Medzhitov and Janeway, 2002). Some of the key structures and cells of the innate immune system include skin, hair, neutrophils, macrophages, dendritic cells and natural killer cells (Potempa and Pike, 2009).

An altered commensal barrier in the gut has been shown to be associated with inflammatory bowel disease (Ott *et al.*, 2004). Arguably, the innate immune system together with the commensals forms an intelligent shield composed of both self and non-self components that act synergistically to prevent the colonization of external pathogens. This results in the maintenance of overall health of the host. As a result of healthy conditions, the commensals also get a chance to survive. Commensals are usually thought to be passive residents, but in reality they are involved in exchanges with the host that is mutually beneficial for both. Therefore, the term ‘commensal’ is a little misleading.

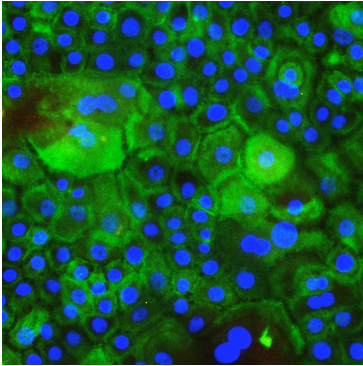
In the following sections, the components of the innate immune system interacting with saliva in oral cavity and epidermis during wound healing are described.

# Epidermal keratinocytes

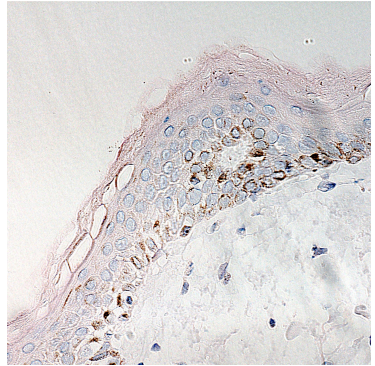
The skin is the largest organ in the human body and is constantly exposed to a plethora of potentially harmful microbes. The primary function of the skin is to separate the host's internal milieu from the external environment and protect against insult and injury. The tight junctions and extremely rigid cytoskeleton prevents water loss as well as protects from mechanical damage. The skin is comprised of the dermis and the epidermis. The epidermis is the outermost layer and is primarily composed of keratinocytes. It consists of the following layers (arranged from the outermost to the inner most layers): stratum corneum, stratum lucidum, stratum spinosum, stratum basale and the basal lamina. Keratinocytes begin their journey from the lower most stratum basale and rest above the basal membrane. These cells possess the ability to divide and have a rather flexible plasma membrane. They are then gradually pushed outward by the underlying layer of dividing cells towards the exterior, during which they begin to differentiate. Finally upon reaching the outermost stratum corneum, as a result of terminal differentiation, they terminate their journey as dead, anucleate and flattened cells called corneocytes. As a result of the terminal differentiation, the proteins and lipids in the cells in the stratum corneum are crosslinked with each other. This protein-lipid crosslinking is vital in making the epidermis impervious to water (Kalinin *et al.*, 2002). The primary function of keratinocytes is the formation of a semi pervious barrier that is impermeable to microbes, but also at the same time allows the secretion of substances like sweat and sebum to facilitate thermal regulation and prevention of cracking of the skin in response to high temperature or dry conditions (Candi *et al.*, 2005).

The underlying dermis is rich in adipocytes macrophages and fibroblasts. This layer also contains sebaceous glands that secrete fatty acids, which provides skin with an oily texture. The fatty acids some of which are produced constitutively make the epidermis impervious to water as well as act as antimicrobials (Cartron *et al.*, 2014; Feingold and Elias, 2014).

(a)



(b)



**Figure 1-** Human keratinocytes (a) Primary human keratinocytes *in vitro*. The actin cytoskeleton of the cells is stained with phalloidin conjugated with alexa 488 (green) and the nuclei are stained with DAPI (blue). (b) Distribution of keratinocytes in healthy human skin. The cells possess a circular nucleus. The dermis is visible as a vacuolated area right below the cellular layer. The stratum corneum can be seen as a flaky exterior layer, immediately above the cellular layer.

# **Wound healing and keratinocyte AMP synthesis**

One of the strategies used by keratinocytes to prevent infection is through the production of peptide antibiotics, called antimicrobial peptides (AMPs). AMPs have been demonstrated to be important tools of the host innate immune response, especially epithelial surfaces that include the hollow of the eyes and the blind sacs of the gut, lungs, the urogenital system and the skin. Some AMPs like human beta defensin-1 (hBD-1) and Rnase7 are constitutively expressed by epidermal keratinocytes, whereas others are induced at sites of inflammation, infection or injury (Roupe *et al.*, 2010).

Wound healing can be broadly classified into four phases namely hemostasis, inflammation, the proliferation and remodeling phases. Injury necessitates the re-establishment of barrier function of skin through growth and differentiation of keratinocytes, fibroblasts and vascular endothelial cells to name a few. Local cutaneous injury results in a rapid response through the activation of coagulation and complement cascades, and an influx of peripheral circulatory immune cells to prevent excessive blood loss and entry of microbes into the host (Proksch *et al.*, 2008).



## ***Hemostatic and inflammatory phase***

The exudation of plasma and subsequent activation of the intrinsic pathway and complement cascades mark the beginning of the first major defensive mechanism after injury (Frick *et al.*, 2006). In the earliest phase of wound healing, about 0-72 hours, vasoconstriction, coagulation and complement cascades, neutrophils, platelets and macrophages are the key players. The complex interplay of immune cells and proteolytic hemostatic cascades orchestrate inflammation by secreting a vast array of cytokines, growth factors and antimicrobial substances (Falanga, 2005). Neutrophils deliver an impressive payload of antimicrobial proteins to the injury site, which includes hCAP18/LL-37, neutrophil elastase,  $\alpha$ -defensins and calgranulins (S100A8/9) (Borregaard and Cowland, 1997; Soehnlein, 2009).

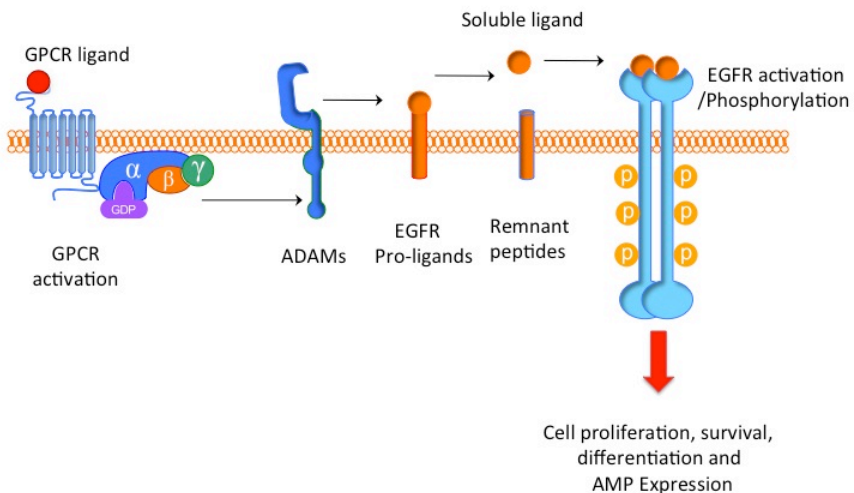
## ***Proliferative phase***

The inflammatory cells gradually begin to withdraw from the wound, marking the resolution of the inflammatory phase and the beginning of the proliferation phase. During the proliferation phase epidermal keratinocytes are involved in reepithelialization but also become a rich source of AMPs (Roupe *et al.*, 2010).

The epidermal growth factor receptor, EGFR, is a key growth factor receptor during wound healing. Apart from direct activation, EGFR is activated through a paracrine process called transactivation, wherein membrane bound EGFR ligands like TGF- $\alpha$ , heparin binding EGF (HB-EGF) and amphiregulin are cleaved by matrix metalloproteases of members of the ADAM (a disintegrin and metalloproteinase) family through activation by G-protein coupled receptors (GPCRs) (Figure 2) (Prenzel *et al.*, 1999). EGFR activation induces expression of a multitude of AMPs including hBD-3, S100A8/9, S100A15, elafin, SLPI, NGAL,

RNase7 and Psoriasin. Although, the human cathelicidin hCAP18/LL-37 has been detected during the inflammatory phase, its presence in keratinocytes during the proliferative phase is scarce. IL-8, which is a potent neutrophil attractant is also synthesized upon EGFR activation has been hypothesized to be involved during neutrophil recruitment and this provides the essential link between injury and neutrophil recruitment in human skin (Buchau, 2010; Roupe *et al.*, 2010). Interestingly, epidermal keratinocytes are able to produce remarkably similar AMPs when compared to neutrophils in the inflammatory phase (Borregaard *et al.*, 2005; Theilgaard-Monch *et al.*, 2004). The following comparative table (Table 1) illustrates the shared AMPs of neutrophils and epidermal keratinocytes.

