Neutrophils and Neutrophil Extracellular Traps in Autoimmune, Occlusive and Infectious Diseases

Neutrophile und extrazelluläre Neutrophilen Fallen bei Autoimmun-, Verschluss- und Infektionskrankheiten

vorgelegt von

Leticija Zlatar (geb. Oreški)
aus Varaždin, Kroatien
Als Dissertation genehmigt von
der Naturwissenschaftlichen Fakultät
der Friedrich-Alexander-Universität Erlangen-Nürnberg

Tag der mündlichen Prüfung: 21.06.2024

Gutachter: Prof. Dr. Thomas Winkler
Gutachter: Prof. Dr. Udo Gaipl
# Table of Contents

1. Abstract / Zusammenfassung ........................................................................... 5

2. Scientific Context ............................................................................................. 7
   2.1. Traditional Aspect of Neutrophils as Simple Players in Innate Immunity .. 7
   2.2. An Ace up Neutrophils Sleeves: Neutrophil Extracellular Traps (NETs) .. 9
   2.3. Good Guys Gone Bad: NETs in Excess ..................................................... 11
   2.4. Current Strategies for Combatting Excessive NET Activity .................... 11

3. Relevant Findings ............................................................................................. 13
   3.1. Neutrophils and NETs in Autoimmune Diseases ...................................... 13
      3.1.1. The Most Frequent Autoimmune Disease in Humans: Rheumatoid Arthritis (RA) .............................................................................................................. 13
      3.1.2. Articular Damage Dealt by Neutrophils and NETs in RA ...................... 13
      3.1.3. Increased Osteoclast Generation and Activity in RA ........................... 15
      3.1.4. K/BxN Murine Model of Arthritis ....................................................... 15
      3.1.5. Could Diet Change the Course of RA by Modulating the Immune System? ......................................................................................................................... 16
   3.2. Neutrophils and NETs in Occlusive Diseases ............................................ 19
      3.2.1. An Unexplored Occlusive Disease: Dacryolithiasis ............................. 19
      3.2.2. The Innate Immunity Drives Dacryolithiasis ....................................... 20
   3.3. Neutrophils and NETs in Infectious Diseases .......................................... 22
      3.3.1. Tuberculosis, Sarcoidosis and the Notorious Granuloma ..................... 22
      3.3.2. Undiscovered Potential of Neutrophils and NETs in the Granuloma ...... 24

4. Concluding Remarks ......................................................................................... 27

5. Abbreviations .................................................................................................. 29
6. Figures..................................................................................................................... 30
7. References.............................................................................................................. 31
8. Publications ......................................................................................................... 41
9. Contributions....................................................................................................... 42
10. Acknowledgments ............................................................................................... 44
1. Abstract / Zusammenfassung

Neutrophils have traditionally been viewed as simple cells limited to pro-inflammatory functions, which are essentially involved in the innate immune response. Even though their main functions include fighting infectious agents and promoting inflammation, their diverse and specialized roles recently gained more attention. Among others, neutrophils produce neutrophil extracellular traps (NETs), specialized structures composed of decondensed chromatin decorated with cytoplasmic and granular proteins. Their primary function is pathogen immobilization, facilitation of phagocytosis and ultimately, pathogen elimination. However, excessive neutrophil infiltration and activation at the site of injury ultimately precipitates chronic inflammation. An imbalance between NET formation and degradation is at the root of various pathologies. To address this issue, various treatment strategies to combat excessive NET activity are currently under development. Yet, the list of neutrophil- and NET-driven pathologies is continuously expanding, and the mechanisms behind many remain elusive.

This thesis discusses our recent work on the role of neutrophils and NETs in autoimmune (rheumatoid arthritis, RA), occlusive (dacryolithiasis) and infectious diseases (tuberculosis, TBC). First, we discuss how a diet rich in salt contributes to arthritis exacerbation, by modulating neutrophils and their effector functions. We investigated the effect of sodium on neutrophils, and detected (I) elevated ROS levels in vitro, (II) arthritis exacerbation in vivo, and (III) increased in vitro activity of bone-resorbing osteoclasts upon the addition of ROS. These data indicate that under high salt conditions, ROS produced by the infiltrating neutrophils promotes bone erosion in RA. Further, we propose the role of neutrophils and NETs within the existing model of gradual formation and growth of mucoprotein concretions in the lacrimal sac, in a process termed dacryolithiasis. Neutrophils contribute to the initial, and NETs to the later stages of this process, the latter presumably by maintaining the pro-inflammatory environment and preventing efficient clearance. Lastly, we report increased neutrophil activation and circulatory NET formation in patients with TBC. Importantly, excessive amounts of NET degradation products can be detected in TBC patients with extensive tissue damage, and in patients with relapse, making NETs parameters discriminatory biomarkers. Further, caseating granulomas in TBC and other granulomatous diseases contain tissue-resident NETs, together with Z-form DNA, resistant to degradation by nucleases. Pulmonary vessels in TBC are occluded with neutrophil aggregates. Taken together, these findings highlight the dual role of neutrophils and NETs in the pathology of various diseases.

2. Scientific Context

2.1. Traditional Aspect of Neutrophils as Simple Players in Innate Immunity

Neutrophils were initially considered simple cells with limited pro-inflammatory functions, essentially involved in the innate immune response (1,2). Even though their main functions include fighting infections and promoting inflammation (3), their diverse and specialized roles have been discovered just recently (1,2). Neutrophils are involved in acute as well as chronic inflammation, and can alter the responses of adaptive immunity. They can suppress T cell proliferation and activity, but also activate B cells in the spleen. At the early stages of infection, neutrophils produce interferon (IFN)-γ, thus promoting adaptive immunity by affecting MHC expression and T helper cell development. Interestingly, neutrophils can also act as antigen-presenting cells (1). Neutrophils produce various cytokines and chemokines, and thereby amplify the inflammatory responses and recruit further immune cells (4,5). Distinct subsets of neutrophils exist under homeostatic and pathological conditions (6). These subsets have specific roles in inflammation, infection and cancer immunology (1). There are no biochemical markers to distinguish between them. Rather, neutrophil subpopulations can be differentiated based on their physical properties, such as density (3). Both pro- and anti-inflammatory phenotypes have been described.

Under physiological conditions, neutrophils are found in the lung, liver, spleen and bone marrow. The differentiation of neutrophils occurs from myeloid precursors in large quantities (up to $2 \times 10^{11}$ cells per day) in the latter (2,6), in a process called granulopoiesis (4,7). During infection, neutrophil production rates can be boosted 10-fold, up to $10^{12}$ cells per day (2). Their differentiation is primarily controlled by granulocyte colony stimulating factor (G-CSF), produced in response to interleukin (IL)-17A and synthesized by T lymphocytes. Neutrophil differentiation is further controlled by transcription factors, mainly PU.1 and CCAAT/enhancer-binding protein (C/EBP) α-ζ (1,2). In humans and mice 50-70% and 10-25% of the circulating white blood cells (leukocytes) are neutrophils, respectively (2,8).

Mature neutrophils are terminally differentiated, their nucleus is segmented, and cytoplasm enriched with granules and secretory vesicles (1). Once maturated, neutrophils are mobilized from the bone marrow into the circulation. In the peripheral blood neutrophils are not capable of dividing and have a half-life of only few hours. Under physiological conditions, only a small percentage (1-2%) of mature neutrophils is released from the bone marrow into the circulation.
CXCR-chemokine receptor (CXCR) 4 plays an important role in neutrophil retention in the bone marrow. During infection or inflammation, the production and release of neutrophils rapidly increases. This process is known as emergency granulopoiesis; the expression of CXCR2 (CXCR4 antagonist) is upregulated to promote neutrophil release into the circulation (7).

Neutrophils are usually the first type of leukocytes recruited to the site of inflammation (1,9). Fully differentiated, they migrate to the site of infection or injury to exert their effector functions. To reach the site of inflammation, neutrophils sense inflammatory cues and extravasate from blood vessels into the tissues (7). This occurs once the tissue-resident sentinel leukocytes release inflammatory mediators such as histamine, cytokines and cysteinyl-leukotrienes after coming into contact with the pathogen. Consequentially, endothelial surface changes. Endothelial cells can further be directly activated by pattern-recognition receptor (PRR)-mediated detection of pathogens. Macrophages and mast cells which reside in tissues also initiate neutrophil recruitment. They control and induce various processes, such as increasing the permeability of local blood vessels and inducing the release of chemokines. The extravasation of neutrophils is promoted by platelets (1). If neutrophils remain inactivated once they reach the tissue, they undergo apoptosis and get cleared by tissue-resident macrophages (4). Elimination of neutrophils also occurs once neutrophil activity is no longer needed at the site of injury (10,11).

Neutrophils contain at least four types of granules filled with pro-inflammatory proteins: primary (azurophilic), secondary (specific), tertiary (gelatinase), and secretory. Primary granules contain neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, proteinase 3, azurocidin, and defensins. NE is also the most abundant enzyme present in neutrophils, it is released into the extracellular spaces by degranulation. Secondary granules contain collagenase, lactoferrin, lysozyme and lipocalin. Tertiary (gelatinase) granules contain collagenase, acyl transferase, cathepsin and matrix metalloproteinase 9 (MMP9, also known as gelatinase B). Secretory granules contain serum albumin and pre-formed cytokines (2,7,8).

The first step to pathogen elimination is pathogen engulfment, termed phagocytosis (1). Phagocytosis is quick and occurs within minutes (2). It is followed by pathogen killing in a process called degranulation by either nicotinamide adenine dinucleotide phosphate (NADPH) oxygenase-dependent (NOX-dependent) mechanisms involving reactive oxygen species (ROS), or by employing antimicrobial proteins and proteolytic enzymes (12). The latter are released from neutrophil granules directly into the phagosomes or into the extracellular environment, in case of infection with intracellular or extracellular pathogens, respectively
Highly activated neutrophils further eliminate extracellular pathogens by the release of neutrophil extracellular traps (NETs), described in more detail in the next chapter (1,12).

Neutrophil immune responses are essential for protecting the host. However, excessive neutrophil infiltration and activation at the site of injury can lead to chronic inflammation, limitation of injury repair, and loss of organ function (7,13). If neutrophils release oxidants, proteases and antimicrobial proteins in an excessive or prolonged manner, the surrounding tissues become damaged, which could chronify acute inflammations (1). Therefore, neutrophils are necessary to drive the initiation of inflammation, but are also required for its resolution (5). When their mission is accomplished, neutrophil activation and infiltration needs to be dampened. This is crucial to prevent damage to the host (8). As they age, neutrophils increase the expression of CXCR4, which marks them for clearance from circulation. This step occurs in the liver, spleen and bone marrow (11).