## EGFR Transactivation



**Figure 2** – Schematic depiction of EGFR transactivation. Upon ligand binding, G protein coupled receptors (GPCRs) are activated, which in turn lead to activation of membrane bound metalloproteases belonging to the ADAM (A disintegrin and metalloproteinase) family. ADAM mediated cleavage leads to the release of membrane bound EGFR ligands such as HB-EGF and TGF- $\alpha$ . The EGFR ligands on binding the EGFR lead to receptor dimerization and phosphorylation. EGFR activation is vital for regulating cell proliferation, survival, differentiation and AMP expression.

## AMPs in neutrophils and keratinocytes

### Neutrophils

*Azuropilic granules*

$\alpha$ -defensins (HNP-1-4)  
Lysozyme

*Specific, gelatinase granules*

Lactoferrin, NGAL, hCAP-18/LL-37  
Transcobalamin, SLPI, lysozyme

*Cytosol*

Calgranulins (S100A8/A9)

*Produced post-extravasation*

Elafin

### Keratinocytes

$\beta$ -defensins (hBD-1-3)  
Lysozyme

Lactoferrin, NGAL, hCAP-18/LL-37  
Transcobalamin, SLPI, lysozyme

Calgranulins (S100A8/A9)

Elafin

*AMPs unique to keratinocytes*

Psoriasin, RNase7, S100A15

**Table 1** - Antimicrobial peptides and proteins common to both neutrophils and keratinocytes.  $\alpha$ -defensins and  $\beta$ -defensins share the same ancestral gene. It is interesting to note that both neutrophils are keratinocytes share very similar antimicrobial peptides. (Sørensen *et al.* 2015)

# Neutrophils

*Searching.... Seek and destroy.*

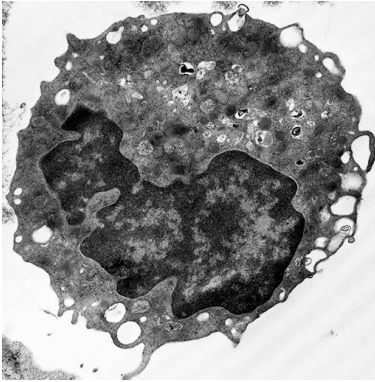
- Metallica, *Seek and destroy* (James Hetfield, Lars Ulrich)

Neutrophils are short-lived, highly motile cells and are the first immune cells to be actively recruited to encounter and prevent dissemination of microbes after injury. About two-thirds of the hematopoiesis or blood cell formation is dedicated to the production of monocytes and granulocytes, which is collectively known as myelopoiesis. Stem cells of myeloid lineage undergo a series of divisions in the bone marrow, in presence of myeloid-specific growth factors. The presence of granules distinguishes granulocytes (neutrophils, basophils and eosinophils) from other leucocyte subtypes. Neutrophil granules are rich in proteins that can either kill bacteria or degrade host tissues. Formation of granules marks the transition from a stem-like to a more differentiated neutrophil phenotype. The granule protein synthesis only takes place in the bone marrow and the general assumption is that proteins synthesized after exit from the bone marrow are not incorporated into the neutrophil granules.

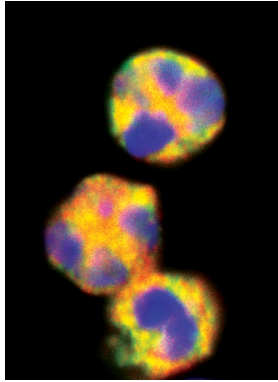
Neutrophil granules can be classified into three distinct subsets, based on the sequential temporal order of appearance during granulocytic differentiation in the bone marrow. These being: primary (azurophilic) granules, secondary (specific) granules and tertiary (gelatinase) granules. Apart from these, neutrophils also contain secretory vesicles, which are not considered to be 'true granules' since they are formed as a result of endocytosis at a much later stage, when the neutrophil is ready to exit the bone marrow. Granules store the effector molecules of the neutrophil such as antimicrobial peptides, membrane-bound components of the NADPH oxidase complex, myeloperoxidase (MPO) and various receptors that help the neutrophil to extravasate through tissue

(Borregaard and Cowland, 1997). This process of migration through tissues is called diapedesis.

(a)



(b)



**Figure 3** - The human neutrophil. (a) Transmission EM image of an unstimulated neutrophil. The central bilobed electron dense region represents the nucleus. The neutrophil granules can be seen scattered in the cytoplasm as small round electron dense regions. Courtesy- Maththias Mörgelin. (b) Neutrophils as visualized by epifluorescence. The blue staining depicts the multilobular nucleus of the cell. The azurophilic granules can be seen distributed in the cytoplasm. The green staining denotes the azurophilic granule membrane marker CD18 and the red staining denotes the core granule protein neutrophil elastase. Both proteins colocalize to produce the visible yellow staining.

After maturation neutrophils traverse tissues and the vascular wall to arrive at sites of injury or at various mucosal surfaces, to exert their defensive functions. Endothelial cells under the influence of pro-inflammatory cytokines like  $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$  and  $\text{IL-17}$ , express P-selectin, E-selectin and various members of the integrin family (ICAMs, VCAMs) for attachment of the neutrophils. Selectins expressed by both the endothelium and the neutrophils are calcium dependent lectins that recognize Sialyl Lewis<sup>x</sup> ( $\text{SLe}^x$ ) presented on the ligands. Endothelial selectins bind the constitutively expressed PSGL-1 (P-selectin ligand 1) and L-selectin on neutrophils. In flowing blood, using these selectin dependent interactions neutrophils are able to roll on the vascular

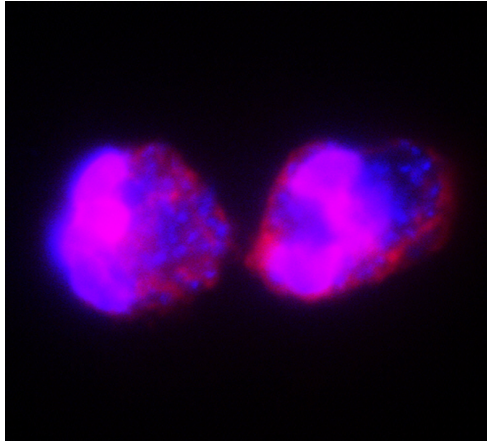
surface. Selectin mediated adhesions are reversible and short-lived. E-selectin which is expressed in higher densities reduces the rolling speed of the neutrophils (McEver, 2015; McEver *et al.*, 1995). The genetic inability to produce functional SLe<sup>x</sup> results in a condition known as Leucocyte Adhesion Deficiency type-II (LAD II), wherein the neutrophils are unable undergo diapedesis and remain confined to the circulation only (Luhn *et al.*, 2001).

The selectin dependent rolling is gradually replaced by the stronger integrin mediated attachment to the vascular surface. LFA-1 (CD11a/CD18) and complement receptor 3 (CD11b/CD18) is present on the neutrophils. Their ligands ICAM-1 (intercellular adhesion molecule-1) and ICAM-2 are present on the endothelial surface. These are crucial molecules that aid in the firm adhesion of the neutrophil and finally the extravasation of the neutrophil (Barreiro *et al.*, 2008). Leucocyte Adhesion Deficiency type-I (LAD I) is a fatal genetic disorder that results from the deficiency of CD18 in the  $\beta_2$  integrin that renders the neutrophil to establish firm adhesion and diapedesis. The disease is characterized by severe soft tissue infections, as the neutrophils cannot migrate. An immediate bone marrow or stem cell transplant is recommended for the patients (Bunting *et al.*, 2002).

After the neutrophils stop rolling, firm adhesion is established. This leads to the polarization in to a leading lamellipodium with a high concentration of receptors for chemokine and phagocytosis. The neutrophils may then choose a transcellular or a paracellular route to cross the endothelium and into various tissues. This requires a complex interplay of intracellular signaling to modify the actin cytoskeleton (Borregaard, 2010).

Once the neutrophils reach the designated site, these are primed due to granule mobilization that coincides with extravasation. Activated neutrophils are a class of ‘trigger happy’ specialised killer sentinels in mammals, perfected through millions of years of evolution. They are

recruited within minutes to the site of injury and are key players in inflammation. They display eight TLRs (Toll-like receptors) (Prince *et al.*, 2011) to sense a variety of PAMPs like LPS or flagellin (Pathogen associated molecular patterns) or DAMPs (Danger associated molecular patterns) like DNA or histones (Matzinger, 1994; Medzhitov and Janeway, 2002). Other important receptors present include Fcγ receptors, fMLF receptors and receptors for anaphylatoxins, PAF (platelet activating factor) and chemokines. Upon encountering the opsonized microbes, the neutrophil actively tries to phagocytose them resulting in a phagosome into which proteases, AMPs, and reactive oxygen species are delivered. The NADPH oxidase complex assembles produces copious amounts of superoxide into the phagosome. The superoxide anion has a short lifespan, resulting in the formation hydrogen peroxide or being broken down by the enzyme superoxide dismutase (SOD). The hydrogen peroxide is finally converted into hypochlorous acid commonly known as bleach by MPO. The granules containing proteases and other antimicrobial peptides are gradually externalized to eliminate microbes that are not phagocytosed (See list of AMPs synthesized by neutrophils in table). This process is termed as degranulation (Segal, 2005). Although, the hierarchy of the order of the granule release is much debated, secretory vesicles are exocytosed the easiest, followed by gelatinase granules and specific granules. Azurophilic granules have the least propensity to be extruded and even upon stimulation are only mobilized partially (Sengelov *et al.*, 1995). Dysregulated neutrophil activation may result in unwanted granule release leading to tissue damage (Dovi *et al.*, 2003).



**Figure 4** – Neutrophils with phagocytosed *S. pyogenes* (AP1). Pictured above are two neutrophils *in vivo*. The PMN nuclei are stained with DAPI (blue) and appear as the larger characteristic multilobed structures. The PMNs are also stained for the azurophilic granule protein, neutrophil elastase (red). The bacterial DNA appears as small round grape-like structures within the cell. Neutrophils employ a variety of measures to eliminate the phagocytosed bacteria (please refer to the current section on neutrophils in the thesis).

### *Role of neutrophils in the oral cavity*

A significant part of the neutrophil present in the peripheral circulation is diverted towards the mucosae. In the oral cavity neutrophil dysfunction commonly results in pathological manifestations such as gingivitis, periodontitis and ulcers (Charon *et al.*, 1985; Hajishengallis and Hajishengallis, 2014). This demonstrates an important role of neutrophils in maintaining oral homeostasis. Patients with neutrophil dysfunctions like cyclic neutropenia, chronic granulomatous disease (CGD) and MPO deficiency develop oral infections (Borregaard *et al.*, 2005; Dar-Odeh *et al.*, 2010; Okuda *et al.*, 1991). This could partly be explained as a lack of the ability of the immune system to control the bacterial overgrowth due to reduced neutrophil numbers.





# Neutrophil extracellular traps (NETs)

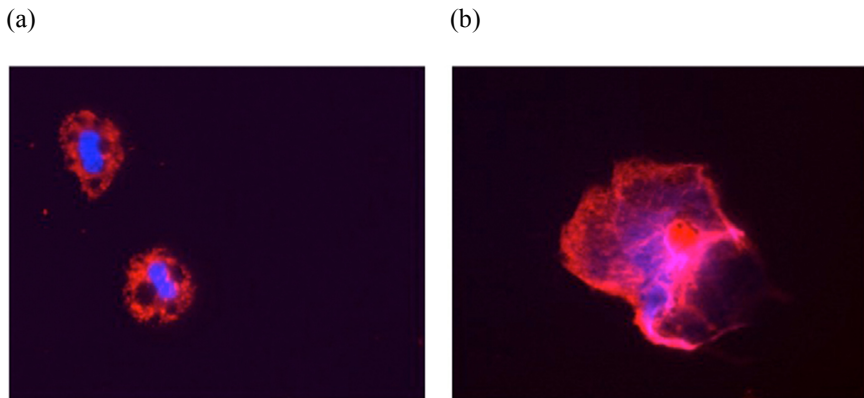
*“There is only one really serious philosophical problem and that is suicide. Deciding whether or not life is worth living is to answer the fundamental question in philosophy. All other questions follow from that”*

- Albert Camus, *The myth of Sisyphus*

Neutrophils kill bacteria either within the phagosome or through the exocytosis of antimicrobial substances. Apart from this, neutrophils have been reported to undergo a unique mode of cell death that results in the release of extracellular DNA strands decorated with antimicrobial proteins of neutrophilic origin, called neutrophil extracellular traps or NETs. Neutrophils that commit towards NET formation also termed NETosis undergo a series of intracellular events that results in nuclear decondensation and subsequent intracellular binding of antimicrobial granule or cytosolic proteins/peptides before being discharged to the cell exterior (Papayannopoulos *et al.*, 2010). Bacteria upon encountering NETs get trapped in the sticky DNA backbone (Brinkmann *et al.*, 2004). According to the current belief the NET-bound bacteria are killed-off owing to the antimicrobial proteins that are bound to the DNA prior to NET release.

NETosis is an active mode of cell death that is distinct from apoptosis or necrosis. NETs are formed in response to pathogens (both gram negative and positive bacteria, protozoans, fungi, viruses), reactive oxygen species or in some cases inorganic crystals like uric acid crystals (Abi Abdallah and Denkers, 2012; Fuchs *et al.*, 2007; Jenne *et al.*, 2013; McDonald *et al.*, 2012; Pilszczek *et al.*, 2010; Schauer *et al.*, 2014; Urban *et al.*, 2009). The partially characterized mechanisms that govern this process is only partially characterized and mostly in vitro. It has been

generally agreed upon that NETosis may be initiated through mechanisms that are either dependent upon NADPH oxidase through generation of reactive oxygen species (ROS) or binding to a combination of TLRs and integrins (Yipp and Kubes, 2013).



**Figure 5** – Neutrophils undergoing NETosis. (a) Unstimulated neutrophils after one hour. The multilobular nuclei (blue) can be noticed along with the azurophilic granule protein neutrophil elastase (red). (b) Neutrophil that has undergone NETosis after 1 hour of treatment with saliva. Due to the nuclear decondensation there is a drastic increase in the diameter of the neutrophil undergoing NETosis. The pink halo demarcates the area under the NET and is due to the colocalization of DNA (blue) and neutrophil elastase (red).

ROS dependent NETosis has mostly been demonstrated *in vitro* through the use of Phorbol 12-myristate 13-acetate (PMA). PMA is derived as a hydrolysis product from croton oil (*Croton tiglium*) and strongly activates the protein kinase C (PKC) in neutrophils. This then leads to the assembly of NADPH oxidase in the neutrophils through the activation of Raf, MEK and ERK kinases (Hakkim *et al.*, 2011). Citrullination of histones by peptidylarginine deiminases (PAD) have also been hypothesized to play a role in the decondensation of the nuclear before the release into an extranuclear environment (Wang *et al.*, 2009). About 90% of human PMNs undergo NETosis after 1-3 hours of

stimulation with PMA *in vitro*. This particular type of NETosis that involves rupture of membranes prior to release has been classified as lytic or suicidal NETs (Papayannopoulos *et al.*, 2010). This *kamikaze* response by the neutrophils renders the cell unfit to be recruited for any other function. PMA seems to be inducing NETs in murine neutrophils to a much lesser extent. The PMA treatment promotes nuclear expansion, but only a fraction of the total population seems to undergo NETosis (Li *et al.*, 2010). Apart from PMA, *C. albicans*, *A. fumigatus*, *M. bovis* and monosodium urate crystals induce NETosis in ROS-dependent manner (Bianchi *et al.*, 2011; Branzk *et al.*, 2014; Schauer *et al.*, 2014; Urban *et al.*, 2009).

Neutrophils also undergo NETosis after engaging bacteria, which results in rapid NET release. The nuclei-free cytoplasts that are observed after this mechanism NETosis are able to undergo chemotaxis and phagocytose bacteria. This is a variety of NETosis where the cytoplasm retains a certain degree of function even after NETosis is known as vital NETosis (Yipp *et al.*, 2012). Vital NETosis has been described in response to *S. aureus* both *in vitro* and *in vivo* (Pilszczek *et al.*, 2010; Yipp *et al.*, 2012), as well as with LPS stimulation in presence of platelets *in vivo* and *in vitro* (Clark *et al.*, 2007; McDonald and Kubes, 2012). Vital NETosis is a rapid process that occurs <10 minutes *in vitro* and between 20-40 minutes *in vivo* after the bacterial challenge and does apparently not rely on the activation of NADPH oxidase (Pilszczek *et al.*, 2010). There seems to be a disparity between the findings between bacteria-mediated NETs *in vivo* and *in vitro*. Pilszczek *et al.* found staphylococcal lysin(s) to be responsible for NET formation *in vitro*. The role of lysins *in vivo* seems questionable since the neutrophils and bacteria have are present in the circulation and, thus, does not allow the significant accumulation of lysins used in the *in vitro* study. The bacteria-induced NETs seems more relevant *in vivo* than PMA generated NETs as these NETs are generated much more rapidly against bacterial pathogens which possess a short doubling period.

Recognition of bacterial patterns from both Gram-negative and -positive bacteria by toll-like receptors combined with integrins on platelets or neutrophils are key to initiate this pathway of NETosis. Toll-like receptor-4 (TLR-4) on platelets interacts with the gram-negative pattern LPS and then these LPS-activated platelets in turn bind the integrin complex lymphocyte function-associated antigen 1 (CD11a/CD18) on neutrophils (Clark *et al.*, 2007; McDonald and Kubes, 2012). In case of gram-positive pathogens, toll-like receptor-2 (TLR-2) and complement receptor 3 (CR3) bind bacteria. CR3 in fact is a combination of integrins CD11b and CD18 (also known as the macrophage antigen complex-1 or Mac-1 complex) and is involved in cellular adhesion, cell-cell interaction and bacterial pattern recognition (Yipp *et al.*, 2012). In contrast to the lytic NETosis, no disruption of the nuclear membrane is observed. Instead, the inner and outer nuclear membranes separate, resulting in an enlarged intra-membrane space. This lumen is gradually filled up with DNA. The DNA filled lumens appear as large dilations within the cytosol, which are trafficked as vesicles to the cell exterior where they lyse to release their contents. This occurs in the later stages, when the vesicles are either fusing with the plasma membrane or after they are in an extracellular environment. The dynamics and exact mechanism of NET release is not clear but it has been proposed that after the DNA release in the cell exterior, the intermixing of the DNA and neutrophil granules takes place in the extracellular environment to form NETs. The intermixing of the DNA filled lumen and neutrophil granules may also take place at the plasma membrane, prior to release into the extracellular space. Due to vesicular DNA release the cell membrane is not harmed and leads to the formation of cytoplasts that are able to undergo chemotaxis and engulf *S. aureus* (Pilszczek *et al.*, 2010). Anuclear cellular structures that carry out important functions have been observed previously in the form of erythrocytes and platelets. During the 1980's it was also observed that anuclear neutrophil cytoplasts generated by treatment with cytochalasin B and isolated with density gradient centrifugation are able to follow a cytokine gradient and are able to trap and kill bacteria (Roos *et al.*, 1983).