### 2.2. An Ace up Neutrophils Sleeves: Neutrophil Extracellular Traps (NETs)

NETs were first described nearly two decades ago (12). Nevertheless, scientific interest in NET formation and their roles in various health issues still continues to be an active area of research. NETs are structures composed of decondensed chromatin (DNA and histones) decorated with antimicrobial proteins from primary, secondary and tertiary granules, such as MPO or NE, lactoferrin or cathepsins, and gelatinase, respectively (2,12). The vast majority of NET-associated proteins are histones. Out of all cytosolic and granular proteins, NE is the most abundant one (14). Although the majority of DNA is of nuclear origin, NETs have been shown to further contain the mitochondrial DNA (15). NET formation appears as an evolutionarily conserved mechanism (16), and extracellular traps have already been described for other granulocytes (basophils, eosinophils, and mast cells), as well as macrophages (17). The primary function of NETs is pathogen immobilization. Bacteria and fungi get entrapped in NETs which prevents spreading and facilitates phagocytosis. Antimicrobial histone and proteases directly kill pathogens (12,18). Some studies confirmed direct killing of microorganisms by NETs, but others did not. This could be either species-specific, or dependent upon the production of deoxyribonucleases (DNases) by certain pathogens. DNases readily dismantle NETs and abrogate their antibacterial activities by degrading the DNA backbone (1,14,16). Some bacteria even use NETs to their advantage, as occurs in biofilm formation (16).
NET formation is spontaneous under homeostatic conditions in low quantities, however it can be further stimulated with IL-8, phorbol myristate acetate (PMA), lipopolysaccharide (LPS) (12), A23187 (19), ionomycin, MSU crystals (20), and others. At least twenty-five different inducers of NET formation have so far been described; PMA is the most frequently used, it is very robust (4,20). More physiological stimuli include immune complexes and cholesterol crystals, fungi and bacteria (4).

Depending on the stimulus, NOX-dependent or NOX-independent NET formation occurs. Most common stimuli which induce the NOX-dependent NET formation are PMA and LPS; A23187 and ionomycin induce the NOX-independent NET formation. In the latter, mitochondrial ROS (mtROS) are produced instead (21). In both pathways, the formation of NETs begins with the production of oxidants which leads to the degradation of the granular and nuclear membrane. Enzyme peptidyl arginine deiminase 4 (PAD4) is implicated in initial chromatin decondensation, it catalyzes the citrullination of arginine residues on histones, causing chromatin relaxation (2,14). NE migrates to the nucleus and employs its’ protease activity to cleave the histones, assisting in chromatin decondensation (4). Once decondensed, chromatin fibers associate with granular and cytoplasmic proteins, NETs are released into the extracellular space (4,19). PAD4 and NE activity are required in many forms of NET formation. PAD4-deficient mice are unable to form NETs, and NET formation does not occur if the elastase activity is inhibited (14). MPO further promotes carbamylation of histones (22). The whole process of is completed in 1-4 hours, and depends on the type of the stimulus (23).

NETs can be visualized by the means of immunofluorescence (12). As NETs are rich in citrullinated proteins, the colocalisation of citrullinated histone H3 (citH3) with extracellular DNA (ecDNA) can be used to detect NETs in human or murine tissues (4,5). A novel method with improved specificity for the detection of ecDNA in the tissue includes the application of an anti-DNA-IgM antibody (24). NETs can further be quantified by measuring the fluorescence intensity of a DNA dye that is excluded from cells with the use of a fluorometer e.g. propidium iodide (12).

In the early stages of neutrophilic inflammation, neutrophils are found in lower densities, and NETs remain solitary structures. They release pro-inflammatory cytokines and chemokines, and act as a source of autoantigens. For example, IL-8 is released, a key chemokine which is involved in the recruitment of neutrophils (5). On the other hand, in fully-blown inflammation, neutrophils are found in comparably higher densities, and NETs become aggregated NETs (aggNETs). These structures degrade the pro-inflammatory mediators and
contribute to the resolution of inflammation (25-27). IL-1β and IL-6 are effectively degraded by aggNETs in vitro by the protease activity of serine proteases associated to aggNETs, disrupting neutrophil recruitment and activation (5,26).

2.3. Good Guys Gone Bad: NETs in Excess

NETs are involved in the initiation, and later in the resolution of inflammation. Both are needed to elicit a balanced immune response with the main functions to protect the host from pathogens and from the damage they may cause (5,17). However, unneeded or excessive NET formation in non-pathogenic conditions may trigger the production of autoantibodies and lead to the development of autoimmune diseases (9,14,19). This increases the chances for systemic organ damage. NET formation is elevated in patients with various autoimmune diseases (28), and an imbalance between NET formation and degradation is at the root of autoimmune diseases (9). Such effects have been described in systemic lupus erythematosus and rheumatoid arthritis (RA) (29). Interestingly, human individuals with impaired NET formation often suffer from chronic inflammatory conditions and/or exacerbated autoimmune diseases, implying that NETs have anti-inflammatory as well as immune-regulatory functions (25).

The pathology associated with the excessive formation of NETs mainly depends on the type of tissue affected (17), and the list of NET associated pathologies is continuously expanding (15,30). NET formation has been reported in diverse forms of vasculitis, (immuno)thrombosis (22,29), transfusion-related acute lung injury and cancer (1). In such pathologies, strategies for eliminating NETs are needed, these are further discussed in the following chapter. Besides vasculitis and (immuno)thrombosis, other NET-driven occlusive diseases have been reported. In such pathologies, occlusions are further calcified; this results in the formation of macroscopic stones. So far, reports on stone formation and corresponding organ obstruction have been described in the biliary system, kidneys, prostate and the appendix. The ducts of exocrine glands can become occluded as well; including ducts of ocular glands, salivary glands, and others (18,31). Further, NETs have been implicated in the formation of peritoneal adhesions, a highly prevalent condition that is poorly understood and can lead to intestinal obstruction (32).

2.4. Current Strategies for Combatting Excessive NET Activity

Mechanisms that lead to the clearance of NETs are still elusive (15). However, certain therapies aimed at NETs and NET-associated proteins are in the pipeline, to be used in treating various diseases (9,17). The first possibility for combatting excessive NET formation and/or
activity is the abrogation of NET formation itself (12,29,30). This can be done by inhibiting the PAD4 activity, essential to many forms of NET formation (15,16). Treatment with PAD4 inhibitors has already had promising results for the treatment of autoimmune diseases (16). Alternative therapeutics include gasdermin D inhibitors. This protein assists in pore formation, it is essential in both NOX-dependent and NOX-independent NET formation, and can reduce the membrane stability (11,21). Another approach is the degradation of NETs by DNases; such as DNase I, a nuclease secreted into the plasma (15,30), followed by phagocytosis of the degradation products by macrophages (15). Apart from DNase I, DNase I-like 3 (DNase 1L3) is also capable of NET degradation (16,33). These two DNases are expressed independently and provide a dual protection to the host (33). DNase I preferentially digests the double-stranded DNA (dsDNA), and DNase 1L3 favors the degradation of chromatin. As the efficient degradation of NETs requires the combined action of both enzymes, recently, a novel form of mutant DNase I, the so-called “dual-active DNase I” has been engineered. This recombinant DNase is superior to the native DNases, as it degrades both dsDNA and chromatin (34).

DNase I was already found effective against various inflammatory diseases (7), its advantage is low toxicity (35). Recombinant human DNase I (Pulmozyme, Roche) is available for the treatment of patients with cystic fibrosis. It was approved by the Food and Drug Administration and is applied by inhaling. It digests the DNA in the alveolar space. Off-label use was effective in patients suffering pulmonary diseases such as asthma, COVID-19 and others (34). It is important to keep in mind that DNases remove DNA from any source; thus the application of DNases is not specific (16). In addition, NET-associated proteins persist long after the DNA has been degraded, indicating that different mechanisms are responsible for their clearance (15). As the accumulation of NETs further causes the production of anti-dsDNA antibodies, another opportunity for combatting the effects of excessive NET activity would be the blockade or selective depletion of these autoantibodies (29). Rituximab and belimumab reduce NET formation by blocking the formation of immune complexes (35).
3. Relevant Findings

3.1. Neutrophils and NETs in Autoimmune Diseases

Autoimmune diseases are characterized by systemic chronic inflammation. Their pathogenesis is often incompletely understood (36). Underlying is the inability of the immune system to differentiate between the self and non-self. This occurs once the loss of immune tolerance takes place (30), and the acute inflammation turns into chronic. Ultimately, extensive damage is dealt to the tissues and organs (11).

3.1.1. The Most Frequent Autoimmune Disease in Humans: Rheumatoid Arthritis (RA)

One of the most frequent autoimmune diseases in humans is RA, its prevalence is about 0.5-1%. Generally, RA is more prevalent in women than in men (37,38). It can occur anytime, but most commonly at the age of 40-70, and its incidence increases with age (38,39). The hallmark of RA and other rheumatic diseases is the chronic inflammation and musculoskeletal tissue damage (40,41), as well as the formation of autoantibodies, of which rheumatoid factor and anti-citrullinated protein antibodies (ACPA) are established (42-44). Such autoantibodies appear years before RA onset (45-47). They are associated with increased bone loss (46,48,49). The predominantly affected tissues in RA are the joints. Inflammation of the surrounding synovia, erosion of bone and cartilage, and ultimately joint deformation occur (43,50). The accompanying symptoms include pain, stiffness and swelling. The progressive articular damage can even lead to permanent disability (11,39). The treatment of RA is not simple, and generally, a life-long therapy is needed. The development of more effective treatments resulted in considerable progress in managing the disease. Despite the rising prevalence and incidence of RA, severity, mortality, and RA-associated comorbidities appear to decrease (37). Still, RA is associated with a higher mortality rate than in the general population. Key therapeutic agents include disease-modifying antirheumatic drugs such as methotrexate. They act by reducing synovial and systemic inflammation. Modern therapy schemes include biological agents, such as inhibitors of tumor necrosis factor, and various further monoclonal antibodies (38,51).

3.1.2. Articular Damage Dealt by Neutrophils and NETs in RA

RA is a multifactorial disorder with a dysregulated function of both innate and adaptive immunity (52), its exact pathogenesis remains unclear (43,53,54). The hallmark of RA is bone
erosion (50,55), a consequence of synovial osteoclast formation (50). Inappropriate activation of neutrophils contributes to the pathogenesis of various systemic autoimmune diseases, including RA (52,53). Neutrophils infiltrate the synovial fluid of the affected joints in RA patients. Their recruitment is promoted by the platelets (56). Neutrophils have been associated with both the onset and the progression of RA. Their pathogenic roles are various and include increased cell survival and migration (54), enhanced inflammatory activity, elevated ROS production (57), and exacerbated NETs release. Their excessive recruitment is thought to be crucial in the early phases of RA development. Proteomic analysis revealed that neutrophils are the most abundant cell type in the inflamed synovial fluid of RA patients (54). Importantly, depletion or inhibition of neutrophils reduces the inflammation and bone damage in experimental arthritis models (53).

Neutrophils are major sources of citrullinated antigens. They produce PAD4 which catalyzes protein citrullination (11); and ACPAs play a key role in RA pathogenesis (9,28,29). Acetylated and carbamylated proteins have been detected in RA patients as well. These form immune complexes in the joints, contribute to RA pathogenesis, and promote inflammation and bone erosion (11). Neutrophils can activate further immune cells, chronify inflammation, and trigger joint destruction (53). Given that histone citrullination is especially abundant during NET formation, NETs play a critical pathogenic role in RA (28,35). RA serum and synovial fluid are strong inducers of NETs (29). Analysis of the peripheral blood and synovial fluid from RA patients and healthy controls revealed increased NET formation in RA patients (28), their concentration is associated with disease severity (22). NET degradation products, such as NE-DNA and MPO-DNA complexes, as well as circulating free DNA (cfDNA), are increased in RA patients (35). NET formation in the joint has been shown to release active PADs, which supply citrullinated autoantigens (58). It is not surprising that also antibodies to citrullinated histones or, rarely, to dsDNA occur (14,22). In many patients, antibodies against PAD4 even precede the clinical onset of RA, indicating that histone citrullination has a role in the initial phases of RA. The levels of NE are further imbalanced in RA (7). Plasma levels of cell-free nucleosomes can be used as a biomarker for the diagnosis of RA patients, the advantages are high specificity and sensitivity (59). As the disease progresses, released NETs exacerbate the inflammation by interacting with further immune cells. Finally, this leads to tissue damage and organ dysfunction and NETs contribute to all stages of RA (22).
3.1.3. Increased Osteoclast Generation and Activity in RA

Bone homeostasis is managed by the bone-resorbing and -generating activities of osteoclasts and osteoblasts, respectively. These processes are balanced under physiological conditions (42,44,60). Under pathological conditions, such as in RA, osteoclast generation and activity are greatly exceeded (42). The rate of bone resorption is much higher than the rate of bone formation (40), leading to the accumulation of articular damage (61). When osteoclasts are effectively blocked or genetically depleted, no erosions are formed, even in the presence of synovial inflammation. Thus, osteoclasts are the only cell type capable of degrading the bone. In this process, calcium resorption is involved (40,50,51). Macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are key cytokines that drive osteoclast differentiation (51,62,63). Both are abundant in the rheumatoid synovial membrane (61). Besides, superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂), are important regulators of osteoclast differentiation and bone homeostasis (60). The immune system interacts with the skeleton, thus affecting bone degradation in RA and other rheumatic diseases. It has previously been reported that T lymphocytes trigger bone loss by inducing the differentiation of osteoclasts (40), however, the effects of other immune cells on the skeleton remain less understood. The regulation of osteoclast formation needs better understanding to define the mechanisms behind bone erosion in rheumatic diseases.

3.1.4. K/BxN Murine Model of Arthritis

There are several murine models of arthritis. They were established by the researchers to analyze the course and treatment of RA. Importantly, neutrophils are essential for the initiation and progression of arthritis in these models. A well-established murine model of arthritis is the K/BxN serum transfer arthritis (STA), its pathology is similar to that of human RA (35,64). K/BxN mice are generated by crossing the autoimmunity-prone non-obese diabetes mice with T cell receptor transgenic mice (KRN-C57BL/6). They develop immunoglobulin G (IgG) antibodies against glucose-6-phosphate isomerase (G6PI), an enzyme involved in the glycolytic cycle. Transfer of the K/BxN serum or purified G6PI-specific IgG leads to the development of arthritis in various mouse strains (64,65). Considering that the autoantibodies are passively transferred, K/BxN STA focuses on the effector phase of arthritis, i.e. the recruitment of immune cells, and joint destruction, rather than the priming phase and loss of tolerance. This model has a rapid and robust onset of arthritis, the incidence is 100% in genetically identical animals, and it can be induced in various mouse strains (65). In the joints of animals with K/BxN
STA, NETs were detected. However, the activity of PAD4 is not required for disease development, as wild-type and PAD4-knockout mice develop K/BxN STA similarly (64).

### 3.1.5. Could Diet Change the Course of RA by Modulating the Immune System?

Genetic predisposition accounts for about 30-50% of the risk for autoimmune disease development, and the other 50-70% can be contributed to environmental factors (38,66). These factors are modifiable, and lifestyle changes are already being incorporated to prevent RA development (67). These factors include smoking, pollution, and importantly, one’s dietary habits (37). Accumulating studies demonstrate the essential role that nutrients play in initiation and progression of RA (54), and an “inflammatory” diet has long been considered to lead to the development of RA. Daily consumption of sodas, rich in sugar, is associated with an increased risk for RA (37). On the other hand, moderate alcohol consumption is a protective factor (66), and so is the intake of dietary fiber (68).

There is a rising interest in the effect of nutrients on the pathogenesis of RA by modulating the activity of immune cells, including neutrophils. Nutrients have been classified into those that exacerbate (carbohydrates, red meat etc.), and those that ameliorate (omega-3 fatty acids, vitamin D etc.) the inflammation, with respect to their influence on neutrophils (54). Sodium plays a vital role in physiology by maintaining extracellular fluid volume, water balance, and cellular membrane potential. However, excessive dietary sodium consumption has been suggested as a risk factor for RA. The interactions between sodium intake and immune-mediated inflammatory diseases are of particular interest (67). Several studies have investigated the effect of increased dietary sodium intake and RA. High sodium intake has been implicated as a contributing risk factor in smokers (69). There is a dose-dependent relationship between daily sodium intake and RA, it is even more clear in the group of nonsmokers (67). However, there is not enough evidence to support one specific diet, and the results are often conflicting; this field of research warrants further studies (37,67).

We investigated the role of neutrophils and NETs in RA in the context of high sodium. We performed both in vitro and in vivo experiments, employing the murine K/BxN STA model of arthritis (70). We observed that high sodium concentrations generally suppress neutrophil effector functions, except for ROS production, including mtROS. Interestingly, NET formation was abrogated in vitro; rather extensive necrosis occurred. Despite the involvement of ROS in the rupture of neutrophil granules (4), we detected increased ROS and mtROS production; but
decreased degranulation and MPO activity. PAD4 activity and NET formation have already been described in the K/BxN STA. In line with these findings, we further detected NETs in intraosseous spaces in these mice. ROS and mtROS production by neutrophils were significantly increased in high sodium concentrations, indicating a substantial degree of stress on neutrophils in such environments. NE activity was also increased. An aggravation of arthritis in mice fed a high salt diet (HSD) occurred, when compared to mice on normal diet (ND). This was indicated by increased inflammation, osteoclast numbers, and bone erosion. Within cellular infiltrates in the proximity to the bone, we detected intact neutrophils. As the in vitro analysis revealed that these cells are highly stressed and release high amounts of ROS, we hypothesized that they alter the microenvironment in the joint. Osteoclasts need ROS for their differentiation and resorption activity, therefore, we tested whether ROS released by neutrophils could have a pro-inflammatory effect on osteoclasts in an in vitro system. We employed H₂O₂, as a downstream product of NOX (4), to investigate whether increase in ROS concentration affects osteoclast differentiation and/or resorption activity. Whereas sodium had anti-osteoclastogenic effects in vitro; the addition of a ROS mimetic (H₂O₂) alone was enough to increase the resorptive capacity of osteoclasts. This revealed a possible link between neutrophils and osteoclasts in RA in the context of a sodium-rich diet. An overview of our findings is displayed in Figure 1. There is a strong positive correlation between serum ROS levels and disease severity in RA patients and arthritic rodent models (71). Further investigation to elucidate this mechanism are needed, this includes neutrophil depletion, or application of ROS scavengers.
Figure 1. Suppression of Neutrophils by Sodium Exacerbates Oxidative Stress and Arthritis.

Increased dietary salt intake leads to accumulation of sodium chloride (NaCl) in joints and other tissues. In a hyperosmolar environment, neutrophil effector functions are inhibited, except for ROS production. Neutrophils undergo extensive cell death by necrosis and NE activity is increased. This pro-inflammatory environment further affects osteoclast function; wherein ROS produced by the infiltrating neutrophils promote osteoclast-driven bone resorption. In an in vivo model of arthritis (K/BxN STA) driven by autoantibodies against glucose-6-phosphate isomerase (G6PI), mice kept on a high salt diet exhibit increased paw swelling, inflammation, osteoclast differentiation, and bone erosion, resulting in arthritis exacerbation. Abbreviations: NET, neutrophil extracellular trap; ROS, reactive oxygen species; H₂O₂, hydrogen peroxide; NE, neutrophil elastase; G6PI, glucose-6-phosphate isomerase; IgG, immunoglobulin G. Created with BioRender.com.
3.2. Neutrophils and NETs in Occlusive Diseases

Occlusive diseases are characterized by the clogging of tubular structures. Examples include the formation of clots within vessels, the obstruction of airways, and the formation of stones within ducts and acini of various exocrine glands. Consequently, reduced flow of bodily fluids occurs, otherwise essential in homeostasis (18,30,31).

3.2.1. An Unexplored Occlusive Disease: Dacryolithiasis

Dacryolithiasis, or the formation of dacryoliths ("lacrimal stones") is an occlusive disease affecting the lacrimal drainage system. Unlike other pathologies caused by occlusions, dacryolithiasis remains vastly unexplored. The cause and mechanism behind it are unclear (72). Several pre-disposing factors leading to dacryolith formation have been described, however, these are still a matter of debate, as conflicting reports on various factors exist. Especially, the effects of age and gender remain elusive (73,74). Early study using tears from patients with partial nasolacrimal duct obstruction, either with or without dacryoliths, revealed altered concentrations of electrolytes and proteins between the two groups. pH of the tears in both groups was higher than pH of the tears in healthy controls (75). Dacryoliths are uncovered and surgically removed during a procedure termed dacryocystorhinostomy (DCR) (72,73). The reported incidence among all DCR surgeries is 6-18% (72,76).

Dacryoliths are commonly classified into two groups: infectious and non-infectious. The former are formed within the canalicular system, thereby also termed “canalicular concretions” or “canaliculoliths”. They are often caused by Staphylococcus, Streptococcus, or Actinomyces species (73,74,77). Mucoprotein concretions (MPC-D) or “mucoliths” are formed in the lacrimal sac and the nasolacrimal duct, their origin is often non-infectious and less understood (73,74,78). A “third group”, encompassing “mixed” concretions with characteristics of both previously described groups, has further been proposed (79).

Under physiological conditions, lacrimal sac and nasolacrimal duct epithelia produce mucins and tre-foil factor peptides to enhance tear transport and antimicrobial properties. However, under pathological conditions, their production is vastly upregulated and accompanied by the infiltration of B and T lymphocytes, macrophages, and “impressive amounts” of neutrophils (76). Compared to other concretions such as salivary gland stones (80) or gallstones (81), MPC-D are soft, as they are composed of organic material, mainly mucoproteins (76,82). In a recent study, proteomic analysis of the organic material in MPC-D was performed.
Identified were proteins, essentially involved in (innate) immune and inflammatory responses, many of which were associated with neutrophils (83).