Other white blood cells such as monocytes (Chow *et al.*, 2010), mast cells (von Kockritz-Blickwede *et al.*, 2008), eosinophils (Schorn *et al.*, 2012) and basophils (Ueki *et al.*, 2013) also seem to be capable to form extracellular traps of nuclear DNA.

### ***Host defence function and relevance in diseases***

The concept of NET formation *in vivo* has been viewed with much skepticism, owing to the manner in which extracellular DNA associated with neutrophil granule proteins was used as a measure of NETosis. This rather indirect manner of detection gave rise to crucial questions like if the observed extracellular DNA was truly derived from neutrophils and not from any other cell type. Secondly, naked DNA tends to bind a lot of host proteins, including those derived from neutrophils, which makes it difficult to assess if the DNA-neutrophil granule protein complexes were formed actively through NETosis or were a result of non-specific binding between exocytosed granule proteins and extracellular DNA released through modes of cell death other than NETosis (Nauseef, 2012). Active NET formation in mice following an infection with both live and heat killed *S. aureus* was eventually demonstrated *in vivo* through the use of live intravital spinning disk confocal microscopy (Yipp *et al.*, 2012). In this experiment, NETs were demonstrated to be able to trap and kill bacteria in mouse blood capillaries.

NET formation as a defensive measure can be classified into trapping and direct microbicidal effects (Brinkmann and Zychlinsky, 2012). NETs have been reported to entrap and subdue a wide variety of harmful microbes to prevent dissemination into the host's internal environment. Utilization of extracellular chromatin as a defense strategy is not only confined to vertebrates but has also been documented in invertebrates (Robb *et al.*, 2014). In case of fungi, immediate phagocytosis is not possible due to the large size of the pathogen and entrapment by

deploying NETs seems to be only viable option to contain the pathogen (Branzk *et al.*, 2014).

The entire purpose of NET formation would be futile, if the microbes possessed DNases that would degrade the DNA backbone. Hence, the expression of DNase as a virulence factor has been linked to several pathogenic bacteria. In mice, injection of DNase during superficial skin infection by *S. aureus* led to bacterial dissemination and increased susceptibility of the infected mice (Berends *et al.*, 2010). *S. agalactiae* is known to bind to siglecs on neutrophils and dampen NET formation (Carlin *et al.*, 2009a). Plants also seem to use extracellular DNA against a soil-borne fungus. DNase treatment of the protective root tip slime enhances infection by the fungus (Driouich *et al.*, 2013). DNA is known to be a major component of biofilms, *P. aeruginosa* has been demonstrated to utilize the extracellular DNA ejected during NETosis and construct biofilms out of it (Walker *et al.*, 2005).

Several reports indicate killing of bacteria and fungi after trapping in NETs. This seems to be the next step in the sequence after trapping. Several neutrophil derived antimicrobial substances that can kill the trapped pathogens cover NETs, including histones, neutrophil elastase, S100A8/9, cathelicidin and pentraxin 3 (Jaillon *et al.*, 2007; Urban *et al.*, 2009). MPO activity on NETs is also crucial in the killing of *S. aureus* (Parker *et al.*, 2012). Even the DNA associated with NETs has been hypothesized to chelate cation that leads to increased susceptibility of opportunistic pathogens (Halverson *et al.*, 2015). CGD neutrophils that lack a functional NADPH oxidase cannot form NADPH-oxidase dependent NETs. These patients often suffer from aspergillosis, where that infecting fungi cannot be phagocytosed. Bianchi *et al* demonstrated that administration of gene therapy targeting PHOX restored NADPH oxidase activity that led to survival of a CGD patient (Bianchi *et al.*, 2009).

In spite of all the aforementioned citations, evidence contrary to the function of NET dependent killing also exists, with the overall importance of NETs also being questioned. Using DNase, Menegazzi *et al* were able to liberate viable bacteria even after co-incubation with NETs, indicating that previous experiments may have only accounted for entrapment and not killing (Menegazzi *et al.*, 2012). Human plasma that leaks out after injury also seems to contain a vast quantity of anti-proteolytic proteins. In fact, one ml plasma contains enough  $\alpha$ 1-proteinase inhibitor to neutralize neutrophil elastase from approximately 500 million neutrophils (Weiss, 1989). Most assays depicting NET mediated killing do not involve the physiologically relevant plasma in the experimental set-up and this raises concerns about the functional effects of NETs in presence of plasma. Sørensen *et al*, also described a patient suffering from Papillon-Lefèvre syndrome (PLS) where a deficiency in functional neutrophil elastase, cathepsin G and proteinase 3 (PR3) was observed. Neutrophil elastase has been demonstrated to be important for ROS dependent NETs (Papayannopoulos *et al.*, 2010). Accordingly, though the PLS patient had normal ROS production the patient's neutrophils form ROS-dependent NETs since these are dependent on elastase activity. In spite of the immune deficiency, the PLS patient had only mild symptoms and PLS neutrophils were able to clear off intracellular bacteria (Sørensen *et al.*, 2014). Hence, the observations made in the PLS patients speak against a major role of elastase-dependent – and thereby NADPH oxidase dependent - NETs in immunity.

### ***NETs, inflammation, autoimmune disease and coagulation***

Dysregulated release of neutrophil granules into the exterior leads to tissue damage. In case of NETs, not only granule proteins, but also the extruded DNA backbone may serve as a danger associated molecular pattern (DAMP) and are highly likely to contribute to inflammation. In psoriasis, DNA complexed with LL-37 (Lande *et al.*, 2011) or HMGB1



(high mobility box group proteins) (Ivanov *et al.*, 2007) has been demonstrated to activate TLR9 in dendritic cells, leading to inflammation. Apart from the seemingly pro-inflammatory chromatin and neutrophil proteases in the extracellular traps, NET-aggregates have also been associated with limiting inflammation in gout by degrading cytokines and chemokines (Schauer *et al.*, 2014).

Systemic lupus erythematosus (SLE) is a non-curable autoimmune disease that affects skin, heart, joints, liver, kidneys and blood vessels. Patients suffer from abrupt states of heightened manifestation of symptoms called flares alternating with withdrawals called remissions. One of the key features of the disease is the failure of the silent clearance of cell debris after cell death, which leads production of autoantibodies against self-antigens (Tsokos, 2011). It is interesting to note that during a flare the serum nuclease activity goes down and neutrophils from SLE patients are more prone to NETosis. This may indicate that once the NETs are formed, they may not be cleared off efficiently and the gradual build up of such NETs may lead to the flares and tissue damage (Hakkin *et al.*, 2010).

The DNA and protein components from NETs like histones and serine proteases initiate blood coagulation and platelet aggregation. NETs act as a scaffold for the activation of coagulation cascade and moreover neutrophil elastase and cathepsin G are known to degrade inhibitors of coagulation. In a flow chamber perfused with whole blood, NETs seem to bind plasma proteins that promote thrombus formation (Fuchs *et al.*, 2010). Therefore, pathological NETosis within the vasculature may result in ischemia and other thrombotic disorders (Martinod and Wagner, 2014)

# Streptococci

*Early in spring, along with the prevailing cold, there were many cases of erysipelas, some from a manifest cause, and some not. They were of a malignant nature, and proved fatal to many; many had sore throat and loss of speech.*

- Hippocrates, *Of the Epidemics*

Streptococci take their name from *Streptos* meaning chain and *coccus* meaning berry in Greek. They are spherical in shape and follow a unidirectional pattern of growth. But rather than separating from each other after cell division, the cocci form chains or pairs which give streptococci their notable twisted chain appearance under the microscope. These non-motile gram-positive bacteria belonging to the phylum Firmicutes are very diverse. Different streptococcal species are known to occupy many niches of the human body including skin, oral cavity, naso-pharynx and the intestines of both animals and humans. Most species reside as innocuous commensals, but some are pathogenic that may cause localized or systemic infections. In 1933, Rebecca Lancefield found that a soluble carbohydrate antigen could be retrieved from beta hemolytic streptococci. The carbohydrate extracted from the wall of the bacteria is known as 'C' carbohydrate. These different C carbohydrate antigens are referred to as *Lancefield antigens*. For instance, all streptococci listed under group A contain a similar type of C carbohydrate compared to Group C streptococci. This was also the first instance for the use of antibodies to detect the changes in an antigen within a genus of bacteria and is known as *serotyping*. The pyogenic or 'pus forming' division contained streptococci that were  $\beta$ -hemolytic and commonly associated with infections in humans and other animals. These included *Streptococcus pyogenes* (Lancefield group A), *Streptococcus agalactiae* (Lancefield group B), *Streptococcus equi* (Lancefield group C), animal pyogenes and human C (Lancefield group

C), minute hemolytic group F (Lancefield group F) and streptococci with Lancefield antigens G, E and H (Lancefield, 1928, 1959, 1962).

Hippocrates in the fifth century B.C wrote about erysipelas that later exacerbated into flesh eating disease (necrotizing fasciitis) in humans. Both the diseases are caused by *Streptococcus pyogenes*. These are one of the best-studied gram-positive pathogens in humans along with *S. aureus*, because of the many common infections they cause. Today, they are identified as one of the top human pathogens responsible for mortality around the globe (Carapetis *et al.*, 2005). Presence of toxins and unique proteins helps the bacteria in outwitting the host humoral and cellular immune effectors like antibodies, complement and white blood cells (Kwinn and Nizet, 2007).

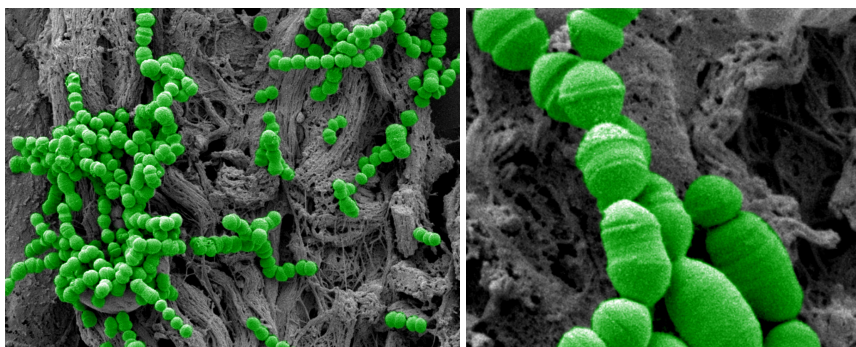
The most famous member of the pathogenic streptococci is the  $\beta$ -hemolytic pyogenic *Streptococcus pyogenes*. *Streptococcus dysgalactiae* subspecies *equismilis* (SDSE) belongs to the Lancefield group G streptococci and is being slowly recognized, as an important pathogen whose repertoire of infections closely resembles that of *S. pyogenes*. SDSE principally exhibits skin and soft-tissue infections, including pharyngitis, pyoderma, cellulitis, wound infections, abscesses, erysipelas, and necrotizing fasciitis (Bramhachari *et al.*, 2010; Brandt and Spellerberg, 2009; Dinkla *et al.*, 2007). It also contains virulence factors related to GAS like streptolysin O, M-like protein FOG (Fibrinogen binding protein of group G streptococci) and streptokinase (Bjorck *et al.*, 1987; Wollein Waldetoft *et al.*, 2014).

Several  $\beta$ -hemolytic streptococci produce a variety of virulence factors including streptokinase (Kwinn and Nizet, 2007). Streptokinase functions by binding and activating host plasminogen into plasmin, a trypsin-like protease that ultimately causes fibrinolysis. This clot-dissolving property of streptokinase has been exploited during treatment of acute myocardial infarctions and embolisms. But due to development

of immunity against the bacterial product by patients and hyperallergenic reactions, it has been substituted by safer alternatives like tissue plasminogen activator (tPA). Virulent bacteria utilize streptokinase to aid dissemination from clots. In mice infected with GAS, inhibition of streptokinase gene expression improves survival (Sun *et al.*, 2012). Streptokinase has also been implicated in the pathogenesis of acute post-streptococcal glomerulonephritis as it has a higher affinity for glomerular tissue (Nordstrand *et al.*, 1998).

(a)

(b)



**Figure 6** – *Streptococcus pyogenes* (a) *S. pyogenes* incubated with deparaffinized sections of human lung. The bacteria appear as pseudo colored green chains. In the background the material from the upper respiratory tract can be observed as a fibrous mesh. (b) At a higher magnification, the individual bacteria (green) appear in their characteristic grape like appearance. The bacterial surface proteins can be seen as white spots on the bacteria. Courtesy- Matthias Mörgelin.

The infections caused by  $\beta$ -hemolytic streptococci in humans can be broadly divided into asymptomatic colonization, superficial symptomatic infection and finally, systemic symptomatic infections. During superficial and systemic symptomatic infections streptococci synthesize a variety of virulence factors that enable invasion of the host tissues. In stark contrast to their virulent form, streptococci are commonly isolated in the pharynx, as asymptomatic colonizers, during which production of virulence factors is low. The streptococci survive as asymptomatic oral

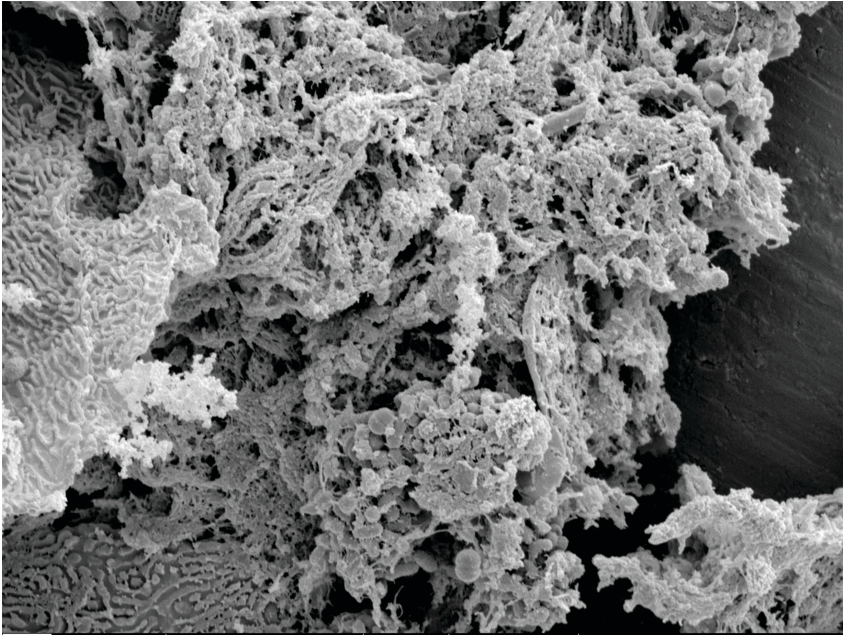
colonizers exploiting the nutrients that plasma carries along with it and replicate to increase their numbers. The real function of the plasma exudate, which is rich in antibodies, complement components and thrombin (Papareddy *et al.*, 2010), is to defend against the overgrowth of oral bacteria. This plasma exudate may in turn facilitate the survival and growth of bacteria that carry with them certain virulence factors like M-protein. M-protein is known to neutralize a range of host defense mechanisms and promote virulence (Staali *et al.*, 2003). Superficial streptococcal infections are much more common than life threatening systemic infections (Wannamaker, 1970). It may be reasoned that asymptomatic colonization or symptomatic superficial infections as an adaptive strategy that leads to better transmissibility and survival of bacteria by doing little damage to the host. The full blown systemic infections can result as a side effect and may be viewed as a non-adaptive strategy as it endangers the survival of both the host and bacteria and is ultimately non-beneficial for the bacteria. Integrating nutritional requirements, bacterial survival strategies and host defense responses may open up new avenues towards a more inclusive and holistic understanding about the complex interplay between host-pathogen interactions in the future (Wollein Waldetoft and Raberg, 2014).

Within the oral cavity, the streptococci as well as the plasma exudate interact with saliva, which is the dominant oral fluid. The interaction between plasma and streptococci in presence of saliva has not been explored. It is also interesting to note that the possible influences of both the host fluids on saliva on bacterial survival and synthesis of virulence factors largely remain unanswered. Bacterial survival and virulence factor synthesis would ultimately provide vital insights into disease transmission, host health as well as host-pathogen interaction.

# The oral cavity

The oral cavity (*cavum oris*) marks the beginning of the alimentary canal. The mucosal lining of the oral cavity consists of keratinized and/or non-keratinized stratified squamous epithelia with blood vessels and an underlying layer of connective tissue. It houses organs necessary for mastication and lubrication of the food bolus namely, teeth, tongue and salivary glands (Squier and Kremer, 2001). As detected by 16S rRNA gene sequencing methods, the mouth plays host to more than 700 species of bacteria, which are primarily commensal with the exception of a few known pathogens (Dewhirst *et al.*, 2010). This is a simple illustration of the vastly diverse resident microflora in the oral cavity. Also, post consumption of food the nutrient levels in saliva go up, providing ample opportunities for the microbes to thrive. Since the oral cavity serves as a passageway for food and air, minor abrasions may serve as a point of entry for microbes.