3.2.2. The Innate Immunity Drives Dacryolithiasis

Current model of dacryolithiasis describes gradual formation, and consensus on four distinct stages has been obtained. First stage involves susceptibility, it is followed by initiation, development, and lastly, maintenance of MPC-D (78). Electron microscopy revealed a fibrillary network in the core, abundant in red blood cells (84). Immunohistochemical analysis further revealed von Willebrand factor, essential in platelet adhesion, and fibronectin, involved in blood clotting, in the core (85). 3D cinematic rendering of MPC-D revealed that the core is compact and dense, with a density gradient from core to periphery (86). Considering, the initiating event was proposed to be a mechanical or chemical insult to the epithelium of the lacrimal sac, followed by blood leakage (84, 86). The blood clot then acts as a nidus for layering of amorphous material and mucopeptides, resulting in cell infiltration, inflammation (78), and swelling (75). Fully formed MPC-D have a macroscopic size, capable of occluding the lacrimal sac and the nasolacrimal duct, resulting in excessive tearing (72).

In our study, we confirmed the layered structure of MPC-D, with only few and scattered calcifications. Cytokeratin staining further revealed the presence of epithelial cells. As we detected heterogeneity between various MPC-D, to facilitate future research we proposed a classification system based on the amount of cellular and acellular material as well as polysaccharides and mucoproteins. Type I MPC-D were classified as abundant in cellular material, type II MPC-D were classified as mostly acellular, and type III MPC-D had intermediate amounts of both cellular and acellular material. Nevertheless, we detected regions containing cells in all types of MPC-D. As it was previously reported that MPC-D are abundant in neutrophils (76), and since neutrophil-derived extracellular traps have already been linked to gallstone (81) and salivary gland stone formation (80), we further investigated the role of neutrophils and NETs in dacryolithiasis. We proposed that neutrophils are involved in the early stages, and NETs in the later stages of MPC-D formation, based on their localization to the inside or the surface of the MPC-D, respectively. On the surface, NETs might contribute to the maintenance of those MPC-D that do no dissolve by maintaining the pro-inflammatory environment (87). All MPC-D contained active NE. In certain areas, we detected protein aggregates, which could still be immunogenic. An overview of these findings in the context of dacryolithiasis is displayed in Figure 2. Whether neutrophils and NETs are also involved in the formation of canalicular concretions, remains unexplored. However, considering that bacterial
infection is involved in this case, it would not be surprising that a similar mechanism, involving neutrophils and NETs, operates. In this context, NETs might prove to be at least initially beneficial, by limiting the pathogen dissemination, and potentially, facilitating its elimination.

**Figure 2. Neutrophil Extracellular Traps Drive Dacryolithiasis.**

Mucopeptide concretion (MPC-D) adopts the shape of the lacrimal sac and nasolacrimal duct in which it is formed. Once it reaches its macroscopic size, MPC-D occludes the sac and the ducts, causing inflammation and swelling. As a consequence of poor drainage, excessive eye tearing occurs. Note the canaliculi (pair), in which the formation of infectious dacryoliths (canalicular concretions) occurs instead. Whether neutrophils and NETs are also involved in their pathogenesis, remains unstudied. At the center of an MPC-D, a blood clot is depicted, it acts as a nidus for MPC-D formation. In the core, neutrophil markers are abundant, however, NETs are detected on the surfaces. Few calcium deposits are shown in green. The removal of an MPC-D is performed in a surgical procedure termed dacryocystorhinostomy. Abbreviations: NETs, neutrophil extracellular traps. Created with BioRender.com.
3.3. Neutrophils and NETs in Infectious Diseases

The deadliest infections in the world are commonly referred to as the “big three”. These include acquired immunodeficiency syndrome, malaria, and tuberculosis (88). There are no examples of sufficient natural immunity that clear the pathogen in any of them. Despite prevention efforts and new drugs, effective vaccines are urgently needed (89).

3.3.1. Tuberculosis, Sarcoidosis and the Notorious Granuloma

Tuberculosis (TBC) is caused by the *Mycobacterium tuberculosis* (MTB) infection. Symptoms are non-specific, and include weight loss, fatigue, back pain, night sweats and breathing obstruction (90). In ~85% of cases, MTB infects the lungs, but cases of extrapulmonary TBC have also been described (91,92). Extrapulmonary TBC can involve any organ; however, lymph nodes and pleura are the most commonly affected tissues (91). Bacillus Calmette-Guérin vaccine is applied worldwide to prevent TBC in infants and young children. However, it is ineffective in controlling the global TBC epidemic (90,93,94).

The disease is commonly divided into latent and active. Latent TBC is asymptomatic and non-transmissible, whereas active TBC is both symptomatic and transmissible (92,93). Various assays are employed for diagnosis, they are based on the type of the disease. These include tuberculin skin test and IFN-γ release assays in case of latent TBC (95); in case of active TBC, chest radiography, computed tomography, sputum smears, culture-based methods and molecular tests including Xpert MTB/RIF test are used (91,96). Despite, there is an urgent need for simpler, low-cost and widely-available screening tests (96,97).

In MTB-infected individuals, substantial and chronic lung disability occurs. Left untreated, TBC has a mortality rate of ~70% in smear-positive individuals. Even though only ~12% of the infected individuals develop the disease (91), more people have died of TBC than any other infectious disease (98). Key risk factors for developing TBC, as well as progressing from latent to active TBC include poverty, overcrowding, undernutrition, alcohol misuse, HIV infection and others (99). TBC mortality has decreased almost in half in the past two decades and the worldwide incidence of TBC appears to decrease. However, TBC incidence in some parts of world such as Africa is still rising, and drug-resistant strains pose a great threat to current treatment strategies. Various types of drug-resistant TBC exist: monoresistant, multi-drug resistant (MDR), pre-extensively drug resistant (pre-XDR) and extensively drug resistant (XDR) (91). Standard TBC treatment includes four first-line drugs: ethambutol, isoniazid,
pyrazinamide, and rifampicin, and resistance to all drugs can occur. MDR TBC includes resistance to at least isoniazid and rifampicin (95,100). Pre-XDR further includes resistance to any fluoroquinolone or a second-line drug, whereas XDR further includes the resistance to both (92,101,102).

A hallmark of MTB infection is the formation of granulomas, organized structures (aggregates) of various immune cells (90,103,104). Once inhaled, MTB is transported to the lung (104) and phagocytosed by the resident alveolar macrophages (92,97,105). Following, the recruitment of additional macrophages and other immune cells occurs (106). Macrophages within the granuloma undergo additional changes, and may differentiate into foam cells, characterized by lipid accumulation, or epithelioid macrophages, which link the adjacent cells. Other cells include dendritic cells, B and T lymphocytes, natural killer cells, and neutrophils. MTB can infect macrophages, dendritic cells, monocytes and neutrophils (98), all involved in the early defense against MTB by utilizing phagocytosis (107).

The formation of granulomas has long been considered to have a protective purpose of containing and restricting the pathogen (97,104,107), by providing a microenvironment in which antigen-specific T cells activate the infected macrophages to inhibit MTB growth (91,108). However, MTB reportedly exploit granulomas during early stages of infection to expand and disseminate (98,106,109). Once MTB is internalized by the local macrophages, it recruits new macrophages and induces their rapid movement within the granulomas. These newly recruited macrophages phagocytose the locally present macrophages which have been infected and are undergoing apoptosis. The expansion of the infected macrophages, as well as MTB within, occurs. Some infected macrophages that exit the primary granuloma contribute to, or even cause, MTB dissemination by establishing remote granuloma formation (98,106).

In TBC granulomas, macrophages undergo (at least) two types of cell death: apoptosis and necrosis. Both can promote or inhibit bacterial growth and proliferation, depending on the circumstances (98). If macrophages undergo necrosis, caseating granulomas with central necrosis are formed. This potentially leads to spread of MTB (108). There is evidence of bacteria being released from necrotic macrophages (98). Caseating granulomas are a hallmark of TBC (90); the “caseum” they contain is necrotic material with a cheesy white appearance. They result from caseous necrosis, a specialized form of necrosis including cellular degradation (98). In animal models of TBC, bacterial growth is rapid in the first two-three weeks after the initial infection, however, it plateaus with the development of adaptive immunity. Thus, the latter is thought to restrict bacterial growth during granuloma formation (98,106).
The complete pathogenesis of TBC is complex, and remains elusive (97). Decoding the mechanisms behind granuloma formation and maintenance present themselves as an important field of research, as granulomas are involved in both the control of infection and pathogen persistence (92). There are further types of diseases associated with granuloma formation. Most closely related with TBC is sarcoidosis (SARC) (110,111), again its exact cause remains unknown. Conflicting reports on MTB as a potential cause of SARC are available, and MTB may play a role in some cases of SARC (112,113). Caseating and non-caseating granulomas without central necrosis are hallmarks of TBC and SARC, respectively (114). Nevertheless, some of the granulomas in SARC are also caseating (115).

3.3.2. Undiscovered Potential of Neutrophils and NETs in the Granuloma

Our understanding of the early phase of MTB infection in humans is very limited. Considering that the exposure to MTB leads to two broad outcomes; either pathogen elimination, or pathogen persistence, further investigations on early immune responses are needed (92). Lungs are enriched in mature, fully differentiated neutrophils. Neutrophils spend more time in lungs than in other organs, such as spleen or bone marrow, and a large number of neutrophils are present in the lung under physiological conditions. There is increasing evidence of rapid and massive neutrophil recruitment to the MTB-infected lungs, and neutrophils reportedly harbor the bacteria (116,117). Neutrophils are the most abundant immune cell type in the MTB-infected lungs during active TBC in humans (118,119). MTB has been shown to induce neutrophil necrosis (117,120), and the uptake of infected necrotic neutrophils by macrophages promotes MTB growth (120). Thus, the importance of neutrophils in lung-associated diseases such as TBC, should not be overlooked (1).

Beyond their role in the resolution of infection, NETs have been implicated in the pathology of a growing number of infectious diseases (34,116,121), as well as various lung diseases, including acute lung injury, acute respiratory distress syndrome (122), asthma, cystic fibrosis, and chronic obstructive pulmonary disease (116,123). It is becoming increasingly clear that an imbalance in NET formation and degradation, rather than the NET formation itself, is responsible for worse disease outcomes (30). Despite, the role of neutrophils and NETs in TBC remains less studied (124), as previous research mainly focused on the macrophages.

In a murine model of pathogen-induced lung injury, PAD4 deficiency reduced NET formation and lung injury, however, increased bacterial load and inflammation (30). Therefore, the current and relevant question is whether the release of NETs in TBC is more beneficial or
harmful to the host (93). Increased circulatory NET formation was detected in patients with active pulmonary TBC (125), and high levels of citH3 were observed in the sera of patients with severe lung damage (121). Plasma MPO and NE levels are elevated in pulmonary TBC, they decrease following anti-tuberculosis treatment (119). NET levels correlate with disease severity, and also decrease with therapy (126). NETs were found in necrotic lung lesions of human pulmonary TBC, as well as TBC-susceptible mice. The formation of NETs was shown to exacerbate the disease (127).

Several bacterial strains are potent inducers of NET formation, including bacteria which cause respiratory tract infections. *Haemophilus influenzae, Klebsiella pneumoniae,* and MTB can induce the formation of NETs (94,128,129). Whether these NETs are capable of killing the pathogen remains an active area of research. NETs can trap MTB, but are unable to kill it (124,129,130). An extensive list of pathogens which successfully evade the entrapment by NETs via NET degradation and other mechanisms is also available (128). Interestingly, these pathogens often first induce NET formation, and then secrete DNases to disassemble NETs, which leads to the release of the pathogen (23,128). Whether nucleases secreted by MTB are also capable of NET degradation, is an interesting area of research that warrants further investigation. So far, the MTB-derived nuclease Rv0888 induced the formation of NETs, despite its nuclease activity (130). Whether it also causes NET degradation, needs to be clarified.

We assessed neutrophil activation and circulatory NET formation in patients with distinct types of TBC by analyzing NE-DNA and MPO-DNA complexes, cfDNA and NE activity (submitted). We detected elevated NET formation and NE activity in TBC when compared to healthy controls. Importantly, NET degradation products were elevated in patients with extensive tissue damage, and patients with relapse. We highlight the importance of balancing NET formation to prevent tissue damage and patient relapse, and further propose the analysis of circulating NET degradation products as a biomarker for patients at risk of relapse. By analyzing biopsies from patients with pulmonary and extrapulmonary TBC, as well as other granulomatous diseases, we detected NETs only in caseating, and not in non-caseating granulomas. The latter rather displayed infiltrates containing degranulating neutrophils. Interestingly, within all granulomas, we detected Z-form DNA, resistant to the degradation by DNases. DNases are usually employed to degrade excessive NET formation or aberrant degradation, and bacteria produce them as an evasion mechanism to escape the entrapment by NETs. Would the NETs in caseating granulomas be handled by DNase digestion; it might prove
ineffective due to the abundance of this DNase resistant form of DNA. Lastly, as TBC is associated with hypercoagulation and (immuno)thrombosis, we analyzed the pulmonary vessels for occlusions, and observed neutrophil-rich vascular occlusions in TBC patients. Taken together, our findings highlight the dual role of neutrophils in TBC pathology.

Figure 3. Neutrophil Extracellular Traps Characterize Caseating Granulomas.
Granuloma formation occurs in pulmonary and extrapulmonary pathologies such as TBC or SARC. Two types of granulomas exist: caseating, containing the area of central necrosis, and non-caseating, without. NET formation occurs only in the caseating granulomas, regardless of the affected tissue or disease type. Non-caseating granulomas rather contain infiltrates rich in degranulating neutrophils. Both types of granulomas are characterized by their content of Z-form DNA, which is resistant to DNase I. DNase-sensitive extra nuclear B-form DNA was to be detected outside the granulomas. Circulatory NET degradation products contribute to the tissue damage, and are elevated in patients with relapse. Abbreviations: DNase, deoxyribonuclease; NETs, neutrophil extracellular traps; TBC, tuberculosis; SARC, sarcoidosis; MTB, Mycobacterium tuberculosis; T\textsubscript{reg}, regulatory T cell; NK cell, natural killer cell; DC, dendritic cell.
4. Concluding Remarks

Neutrophils and NETs are essential in eliciting appropriate immune responses, especially in the early stages of pathogen elimination. However, insufficient or excessive neutrophil activation and elevated and prolonged NET formation are rather detrimental to the host. The consequences are damage to the healthy tissues. This is an underlying cause among various pathologies, such as autoimmune, occlusive and infectious diseases. Whereas it is logical that neutrophil overactivation and NET formation are inappropriate in sterile inflammation, here we discuss that this further applies to infectious diseases, that otherwise require the pro-inflammatory response for efficient pathogen elimination.

The most important findings discussed in this thesis include:

(I) Neutrophils produce excessive amounts of ROS in a high sodium environment; ROS further increase the activity of bone-resorbing osteoclasts in arthritis (70).

(II) Neutrophils act in the early stages of mucoprotein concretions formation, and NETs promote their growth by settling on their surfaces (87).

(III) Neutrophils are highly activated in TBC patients, and excessive NET formation in the caseating granulomas contributes to lung destruction, and patient relapse (submitted).

NETs are generally beneficial, and of utmost importance during infections. However, NETs formed in excessive amounts, or inappropriately cleared contribute to development and pathogenesis of various autoimmune, occlusive and infectious diseases. Timely intervention to restore the balance is needed. This can be achieved by blocking the pathways leading to excessive NET formation, or by dismantling of existing NETs and their aggregates.
Figure 4. Neutrophils and NETs in Autoimmune, Occlusive and Infectious Diseases.

An overview of neutrophils and NETs implications in various pathologies, including RA, dacyrolithiasis, and TBC. Neutrophils, as the effector cells of the innate immune system, are essentially involved in containing and eliminating invading pathogens. Neutrophils often release NETs, structures containing decondensed chromatin and various granular and cytoplasmic proteins. NETs can trap and, in some cases, eliminate the pathogen. However, excessive neutrophil infiltration and activation, as well as unbalanced NET activity, are at the root of various pathologies, including autoimmune, occlusive and infectious diseases. Hence, it is important to continue studying the mechanisms involving neutrophils and NETs in various contexts, so that the appropriate balance can be achieved and new immunotherapies developed. Abbreviations: NETs, neutrophil extracellular traps.
## 5. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregated NET</td>
<td>aggNET</td>
</tr>
<tr>
<td>Anti-citrullinated protein antibody</td>
<td>ACPA</td>
</tr>
<tr>
<td>Circulating free DNA</td>
<td>cfDNA</td>
</tr>
<tr>
<td>Citrullinated histone H3</td>
<td>citH3</td>
</tr>
<tr>
<td>CXC-chemokine receptor</td>
<td>CXCR</td>
</tr>
<tr>
<td>Dacryocystorhinostomy</td>
<td>DCR</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>DNase</td>
</tr>
<tr>
<td>Deoxyribonuclease I-like 3</td>
<td>DNase 1L3</td>
</tr>
<tr>
<td>Double-stranded DNA</td>
<td>dsDNA</td>
</tr>
<tr>
<td>Extensively drug resistant</td>
<td>XDR</td>
</tr>
<tr>
<td>Extracellular DNA</td>
<td>ecDNA</td>
</tr>
<tr>
<td>Glucose-6-phosphate isomerase</td>
<td>G6PI</td>
</tr>
<tr>
<td>Granulocyte colony stimulating factor</td>
<td>G-CSF</td>
</tr>
<tr>
<td>High salt diet</td>
<td>HSD</td>
</tr>
<tr>
<td>Immunoglobulin G</td>
<td>IgG</td>
</tr>
<tr>
<td>Interferon</td>
<td>IFN</td>
</tr>
<tr>
<td>Interleukin</td>
<td>IL</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>LPS</td>
</tr>
<tr>
<td>Macrophage colony-stimulating factor</td>
<td>M-CSF</td>
</tr>
<tr>
<td>Matrix metalloproteinase 9</td>
<td>MMP9</td>
</tr>
<tr>
<td>Mitochondrial ROS</td>
<td>mtROS</td>
</tr>
<tr>
<td>Mucoprotein concretion</td>
<td>MPC-D</td>
</tr>
<tr>
<td>Multi-drug resistant</td>
<td>MDR</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>MTB</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>MPO</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>NE</td>
</tr>
<tr>
<td>Neutrophil extracellular trap</td>
<td>NET</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide phosphate</td>
<td>NADPH</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide phosphate oxygenase</td>
<td>NOX</td>
</tr>
<tr>
<td>Normal diet</td>
<td>ND</td>
</tr>
<tr>
<td>Pattern-recognition receptor</td>
<td>PRR</td>
</tr>
<tr>
<td>Peptidyl arginine deiminase</td>
<td>PAD</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate</td>
<td>PMA</td>
</tr>
<tr>
<td>Pre-extensively drug resistant</td>
<td>Pre-XDR</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>ROS</td>
</tr>
<tr>
<td>Receptor activator of NF-κB ligand</td>
<td>RANKL</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>RA</td>
</tr>
<tr>
<td>Sarcoïdosis</td>
<td>SARC</td>
</tr>
<tr>
<td>Serum transfer arthritis</td>
<td>STA</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>TBC</td>
</tr>
</tbody>
</table>
6. Figures

Figure 1. Suppression of Neutrophils by Sodium Exacerbates Oxidative Stress and Arthritis. ................................................................. 18

Figure 2. Neutrophil Extracellular Traps Drive Dacryolithiasis. .................................................. 21

Figure 3. Neutrophil Extracellular Traps Characterize Caseating Granulomas. ....................... 26

Figure 4. Neutrophils and NETs in Autoimmune, Occlusive and Infectious Diseases. .......... 28
7. References


8. Publications

First authorships (included in this dissertation):

Co-authorships (not included in this dissertation):

Review (not included in this dissertation):
9. Contributions

  o Leticija Zlatar has performed the experiments, analyzed the data, and written the original manuscript, including:
    ▪ Sample preparation and analysis (isolation of human neutrophils, 4 color death staining, ROS and mitochondrial ROS production, phagocytosis, degranulation, MPO activity, NE activity, quantification and imaging of NETs, K/BxN-STA model for human inflammatory arthritis, histology, murine osteoclast differentiation)
    ▪ Data collection and evaluation, including statistical analyses (4 color death staining, ROS and mitochondrial ROS production, phagocytosis, degranulation, MPO activity, NE activity, quantification and imaging of NETs, K/BxN-STA model for human inflammatory arthritis, histology, murine osteoclast differentiation)
    ▪ Visualization of the data analyzed (4 color death staining, ROS and mitochondrial ROS production, phagocytosis, degranulation, MPO activity, NE activity, quantification and imaging of NETs, K/BxN-STA model for human inflammatory arthritis, histology, murine osteoclast differentiation)
    ▪ Preparation of the manuscript (literature research & original draft preparation, including the preparation of supplementary materials, submission, revision, and final draft preparation)

  o Leticija Zlatar has performed the investigation, formal analysis, validation and visualization of the data presented in this manuscript, including:
    ▪ Sample preparation for the analysis (mass spectrometry, neutrophil elastase activity measurement, histochemistry/immune fluorescence, macroscopy and fluorescence microscopy)
    ▪ Sample analysis (neutrophil elastase activity measurement, histochemistry/immune fluorescence, macroscopy and fluorescence microscopy)
- Data collection and evaluation (mass spectrometry, neutrophil elastase activity measurement, histochemistry/immune fluorescence, macroscopy and fluorescence microscopy)
- Visualization of the data analyzed (mass spectrometry, neutrophil elastase activity measurement, histochemistry/immune fluorescence, macroscopy and fluorescence microscopy)
  - She has written the original manuscript, and was responsible for the submission, revision and correspondence with the Journal as the corresponding author, which included:
    - Preparation of the manuscript (literature research & original draft preparation, including the preparation of supplementary materials, revision, correspondence with the Journal, and final draft preparation)
10. Acknowledgments

To **Georg Schett**, for employing me at the University Hospital Erlangen, so that I could do the work for my PhD Thesis while studying at the Friedrich Alexander University Erlangen-Nürnberg.