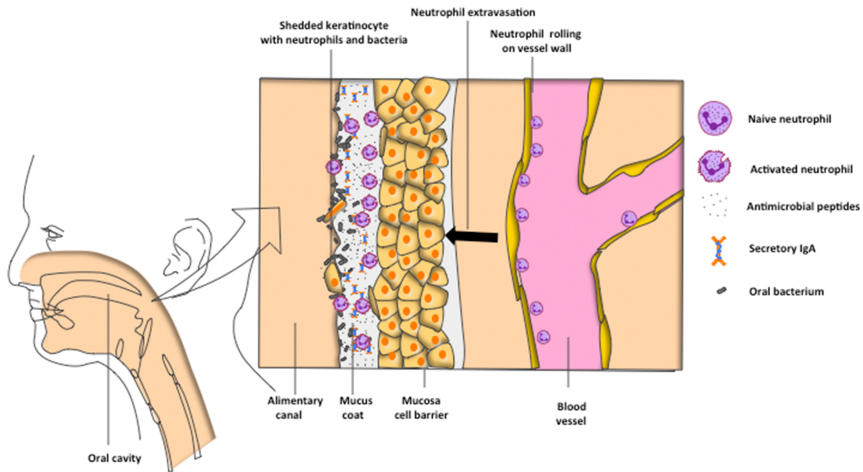
The keratinocytes of the mucosal lining and neutrophils comprise the primary cellular barrier against microbial invasion. The external epithelial layer of the oral mucosa is regenerated constantly, to prevent the overgrowth of attached bacteria. The shedded epithelial layer(s) to which bacteria are attached are cleared off continuously by swallowing, only to be neutralized by the acidic gastric pH, thus reducing the bacterial load at the mucosal surface. The barrier function exhibited by these cells also prevents the entry of toxins from the oral bacteria. Furthermore, oral keratinocytes synthesize a plethora of antimicrobial peptides like human beta defensins (hBD-1, -2, -3) (Mak *et al.*, 2009; Steinstraesser *et al.*, 2008), calgranulins (S100A8, 9, 10), and cystatin C ([www.proteinatlas.org](http://www.proteinatlas.org)) to keep microbes at bay.



**Figure 7** – Shedded epithelial cells attached to mucin enmeshed bacteria in saliva as visualized by scanning EM. The oral cavity contains a large and diverse microflora. In order to keep the bacteria at bay, the epithelial layer is shedded with bacteria entrapped in mucins. Epithelial cells in the image can be seen on the far left as large flakes with intricate ridges on the surface. Mucins can be seen as strands surrounding bacteria in the form of cocci (round shaped) and bacilli (rod shaped). Courtesy- Matthias Mörgelin.

There is a constant influx of neutrophils from the gingival crevices, into the oral cavity. The rate of neutrophil migration can be as much as  $0.5\text{--}2 \times 10^6$  cells/minute, assuming the rate of saliva flow to be 1 ml/minute (Thomas *et al.*, 1994; Woolweaver *et al.*, 1972). The breach of barrier after injury results in the leakage of blood, that leads to the activation of the coagulation system. Activation of the coagulation system prevents blood loss, promotes inflammation and contributes to innate immune functions. Despite the presence of pro-inflammatory stimuli in the form of bacteria and inflammatory mediators of the immune system like neutrophils and dendritic cells, there is surprisingly very little or no

inflammation in the oral cavity during wound healing. Wounds in the oral cavity are also known to heal faster without scarring in comparison to epidermis (Mak *et al.*, 2009).

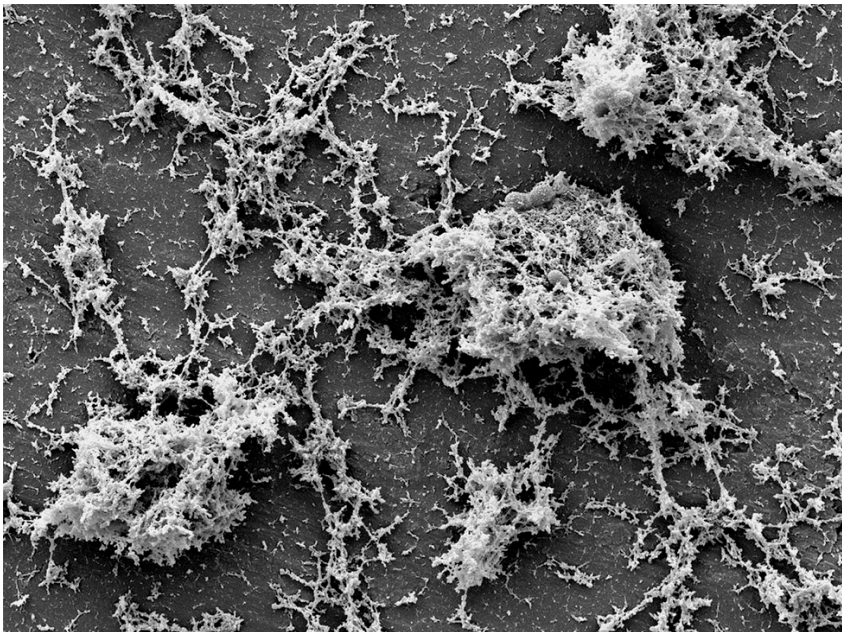


**Figure 8** – A representative model of the bacterial clearance mechanism in the oral cavity. The mucus coat covers the entire length of the oropharyngeal cavity and forms a gel like layer over the mucosal surface. Shedded keratinocytes, neutrophils that have migrated out into the oral cavity and the oral bacteria remain embedded within the gel-like mucus coating. Secretory IgA and various antimicrobial peptides are other notable components of the mucus coat. The origins of the antimicrobial peptides that are found can be traced back to the salivary glands, keratinocytes or neutrophils. This layer is recycled frequently through the act of swallowing. The mixture of bacteria, cells, host defense proteins and mucus are passed into the alimentary canal. Finally everything is eliminated into the stomach, where the bacteria and potentially harmful toxins are neutralized by the acidic gastric pH.

Another important component of mucosal defenses is the gel-like mucous layer that coats the oral surfaces. The mucous consist of mucins that contain several non-immunoglobulin bacterial aggregation sites that aid in binding and trapping oral bacteria or their harmful secreted products. The viscous mucin coat acts as a mesh within which there are contained secreted IgA, various antimicrobial substances including



AMPs and professional phagocytes along with trapped bacteria. This limits bacterial overgrowth and preventing direct microbial contact with the underlying epithelium (Tabak *et al.*, 1982). This complex mucin coat containing both bacteria and host defense components is recycled frequently through swallowing, thereby reducing overall the oral bacterial load.



**Figure 9** – Mucin coated PMN debris with bacteria trapped. Using scanning EM, mucins can be visualized as white strand-like structures and aggregates. Cocci (bacteria) can be seen trapped and in contact with the PMN remnants (large central cell) and mucin network. Courtesy- Matthias Mörgelin.

Saliva interacts with other innate immune system components in the oral environment. Therefore, we examined the effects of saliva on components of the innate immune system.



# *Present investigation*

Previous studies have described the presence of several antimicrobial functions of saliva like killing and entrapment of oral bacteria (Murakami *et al.*, 2002; Oudhoff *et al.*, 2009; Prakobphol *et al.*, 1998; Thomsson *et al.*, 2002). This compelled us to investigate whether saliva had any influence on the cells or plasma exudation that are commonly associated with defense at the oral mucosa.

## *Paper I*

*Paper I*, describes the influence of human saliva on AMP gene expression in primary keratinocytes, the entity responsible for AMP expression and the subsequent saliva-induced clearance of invasive bacteria intracellularly. The constant exposure of the oral to the bacterial flora requires some form of constant defense mechanisms in place to prevent bacterial overgrowth. Due to constant changes in the oral microflora, innate immune mechanisms that target a wide variety of microbes non-specifically are better suited for the purpose. We found a more prominent expression of AMPs by immunohistochemistry in paraffin embedded sections of healthy oral tissue than in healthy epidermis that is unexposed to saliva. Upon examining the effect of saliva on primary keratinocytes *in vitro*, it was discovered that saliva stimulation induced AMP induction. The direct influence of gene expression by saliva in human keratinocytes is not previously documented. This effect was found to be dependent upon the epidermal

growth factor receptor (EGFR), which has previously been described to stimulate AMP induction.

Although human saliva lacks in the amount of ligands that are known to bind the EGFR directly, saliva was able to induce the transactivation of the EGFR, which is an effective mode of activation in the absence of direct binding EGFR ligands. A lack of peptide EGFR ligands and the involvement of G protein coupled receptors led us to investigate the involvement of lipids that are present in significant amounts in saliva. The lipid fraction from whole saliva was able to induce AMP synthesis in an EGFR transactivation dependent fashion. The AMP enhancing effect could not be narrowed down to a single lipid, but the results can be interpreted as being indicative towards the involvement of a complex mixture of biologically active lipids or a poor recovery of the active lipid(s).

We then examined the role of saliva in strengthening the antimicrobial function of keratinocytes and our results demonstrated that saliva aided keratinocytes in clearing off internalized *S. aureus* due to EGFR-activation. To the best of our knowledge this saliva-induced clearing off internalized bacteria by keratinocytes has not been described previously.

The data described in the manuscript implicates the defense role of healthy human saliva in preserving the oral tissue and also during the phenomenon of wound licking, where animals instinctively apply saliva to the region of injury by upregulating AMP synthesis.

## ***Paper II***

The neutrophil-based response is one of the key innate immune responses against invasive pathogens upon injury or keeping at bay the

commensal flora at various human mucosal surfaces. This is very evident when disordered oral homeostasis is seen in patients who suffer from neutropenia or defects in neutrophil function. During healthy conditions, the neutrophils continuously migrate into the oral cavity and neutrophil contents have been demonstrated to be present in saliva. Trapping bacteria in order to prevent dissemination, while simultaneously arresting growth has been attributed to neutrophil extracellular traps, which utilize extruded DNA and antimicrobial enzymes like MPO for the purpose. We were able to isolate NETs from morning saliva of healthy individuals and whole sterile filtered human saliva was able to induce NETosis in PMNs *ex vivo*. This involved sialyl-Lewis<sup>x</sup> (SLe<sup>x</sup>) residues present in salivary mucins under the specific ionic conditions present in saliva by interacting with L-selectin on neutrophils and inducing NETs in a ROS and elastase independent manner.

Saliva initiated a series of unique intracellular events in neutrophils that were distinct from other previously described events during NETosis. Saliva induced NETosis rapidly. This led to disruption of the nuclear membrane and granules with intracellular release of granule content prior to extracellular DNA release. Saliva-mediated NETosis did not require the activity of neutrophil elastase or the NADPH oxidase. Lack of the integrin CD18 results in type-1 leukocyte adhesion deficiency (LAD-1), where neutrophils are unable to extravasate or phagocytose bacteria, as they cannot form the Mac-1 complex (Bunting *et al.*, 2002). In theory LAD-1 neutrophils cannot form NETs in response to bacteria. Surprisingly, LAD-1 neutrophils were able to undergo NETosis in response to saliva. This also indicated that Mac-1 does not play a role in the formation of saliva-mediated NETs.

Compared to other types of NETs, the saliva-induced NETs were resistant to DNase degradation. This is important in the preservation of the trapping function of NETs in the oral cavity as DNases endogenous to either saliva or the resident bacterial flora proved detrimental to the structural integrity of the NETs induced by either bacteria or PMA,

thereby hindering the trapping function. Furthermore the saliva-induced NETs were able to bind and kill more bacteria in comparison to NETs generated by PMA or bacteria. Intracellular pathogens like *S. pyogenes* can survive intracellularly and may escape from the clutches of the neutrophil as the NETs are being thrown out. We observed killing of intracellular bacteria in neutrophils undergoing saliva-mediated NETosis. This may provide a novel insight into a clearance mechanism of a pathogen that is known to survive intracellularly and is a resident of the oral cavity. Very little complement was deposited on the saliva-induced NETs as well in contrast to PMA-induced NETs, demonstrating that saliva-induced NETs do not promote inflammation through complement activation.

Neutropenia and neutrophil dysfunction is associated with oral disease often manifested as oral ulcers. Behçet's disease and recurrent aphthous stomatitis are conditions with normal neutrophil counts and function but with benign non-contagious oral ulcers. The actual trigger for the ulceration in these diseases is unknown. During Behçet's disease, ulcers appear in the genitalia, uvea and in the oral cavity. Other symptoms manifest in the form of inflamed bowels, lungs, deep vein thrombosis and neurological symptoms. The causes of the disease are unknown and it is hypothesized to be a form of autoimmune disease due involvement of Th1 cells. It is treated with anti-inflammatory drugs like colchicine or corticosteroids, similar to autoimmune disease therapy. Similar to Behçet's disease, recurrent aphthous stomatitis is another such disease of unknown etiology. Ulcers are confined to the buccal cavity, with ulcerations occurring periodically.

The altered microflora in both the diseases may also play a vital role. Sialidase producing bacteria that metabolize SLe<sup>x</sup> can sequester these from salivary mucins and potentially reduce the salivary NET-inducing capacity. SLe<sup>x</sup> residues were found to be intact in Behçet's disease and the defect in NET formation was due an unknown factor in the saliva that inhibited the capacity of mucins to induce NETs. The NET forming

capacity of saliva from Behçet's disease could then be restored by buffer change to buffer with similar ion composition as saliva. During ulceration in the recurrent aphthous samples the amount of SLe<sup>x</sup> bound to mucins was greatly reduced. SLe<sup>x</sup> is the responsible agent in saliva that induces NETosis and changing the ionic environment in which the mucins resided did restore the capability to induce NETosis. The capability of saliva to induce NETosis was restored during the recovery phase of aphthous ulceration and it also coincided with the reoccurrence of the SLe<sup>x</sup>.

The prime function of neutrophils is to reach the site of injury and limit any intrusion by microbes. Therefore, they play an important role in restricting invasion of the oral microflora. In this manuscript, we describe a rapid NETosis mediated by a novel pathway requiring salivary glycans that leads to NETs with unique functional capabilities. The saliva-induced DNase resistant NETs hence formed had the ability to bind and kill bacteria. And lastly, we were able to identify reduced NET formation in saliva from patients suffering from Behçet's syndrome and recurrent aphthous stomatitis. In light of these findings, it would be interesting to examine the function of mucin-mediated NETosis at other mucosal surfaces and if this is a common fate by neutrophils at all mucosal surfaces.

### ***Paper III***

Streptococci are responsible for some of the most prevalent infections. During pharyngitis, plasma exudes out into the oral cavity inhabited by a diverse microflora that includes  $\beta$ -hemolytic streptococci and mixes with the present saliva. We found that that saliva-plasma intermixing led to the formation of a fibrin clot by the activation of both arms of the coagulation system. Mass spectrometry was performed on the clots and it was revealed that the clot was comprised fibrinopeptides, IgG,



complement components, S100 proteins and statherin. We also observed that the fibrin mesh entrapped streptococci. This trapping mechanism may help prevent the dissemination of bacteria or toxic bacterial products in the pharynx. The clot surrounding the bacteria may also have an antimicrobial function as products of the complement cascade, S100 proteins, statherin and IgG were detected in it. The activation of the coagulation cascades as well as the complement system was dependent on the low osmolality of saliva. The three dimensional structures of the factors modulating the coagulation cascades like factor XII and plasma kallikrein (PK) were found to be affected by the low salivary ionic strength. The bacteria trapped in the resulting fibrin mesh may well be eliminated by swallowing, ultimately leading to bacterial neutralization in the acidic gastric pH.

In pharyngitis, this phenomenon of clot formation may represent a novel bacterial entrapment mechanism facilitated by saliva. In contrast to the commensal oral flora, the entrapped *S. pyogenes* dissolve the clot through streptokinase, a classic virulence factor in the repertoire of the streptococci. Streptokinase binds host plasminogen and converts it into its active form plasmin, which then cleaves the clot allowing the bacteria to escape. Overt plasmin activation by the streptococci may lead to destruction of host tissue and may result in the bacteria transforming into a more invasive phenotype.

The oral microflora commonly includes the presence of  $\beta$ -hemolytic streptococci belonging to group G and group A. During plasma exudation these may get trapped. They subsequently synthesize the virulence factor streptokinase that binds plasminogen and converts it into plasmin, a potent serine protease. Using the surface bound activated plasmin the bacteria were able to dissolve the clot and escape. Streptokinase plays an important role during invasive disease caused by streptococci. It is tempting to hypothesize that the escape by streptococci from the saliva-plasma clot using streptokinase and plasminogen activation may symbolize an adaptive function for streptokinase in

primarily promoting superficial disease like pharyngitis. In order to verify this proof of concept, we devised a model that mimicked oral epithelium during pharyngitis. We added sterile saliva to plasma in the presence of fluorescently labeled bacteria. The bacteria-saliva-plasma mixture was then added to keratinocytes. We observed that the saliva-plasma clot trapped the bacteria on the surface of the monolayer. Inhibition of bacterial protein synthesis that would affect streptokinase production led to the bacteria being trapped in clots.

To summarize the results from this paper, saliva was found to accelerate coagulation by influencing both branches of the system. The fibrin rich clot hence formed was able to entrap bacteria within it. The pathogenic *S. pyogenes* through the use of streptokinase were able to degrade the clot, thus, thus demonstrating that streptokinase is potentially an important virulence factor in the oral cavity/pharynx.



# *Discussion*

Saliva is a mucosal fluid that performs a host of functions in the oral cavity. The role of saliva in carrying out several innate immune functions is well known. Antimicrobial peptides present in saliva prevent overt bacterial overgrowth in the oral cavity. Salivary mucins are also known to aggregate bacteria and prevent direct contact with the oral mucosa. sIgA is the primary immunoglobulin present in saliva. The present thesis elucidates how saliva interacts with other component of the innate immune system and thereby elicits innate immune responses.