To **Martin Herrmann**, for providing me with the opportunity of doing my PhD Thesis in his laboratory, under his experienced supervision and guidance. I have learned a lot from you, thank you.

To **Jasmin Knopf**, for taking care of everything in the laboratory during her time as a Postdoctoral student in the lab of Prof. Herrmann, especially for closely supervising my work, and providing critical feedback.

To **Luis Muñoz**, for helping with all experiments, statistical analyses, and participating in discussions together with Dr. Knopf.

To **Thomas Winkler**, **Andreas Burkovski**, and **Udo Gaipl**, for agreeing to be the Members of the Defense Committee, as their participation and the time invested were essential to the completion of the procedure.

To **all the lab members** during my time at the University Hospital Erlangen; for all the wonderful times we had together in the past three years.

To **Matej**, for without him, I would not have moved to wonderful Bavaria, started and completed my PhD Thesis. This one’s for you.

To my parents, **Aleksandra** and **Marijan**, for supporting me in my academic endeavors since the day one. I am forever grateful to you. To my grandparents, for cheering me on just as strongly. I know how much this means to you.

To **everyone else** who remain unmentioned for the purpose of concision, but have been a part of my PhD journey nonetheless:

**Hvala! Danke! Thank you! Gracias! धन्यवाद! شكرا لك! 謝謝! Teşekkür ederim!**
Suppression of neutrophils by sodium exacerbates oxidative stress and arthritis

Leticija Zlatar¹,², Aparna Mahajan¹,², Marco Muñoz-Becerra¹,², Daniela Weidner³, Galyna Bila³, Rostyslav Bilyy⁵, Jens Titze⁴,⁵, Markus H. Hoffmann⁶, Georg Schett¹,², Martin Herrmann¹,², Ulrike Steffen¹,², Luis E. Muñoz¹,²* and Jasmin Knopf¹,²,⁷

¹Department of Internal Medicine 3 – Rheumatology and Immunology, Friedrich-Alexander-University Erlangen-Nürnberg (FAU) and Universitätsklinikum Erlangen, Erlangen, Germany, ²Deutsches Zentrum für Immuntherapie (DZI), Friedrich-Alexander-University Erlangen-Nürnberg and Universitätsklinikum Erlangen, Erlangen, Germany, ³Department of Histology, Cytology, Embryology, Danylo Halitotsky Lviv National Medical University, Lviv, Ukraine, ⁴Division of Nephrology and Hypertension, Universitätsklinikum Erlangen, Erlangen, Germany, ⁵Programme in Cardiovascular and Metabolic Disorders, Duke-NUS Medical School, Singapore, Singapore, ⁶Department of Dermatology, Allergology, and Venereology, University of Lübeck, Lübeck, Germany, ⁷Department of Pediatric Surgery, University Medical Center Mannheim, University of Heidelberg, Mannheim, Germany

Introduction: Typical Western diet, rich in salt, contributes to autoimmune disease development. However, conflicting reports exist about the effect of salt on neutrophil effector functions, also in the context of arthritis.

Methods: We investigated the effect of sodium chloride (NaCl) on neutrophil viability and functions in vitro, and in vivo employing the murine K/BxN-serum transfer arthritis (STA) model.

Results and discussion: The effects of NaCl and external reactive oxygen species (H₂O₂) were further examined on osteoclasts in vitro. Hypertonic sodium-rich media caused primary/secondary cell necrosis, altered the nuclear morphology, inhibited phagocytosis, degranulation, myeloperoxidase (MPO) peroxidation activity and neutrophil extracellular trap (NET) formation, while increasing total ROS production, mitochondrial ROS production, and neutrophil elastase (NE) activity. High salt diet (HSD) aggravated arthritis by increasing inflammation, bone erosion, and osteoclast differentiation, accompanied by increased NE expression and activity. Osteoclast differentiation was decreased with 25 mM NaCl or 100 nM H₂O₂ addition to isotonic media. In contrast to NaCl, external H₂O₂ had pro-resorptive effects in vitro. We postulate that in arthritis under HSD, increased bone erosion can be attributed to an enhanced oxidative milieu maintained by infiltrating neutrophils, rather than a direct effect of NaCl.

KEYWORDS
neutrophils, sodium chloride, reactive oxygen species, neutrophil extracellular traps (NETs), osteoclasts, K/BxN serum transfer arthritis
1 Introduction

Neutrophils are the most abundant white blood cells, and the first line of defense in innate immunity. Upon activation, they migrate to the site of inflammation, to execute various effector functions: phagocytosis, degranulation, reactive oxygen species (ROS) and cytokine production, and neutrophil extracellular traps (NETs) release (1, 2). In neutrophils, azurophilic (primary) granules containing enzymes such as myeloperoxidase (MPO) or neutrophil elastase (NE) fuse with the phagosome during phagocytosis. MPO is a heme enzyme with peroxidase-like and halogenizing activities (3), it utilizes the H$_2$O$_2$ as a substrate to generate hypochlorous acid (HClO). The latter kills pathogens on the expense of tissue destruction (4). The formation of NETs is commonly initiated in neutrophils undergoing an oxidative burst (5), followed by chromatin de-condensation and nuclear membrane disintegration (6), NE and MPO access to the nucleus, histone modification, cellular membrane rupture, and chromatin expulsion (7). NETs can exert pro- or anti-inflammatory activities depending on the intensity of neutrophil infiltration, and are eventually dismantled by serum DNases and cleared by phagocytes.

Neutrophils contribute to the pathogenesis of many diseases. One example is rheumatoid arthritis (RA) (8), a chronic inflammatory autoimmune disease characterized by persistent and progressive joint and synovial membrane damage (2, 7). In patients with RA, immune complexes deposited in synovial tissues trigger inflammatory responses driven by neutrophils and NETs (2). A well-established murine model for RA is K/BxN serum transfer arthritis (STA) (7, 8). The inflammation after transfer of serum or purified glucose-6-phosphate isomerase (G6PI)-specific IgGs from arthritic transgenic K/BxN mice is driven by autoantibodies against G6PI (9). In this model neutrophils are an essential player in the pathogenesis (10).

A Western diet, rich in salt, has various effects on the innate immune system. It has long been considered a risk factor for autoimmune diseases (11–14). NaCl aggravates inflammatory arthritis via induction of pathogenic CD4+ T helper cells which produce interleukin-17 (IL-17) (15). The effect of salt on neutrophils, however, hasn’t been fully elucidated, as many publications provide conflicting information. It was reported that NaCl-induced hyperosmolarity suppresses some leukocyte functions, such as chemotaxis, phagocytosis, intracellular killing of bacteria and superoxide production (16). More recently, it was reported that a high salt environment suppresses ROS-dependent NET formation (17) while Krampert et al. reported that “high salt” (40 mM additional NaCl) reduced neutrophil movement, degranulation and ROS production with no changes in cell viability or NET formation. The latest publication showed that the effect of additional 50 mM NaCl was time-dependent and caused a delayed activation of human neutrophils. In short-term and long-term neutrophil cultures, production of IL-8 and respiratory burst were inhibited or augmented, respectively (18).

The ionic strength in the bodies of mammalians is strictly regulated (19). The overall ionic strength of cell bodies and interstitial fluids is approximately 150 mM corresponding to 300 mOsm/kg. Under physiological conditions, sodium plays an important role in maintaining the volume of extracellular fluids and generating cells’ membrane potential (20). It also plays an important role in muscle contraction and is directly related to blood pressure (21). In the kidney, which is involved in osmoregulation (22), this osmotic toxicity is locally greatly exceeded. On the way of the urine from the blood to the inner medulla osmolality gradually increases from 300 to 1200 mOsm/kg. The toxicity of the corresponding interstitial spaces follows the increase inside the tubules (23). In addition to these osmolar active ions, bones constitutively contain much sodium adsorbed to the apatite in an osmolar inactive manner. In vivo sodium quantification in the healthy human wrist by the means of MRI revealed average sodium concentrations ranging from 115 to 150 mmol/L in noncartilaginous regions, and from 200 to 210 mmol/L in cartilaginous regions (24). However, the exact tissue concentration of sodium in many pathological tissues, as in those of RA patients, has not been determined yet. In patients with human brain tumors, MRI showed increased sodium concentration in tumors relative to that in normal brain structures (25). High sodium intake has been associated with rheumatoid arthritis (26), and patients with rheumatoid arthritis showed increased sodium excretion (27). Furthermore, hypertonic sodium rich environment can be found in the lymphatic organs, skin, and the inflamed tissue, under both physiological and pathological conditions. In inflamed tissues of patients with skin infection the sodium accumulates at the site of infection (23, 28). Whether this sodium increases the local toxicity is not fully clarified. Measurement of the tissue osmolality in lymphoid tissues also revealed the hyperosmolar environment (29).

Here we examined the effects of hypertonic sodium-rich media on neutrophil viability, nuclear morphology and various cell activities, as well as osteoclast differentiation and resorption activity, in the context of inflammatory arthritis. We observed that hypertonic sodium-rich media changed the morphology of neutrophil’s nuclei, greatly reduced neutrophil viability by primary or secondary necrosis, and inhibited neutrophil effector functions, except, it fostered ROS production. In vivo, HSD aggravated STA by increasing tissue inflammation, bone erosion and osteoclast count, and was accompanied by enhanced neutrophil infiltration in the hind paws. Additional 25 mM NaCl decreased osteoclast differentiation in vitro without altering their resorption activity, whereas external ROS (H$_2$O$_2$) increased resorption activity, despite decreased cell differentiation in these conditions.

2 Materials and methods

2.1 Ethical issues

 Investigations on human material were performed in accordance with the Declaration of Helsinki and with the approval of the ethical committee of the University Hospital Erlangen (permit 243_15 B). A written informed consent was given by each donor. All animal experiments and procedures
were performed according to institutional guidelines on animal welfare and were approved by the local Animal Care and Use Committees of the Danylo Halytsky Lviv National Medical University (permit numbers 20191219/10 and 20201221/9).

2.2 Isolation of human neutrophils

Whole blood from healthy human donors was freshly drawn into EDTA tubes (Sarstedt). We isolated neutrophils by density gradient centrifugation at 350 g for 30 minutes at room temperature (RT) with Lympholot Ficoll-Diatrizoate (Bio-Rad). The high-density layer was subjected to hypotonic erythrocyte lysis. We resuspended pelleted neutrophils in Dulbecco’s Phosphate Buffered Saline (DPBS, ThermoFisher Scientific) and determined the viable cell concentration by staining with acridine orange/propidium iodide (Logos Biosystems) employing the Luna-FL™ Dual Fluorescence Cell Counter (Logos Biosystems).

2.3 4 color death staining

We incubated freshly isolated neutrophils in DPBS-NaCl buffers at various osmolarities (1) isotonicity 137 mM NaCl, (2) moderate hypertonicity 200 mM NaCl, and (3) high hypertonicity 300 mM NaCl, with or without addition of 10% heat-inactivated fetal calf serum (FCS, c.c.pro GmbH), at different time points. Corresponding buffers were prepared by addition of 3.4 M NaCl solution (Merck) to the standard DPBS buffer. Cell viability was assessed by Gallios Flow Cytometer (Beckman Coulter) after 30-minute staining with Annexin A5-FITC (Immunotools, 0.5 µg/ml), PI (Sigma-Aldrich, 1 µg/ml), DiIC1(5) (Invitrogen, 1.67 nM) and Hoechst33342 (Molecular Probes, 1 µg/ml) in Ringer’s solution (Delta Select) at 4°C as reported previously (30). The analysis was performed in Kaluza Analysis 2.1 software (Beckman Coulter).

2.4 ROS and mitochondrial ROS production

We pre-incubated freshly isolated neutrophils at 37°C with general oxidative stress indicator 2 µM CM-H2DCFDA (ThermoFisher Scientific) or mitochondrial superoxide indicator 5 µM MitoSOX Red (ThermoFisher Scientific) for 20 or 10 minutes, respectively, and incubated them in respective DPBS-NaCl buffers (137, 200 or 300 mM) without serum upon Phorbol 12-myristate 13-acetate (PMA, 100 ng/mL) or Pyocyanin (10 µM or 50 µM, respectively) stimulation for another 20 minutes. We stained the neutrophils with CD15 APC (BioLegend, 1:150) and CD16 PB (BD Pharmingen, 1:150) for 30 minutes at 4°C and quantified the signal using the Gallios Flow Cytometer (Beckman Coulter). The analysis was performed in Kaluza Analysis 2.1 software (Beckman Coulter).

2.5 Phagocytosis

We coated the Fluoresbrite YG Carboxylate Microspheres, 1 µm (Polysciences, 15702-10) with 2 mg/mL human immunoglobulins (IVIg, Gammunex-C) or 2 mg/mL human serum albumin (BSA, Sigma-Aldrich) and stored them at 4°C in a DPBS-NaCl buffer with 10 mg/mL bovine serum albumin (BSA, Santa Cruz Biotechnology). We then centrifuged the microspheres and replaced the supernatant with 50% autologous serum diluted in DPBS-NaCl buffers of various salt concentration (137, 200 or 300 mM). We vortexed the microspheres for 5 minutes and washed them with 1% BSA in DPBS. We resuspended them in hypertonic sodium-rich media (137, 200 or 300 mM). The microspheres were sonicated for 10 minutes, and vortexed for 5 minutes before immediate use. We isolated the neutrophils as described in 2.2., and resuspended them in respective DPBS-NaCl buffers (137, 200 or 300 mM). We added the final microsphere suspension to the cells and incubated them for 1 hour at 37°C and 5% CO₂. We stained the cells with CD15 APC (BioLegend, 1:150) and CD16 PB (BD Pharmingen, 1:150) for 30 minutes at 4°C and quantified the signal using the Gallios Flow Cytometer (Beckman Coulter). The analysis was performed in Kaluza Analysis 2.1 software (Beckman Coulter).

2.6 Degranulation

We isolated neutrophils as described in 2.2., and incubated them for 1 hour with PMA (100 ng/mL) or Pyocyanin (10 µM) in various DPBS-NaCl buffers (137, 200 or 300 mM). We stored the supernatant and later used it for MPO or NE activity measurement (2.7 and 2.8, respectively). The remaining cell pellet was resuspended and stained with anti-CD66b-FITC (Immunotech, 1:1000) for 30 minutes at 4°C and quantified using the Gallios Flow Cytometer (Cytoflex S) to assess degranulation. The analysis was performed in Kaluza Analysis 2.1 software (Beckman Coulter).

2.7 MPO activity

To assess the MPO peroxidation activity, reaction was developed by adding the TMB substrate set (BioLegend) to the collected supernatants. The reaction was stopped by adding 25% sulfuric acid (PanReac AppliChem). The optical densities (ODs) were read at 450 nm with a reference at 620 nm using the SUNRISE microplate reader (Tecan). To assess the MPO chlorination activity, we used the EnzChek™ Myeloperoxidase (MPO) Activity Assay Kit (ThermoFisher Scientific) according to manufacturer’s instructions. The fluorescence intensity was read in an Infinite F200 PRO plate reader.
reader (Tecan; ex. 485 nm, em. 530 nm). We used RIPA buffer (ThermoFisher Scientific) for complete cell lysis representing maximum MPO activity in the cell supernatant.

2.8 NE activity

To assess the NE activity, reaction was developed by adding the NE fluorogenic substrate (MeOSuc-AAPV-AMC, Santa Cruz Biotechnology, 1:10) to the collected supernatants. The fluorescence intensity was measured for 12 hours at 37°C in an Infinite F200 PRO plate reader (Tecan; ex. 360 nm, em. 465 nm). We used RIPA buffer (ThermoFisher Scientific) for complete cell lysis representing maximum NE activity in the cell supernatant. Endpoint values were analyzed.

2.9 Quantification and imaging of NETs

We employed SYTOX™ Green Nucleic Acid stain (ThermoFisher Scientific, 1:1200) to detect extracellular DNA (ecDNA) in neutrophils cultures. Neutrophils were stimulated by PMA (100 ng/mL) or Pyocyanin (10 µM), and DPBS as control for NET formation in various salt conditions. 150,000 neutrophils were seeded per well, and the fluorescence intensity of SYTOX™ Green Nucleic Acid stain was measured for 4 hours at 37°C and 5% CO2 in an Infinite F200 PRO plate reader (Tecan; ex. 485 nm, em. 535 nm). Endpoint values were analyzed.

To visualize NETs, we cultured neutrophils in chamber slides (Permanox, Thermo Fisher) for 4 hours at 37°C and 5% CO2 with PMA (100 ng/mL) or Pyocyanin (10 µM), or without stimuli (DPBS). We fixed the cells with 2% paraformaldehyde (PFA, Merck), permeabilized with 0.1% Triton X-100 (Merck), and blocked with blocking buffer (10% FCS, 2% BSA, 0.1% Triton X-100, 0.05% Tween 20 in DPBS) for 1 hour. Next, we stained them with primary rabbit anti-human neutrophil elastase (NE, Invitrogen, PA5-87158, 1:50), secondary goat anti-rabbit Cy5 (Jackson Lab., 111-175-144, 1:400) and DNA stain Hoechst33342 (Molecular Probes, 1µg/ml), and mounted them with DAKO fluorescence medium (Agilent). Controls were stained with Hoechst33342 and secondary goat anti-rabbit Cy5 only. We took the microphotographs with fluorescence microscope BZ-X710 (Keyence Corporation). Fluorescence microscopy pictures of NETs were analyzed using Adobe Photoshop CC 2018 to quantify the intensity of NE.

2.10 K/BxN-STA model for human inflammatory arthritis

We initially transferred the sera from arthritic K/BxN mice into healthy 2-month-old C57BL/6 female mice by intraperitoneal (i.p.) injection. The 3R principles (Replace, Reduce, Refine) were considered for the calculation of the minimum required sample size and it was determined that to have an 80% chance of detecting a drop of 4 points in the arthritis score at the 5% level of significance using 2-sided t-test, at least 5 mice per group were required to be included in this study. Simple (unrestricted) randomization of all mice into 4 groups followed. One group (n=8) was fed a diet with normal salt content (0.24% sodium) in food (sniff Spezialdiäten GmbH, V1534-000) and water, while the other group (n=8) received a diet with high salt content: 4% NaCl-containing pellets (sniff Spezialdiäten GmbH, E15431-34) and 0.9% NaCl-containing water one week before injection of the K/BxN serum and during the observation period. We followed the onset of arthritis for 43 days post-injection by measurement of ankle thickness with caliper and estimation of ankle thickness (clinical) by scoring of joint swelling of front and hind paws as follows: each paw was individually scored using a 4-point scale (0, normal paw; 1, swelling and redness of one joint type; 2, swelling and redness of two joint types; 3, swelling and redness on three joint types; 4, maximal swelling and redness which leads to complete joint deformity). Scoring was performed blindly and the measuring order of the cages was randomly alternated to avoid observer bias. Once the acute and resolution phases were defined, we induced arthritis to additional two groups (n=5) and euthanized at the peak of joint inflammation at day 10. The collected data were thereafter matched with the corresponding groups and analyzed accordingly.

2.11 Micro-computed tomography (µCT)

In order to evaluate bone erosions at distal sites from inflammation, we performed µCT imaging of tibiae collected after 43 days of STA was performed using the cone-beam Desktop Micro Computer Tomograph “µCT 40” by SCANCO Medical AG, Brüttisellen, Switzerland. The settings were optimized for calcified tissue visualization at 55 kVp with a current of 145 µA and 200 ms integration time for 500 projections/180°. For the segmentation of 3D-Volumes an isotropic voxel size of 8.4 µm and an evaluation script with adjusted greyscale thresholds of the operating system “Open VMS” by SCANCO Medical was used. For evaluation of the trabecular and bone structure of the proximal tibia metaphysis the volume of interest was determined as starting 0.42 mm from the middle of the growth plate and extending 1.680 mm (200 tomograms) distally. The segmentation of cortical and trabecular bone was carried out manually, based on a threshold dependent Open VMS-auto-contouring script provided by SCANCO Medical.