Progress into investigating the role of saliva may have been hindered by the fact that saliva is not an easy body fluid to work with. Salivary mucins are large and heavily glycosylated molecules that have non-specific affinity towards several smaller molecules. This serves as a major hindrance for the functional characterization of individual salivary components. The N-linked glycosylations can be removed to reduce simplify the analyses. But salivary mucins predominantly contain O-linked glycans. Currently enzymatic removal of the O-linked glycosyl residues is unknown and one has to resort to chemical methods (Merry and Astrautsova, 2003). Such removal strategies of salivary mucins require severe degradation of saliva that leads to loss of biological activity of the effector molecules. Saliva contains several bioactive lipids. But due to low recovery by conventional techniques their relevance is understudied.

Although EGF was discovered in murine saliva, human salivary EGF levels are very low (Bodner, 1991), approximately 1 ng/ml. This level is

not sufficient enough to induce AMP expression in primary human epidermal keratinocytes (Sorensen *et al.*, 2005). However, we found that the expression of several EGFR-dependent AMPs in the oral mucosa was higher in the oral mucosa compared to epidermis. An effort has been made in our part to link between upregulated AMP expression in both human keratinocytes and skin *ex vivo* with saliva. We found the AMP inducing activity of saliva to be associated with salivary lipids. Due to technical limitations we were not able to find a single lipid that was responsible. We therefore propose that it is a combination of several lipids that act in concert to upregulate the AMP expression. We also do not rule out the involvement of other salivary components such as lipopeptides or glycolipids that purify together with the lipid fraction of saliva.

During wakeful conditions, there is a constant flow of saliva and swallowing clears the accumulated saliva. The immune response is also much more active. On the contrary, during sleep, the saliva flow and swallowing are reduced (Nishino, 2012). The immune responses are also dampened. This may provide the bacteria with better growth conditions and an increase in the risk for infection. We were able to observe a higher number of NETs associated with bacteria in saliva samples collected immediately after waking up. It is therefore tempting to hypothesize that during sleep, when various innate immune responses are reduced, saliva mediated NETs may play a role in keeping bacterial numbers in check by entrapment and killing.

NETs are a combination of DNA and proteins. Both the entities can be stained using fluorophores and the degree of NET formation can be estimated by fluorescence microscopy (Brinkmann *et al.*, 2012). Along with DNA, other NET associated proteins such as histones, neutrophil elastase and LL-37 present extracellular have been used as markers for NET formation. Just nuclear expansion or extracellular DNA may is not ideal for quantification of NET formation. This is because many events unrelated to NETosis may lead to nuclear expansion as well extrusion of

DNA. Generation of NETs is an active process that results in the intermixing of DNA and neutrophil granule protein. Quantifying the area stained for DNA-associated neutrophil granule protein as we did is therefore - in our opinion - a better and more suitable method of NET quantification.

Several authors have reported the direct killing activity of NETs without the matter been settled. Menegazzi et al demonstrated that viable bacteria could be released from NETs after DNase digestion. They advocated that a reduction in the number colonies observed after performing viable counts on NETs could be due to the fact that the bacteria may aggregate due to the presence of DNA. These aggregated colonies may give a false impression that NETs reduce the bacterial colonies due to killing, whereas in reality the bacterial viability remain largely unaffected and there are just lesser number of colonies observed due to NET-mediated bacterial aggregation. It could also be argued that in the paper by Menegazzi et al., 100U/ml of DNase was used to digest the NETs. This is a significantly high concentration of enzyme. In theory, the bacteria can use this high concentration of protein as a nutrition source and cause overgrowth leading to increased colonies, where killing might have been observed. We demonstrated that the use of a live/dead bacterial viability kit might provide a better idea of the percentage of bacteria that are killed off due to direct contact with the NETs. Also, killing per individual NET is taken into account eliminating variation due to different degrees of NETosis, thus, providing a much more accurate estimate of killing of bacteria by NET. This method may be inadequate in demonstrating the number of bacteria killed by NETs generated by bacteria, since some of the NET-bound dead bacteria may have been killed before binding to the NETs.

Histones are known to be highly antimicrobial and are found abundantly on NETs. Histone citrullination is a post-translational modification that is thought to be important prior to NET release. This modification is catalyzed by the enzyme peptidyl arginine deiminase (PADI). PADI

converts cationic peptidyl arginine to neutral peptidylcitrulline on the histones, which helps in loosening of the DNA coils (Wang *et al.*, 2009). Kilsgard *et al.* have previously reported that the citrullination of the antimicrobial peptide LL-37 leads to a loss of antimicrobial activity and enhances neutrophil chemotactic activity. The citrullinated LL-37 was even more prone to degradation by bacterial proteases compared to the native form (Kilsgard *et al.*, 2012). Similar to the findings of Kilsgard *et al.*, Wang *et al.* (Wang *et al.*, 2009) demonstrate that citrullinated histones possess reduced antimicrobial activity compared to native histones. Future studies must aim at finding if NETs that involve hypercitrullination are actually antimicrobial.

PLS patients suffer from a mutation in the cathepsin C gene. This results in defective N-terminal trimming of neutrophil elastase, leading to disappearance of the protease as the neutrophil matures. Neutrophil elastase is essential for direct antimicrobial activity as well as the generation for ROS-dependent NETs. In spite of the lack of elastase and inability to form NETs, PLS neutrophils were able to kill off bacteria. The PLS patient was also surprisingly healthy without major immune dysfunction, despite the loss of a major neutrophil antibacterial protein and NET formation. PLS patients suffer from loss of teeth as a consequence to their immunosuppression. It can be argued that this loss of teeth may be due to a lack of ROS-dependent NETs. On the other hand CGD neutrophils, where NETs are not formed due to the lack of functional NADPH oxidase, loss of teeth is not a common phenomenon. This shows a non-redundant function of ROS-dependent NETs in humans.

MPO is responsible for the production of hypochlorite (HOCl) that is highly antimicrobial in nature and has been implicated in NET formation (Metzler *et al.*, 2011). MPO deficiency represents the most common neutrophil deficiency. This is mostly noticed as a partial loss of function and patients suffering from this condition rarely get severe infections. On the other hand a total MPO deficiency leads to profound sickness due

infections. Arguably this can be attributed more to the absence of HOCl production that is the major antimicrobial agent in neutrophils than NET formation.

It is interesting to note that during diseases like recurrent aphthous stomatitis and Behçet's disease, compositional changes in the salivary constituents and the microflora are observed (Bankvall *et al.*, 2014; Saadoun and Wechsler, 2012). How the changed microflora affects the manifestation of the disease remains to be seen. Mucins are the most abundant organic salivary constituents that endow saliva its characteristic visco-elastic properties. We observed a reduction in the amount of SLe<sup>x</sup> and the ability to form NETs associated with periods of ulceration in patients suffering from recurrent aphthous stomatitis. Levels of SLe<sup>x</sup> comparable to healthy controls were observed in saliva during the non-aphthous periods in these patients. These observations indicate that a loss of NET forming components and NETs are paralleled with disease periods. Saliva from patients with Behçet's disease was incapable of inducing NETosis even though SLe<sup>x</sup> was present. We found that buffer change endowed saliva from patients with Behçet's disease the capability to induce NETosis and that mucins resuspended in protein-free saliva from patient with Behçet's disease did not induce NETosis. We hypothesized that loss of NET formation may have been partly due to disturbances in the ionic composition of saliva or other low molecular weight substance that interacts with the mucins. . We found both in patients with Behçet's disease and aphthous stomatitis that a deficient NET formation is associated with oral ulcers. Future studies will demonstrate whether the deficient NET formation led to reduction of bacterial killing in saliva and how this may be linked to the oral ulceration.

Saliva often reflects the state of health of the internal environment of the host. Previous studies have examined the role of saliva as an easily accessible body fluid for quantifying various biomarkers during disease (Rathnayake *et al.*, 2013). But the potential innate immune boosting



effects that saliva may have by stimulating keratinocytes, neutrophils and the clotting cascades in plasma to prevent overt infections, have not been explored. This when integrated with disturbances in the commensal flora and presence of pathogens may lead to a better understanding of the complex interactions that reflect the overall health of the host, and provide us with better treatments.

## **Concluding remarks**

We examined the role of saliva in promoting keratinocyte, neutrophil and coagulation cascade based innate immune responses.

In our studies we examined the resulting antimicrobial gene expression in keratinocyte after saliva exposure and this was found to be dependent on the bioactive lipids found in saliva. Terminal SLe<sup>X</sup> residues on salivary mucins were found to prompt neutrophils to undergo NETosis. This mechanism is disrupted in patients suffering from aphthous stomatitis and Behçet's disease. We were able to restore NET formation in both patients. Finally, we looked at how the low ionic strength of saliva influences activation of the coagulation cascades in plasma, resulting in a clot that was able to trap oral bacteria. Streptococci then using streptokinase were able to escape from the clot.

This thesis documents an important role of saliva by interacting with other component of the innate immune system by regulating the function of some of its vital sentinels, namely keratinocytes, neutrophils and plasma thereby shedding light on some of the molecular mechanisms of innate immunity involved in health and disease.

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