2.12 Histology

We euthanized 10 mice (5/group) on day 10 and fixed the paws overnight (ON) at RT in 4% PFA (Merck), washed them ON in 70% ethanol, and decalcified them in Teitel buffer for 2 weeks. Histological sections were prepared by embedding in paraffin and staining for hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP). We took pictures with the fluorescence scanner (Aperio Versa 8, Leica Biosystems). We quantified the tissue area (T.Ar.), bone area (B.Ar.), inflammation area (Inf.Ar.), eroded area (Er.Ar.), and osteoclast count (N.Oc.) using the Aperio...
2.13 Murine osteoclast differentiation

We isolated macrophage-like osteoclast precursor cells from female C57BL/6J mice (6-14 weeks old) by flushing the bone marrow from femora and tibiae. We incubated the cells in a Petri dish at 37°C and 5% CO₂ overnight. The next day, we collected the non-adherent cells and plated them on 96-well plates coated with calcium phosphate, at 1 x 106/mL (200 µL/well) in α-MEM (Gibco) containing 10% FCS and 1% Penicillin/Streptomycin, supplemented with 30 ng/mL recombinant murine M-CSF (Peprotech, 315-02) and 50 ng/mL recombinant murine sRANK Ligand (Peprotech, 315-11). Where stated, 5, 15, or 25 mM NaCl and/or 100 nM H₂O₂ were added in the medium change (every 2 days). On day 9, we fixed and stained the fully differentiated osteoclasts using the TRAP staining Kit (Merck, 387A-1KT). We identified purple colored cells with more than 3 nuclei as osteoclasts. One field per well was imaged. We quantified the osteoclasts employing the Carl Zeiss microscope equipped with a camera (Osteomeasure; Osteometrics). After TRAP analysis, cells were lysed with ddH₂O, and we performed Von Kossa staining (Resorption Assay). We took the microphotographs of all wells using the fluorescence microscope BZ-X710. We identified the resorbed area as white patches on black background, and performed the morphometry analysis in ImageJ. We pooled the results from 5 mice.

2.14 Statistical analyses

We performed the statistical analyses in Excel 2019 (Microsoft) or in Graphpad Prism 9. We performed Mixed Effects Analysis, 2-way ANOVA, ordinary one-way ANOVA, Multiple t-tests, Unpaired t-test, Mann-Whitney test or Kruskal-Wallis test, as indicated in the figure legends. All data are presented as mean ± standard deviation (SD).
control (Figures 3A, B). We measured DNA externalization using the cell-impermeable SYTOX™ Green Nucleic Acid stain. NET formation was decreased with increasing salt concentrations. Upon PMA or Pyocyanin stimulation, NET formation was already reduced at 200 mM when compared to isotonic media. To confirm the suppression of NET formation, we employed fluorescence microscopy after immunostaining for NE in the presence of Hoechst33342 (Figure 3B), and analysed nuclear morphology and intracellular colocalization of DNA and NE. Unstimulated neutrophils in various salt conditions were also

![Flow cytometric dot plot analysis showing the gating strategy used to identify viable, apoptotic, and necrotic cell populations; (B) culture without (w/o) serum; (C) culture with (w/) 10% heat-inactivated FCS. Data were obtained from 3-5 healthy individuals and are presented as mean ± SD.](image)

**TABLE 1** Cell viability.

<table>
<thead>
<tr>
<th></th>
<th>P value (w/o serum)</th>
<th>P value (w/serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>A</td>
</tr>
<tr>
<td>137 vs. 200 mM, 1h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>137 vs. 300 mM, 1h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>200 vs. 300 mM, 1h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>137 vs. 200 mM, 2h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>137 vs. 300 mM, 2h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>200 vs. 300 mM, 2h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>137 vs. 200 mM, 4h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>137 vs. 300 mM, 4h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>200 vs. 300 mM, 4h</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

1Statistical analysis was performed in Graphpad Prism 9 using Mixed Effects Analysis. All P values were calculated applying the Bonferroni correction; n.s., not significant.
morphometrically evaluated (Figure 3C). To this end we compared area size, circularity and DNA content of the neutrophils’ nuclei (Figure 3D), and observed that the neutrophils lost their lobulated nuclear morphology (left panel) at moderate hypertonicity (middle panel). At high hypertonicity the nuclei shrunk and displayed increased Hoechst33342 fluorescence intensities (right panel).

Next, we performed the murine STA model with a normal (NSD) or a high salt diet (HSD), to investigate the effect of salt on the development of arthritis in vivo. The course of arthritis was time-monitored (Figure 4A). Mice kept on HSD developed more severe arthritis, reflected by increased paw swelling and increased clinical score. The tibiae were scanned by µCT to investigate whether the inflammation of the paws caused changes in the proximal bone (Figure S2). Since µCT analysis revealed no long-term effect on tibial bone remodeling in HSD mice, we investigated the acute phase of arthritis by analyzing the hind paws with HE (Figure 4B) or TRAP staining (Figure 4C). Both techniques revealed significant morphological changes in mice on HSD with extended tissue inflammation (Figure 4B), increased bone erosion and osteoclast count (Figure 4C).

We analyzed hind paw sections for NE, Ly6G (Figure 5) and citrullinated histone H3 (citH3) by fluorescence microscopy (Figure S3). Between the carpal bones we detected extracellular DNA, co-located with citH3 or NE, namely NETs (Figure S3A–D, respectively). Importantly, we also detected intact neutrophils (Ly6G positive cells) infiltrating the carpal bones of both mice kept on NSD or HSD (Figures 5C, D). Infiltrating neutrophils displayed a granular phenotype with no obvious signs of NET formation. However, quantification of similar sized bone infiltrates from mice on NSD or HSD revealed that the frequency of NE positive cells tended to be higher in HSD mice, and that the intensity of NE expression at the single cell level was increased in the HSD group (Figures 5A, B). Accordingly, the NE activity was increased in the paws of HSD mice (Figure 5E).

Since neutrophil infiltrations in a high salt environment were accompanied by more severe arthritis, increased osteoclast counts and bone erosion, and neutrophils responded to high salt with increased ROS production, we analyzed the effect of NaCl, H2O2 (ROS mimetic) or both on osteoclast differentiation (Figures 6A, B) and their resorptive activity (Figures 6C, D) in vitro. The addition of 25 mM NaCl or 100 nM H2O2 decreased total osteoclast counts when compared to controls. Whereas NaCl alone had no effect, the addition of H2O2 increased osteoclast resorption.

4 Discussion

Diet together with other environmental factors accounts for 70% of the risk towards developing an autoimmune disease (31). HSD is considered to be generally pro-inflammatory, but more recent findings imply that it has anti-inflammatory effects as well, such as polarizing the medullary mononuclear phagocytes, and inducing neutrophil apoptosis (32, 33). High salt conditions not only cause osmotic and ionic imbalance, but also oxidative stress and metabolic changes (34). In our work, we assessed the effect of hypertonic sodium-rich media on neutrophil viability and activity in various salt conditions in vitro. Our data show that the high salt content compromised neutrophil-driven immune response by decreasing neutrophil viability, degranulation, phagocytosis, MPO...
activity and NET formation; processes crucial for immune defense (35). However, high salt content increased oxidative burst activity, an effective but damaging mechanism of defense. Moderate salt content increased mitochondrial ROS production. Nuclear morphometry of unstimulated neutrophils revealed that at 200 mM NaCl the nuclei rounded up and swelled. At 300 mM NaCl nuclei shrunk, got more circular, and displayed a higher fluorescence intensity, indicating higher DNA densities in the pyknotic nuclei. Pyknosis is known as the irreversible condensation of chromatin in the nucleus of a cell undergoing cell death and precedes karyorrhexis (36, 37). Our data indicate that neutrophils in hypertonic environment initiated the apoptotic program with preserved membrane integrity, rapid loss of mitochondrial potential, and shrinkage of the nucleus before they progressed to secondary necrosis. Interestingly, under these conditions the release of DNA was abrogated.

In the work of Nadesalingam et al., hypertonic saline induced neutrophil apoptosis along with decrease in ROS production and NET formation (17). Our data rather suggest that while the hypertonic environment promoted apoptotic cell death and suppressed NET formation, the ability to produce ROS was preserved, or enhanced. An enhanced ROS production due to metabolic imbalances is used by cells to sense stress and was also reported by other authors due to osmotic stress and increased salinity (34, 38). Superoxide production was lower when neutrophils were pre-treated with hypertonic saline and then activated, and highest, when cells were first activated, and then treated with hypertonic saline. Meaning, the increased ROS production driven by high salt depends on prior activation of the neutrophils. Neutrophil cytotoxicity is therefore increased by higher salinity only if the system has already been primed by inflammation and cells were pre-activated (39). In our settings, neutrophils were simultaneously
activated by PMA/Pyocyanin and treated with high salt; these conditions augmented ROS production in high hypertonicity. This is in line with recent findings where NaCl initially inhibits (up to 2 hours) (18, 40) but later (6 to 18 hours) fosters ROS production (18), even at lower osmolarities (190 - 200 mM). Our higher salinity was enough to induce oxidative stress in short-term cell cultures. Whereas high hypertonicity (300 mM) increased the oxidative burst, already moderate hypertonicity (200 mM) increased the ROS production in the mitochondria of neutrophils. Interestingly, NET formation, in which also mitochondrial ROS can be used as an alternative ROS source in the absence of functional NADPH-oxidase (41), was decreased. Both NOX-dependent and NOX- independent NET formation (42) were abrogated despite increased levels of ROS, and mitochondrial ROS, respectively. Mitochondrial ROS also plays an important role in cells’ signalling pathways, including the regulation of immune responses, apoptosis, autophagy and inflammation (43).

Once the cellular homeostasis is disrupted, as with increased NaCl content in our settings, mitochondrial ROS facilitate crosstalk to determine the cell’s fate. Taken together, neutrophils display a higher oxidative stress level and a rapid progression to secondary necrosis in high hypertonicity.

In RA, several immune-mediated mechanisms affect the balance of bone-forming osteoblasts and bone-resorbing osteoclasts (44). Excessive osteoclast activity has been linked to bone erosion, joint destruction and disability. Recent findings revealed that for osteoclast differentiation and resorption activity, ROS are
Hypertonic NaCl, as well as external ROS (H2O2) impair the maturation of murine osteoclasts in vitro. H2O2 increases osteoclast resorption activity in vitro.

FIGURE 6
Hypertonic NaCl, as well as external ROS (H2O2) impair the maturation of murine osteoclasts in vitro. H2O2 increases osteoclast resorption activity in vitro. (A) TRAP-stained osteoclasts (n=5 per group) in purple (>3 nuclei) and (B) their quantification. Statistical analysis was performed using Kruskal-Wallis test; N. Oc., osteoclast count; bar represents 50 µM; (C) von Kossa staining of the corresponding area resorbed by the osteoclasts (white) and (D) quantification per osteoclast (%), n=5. Data are presented as mean ± SD. Statistical analysis was performed using Kruskal-Wallis test. N.Oc., osteoclast count; bars represent 500 µM.
necessary. The application of oxidant scavengers, such as N-acetylcycteine (NAC) or diphenylene iodonium (DPI) suppressed RANKL-mediated ROS production, and consequently inhibited osteoclast differentiation (45). Importantly, RANKL KO mice are protected from bone erosion after the transfer of K/BxN serum (46). As our in vitro data showed an increased ROS production by neutrophils in high hypertonicity, we examined the effect of HSD in the STA arthritis model. In line with previous findings, our data show that HSD boosted osteoclast differentiation and bone erosion as seen in bone histology of osteoclast-specific TRAP staining (47). The aggravated arthritis in the STA mice on HSD was accompanied by high osteoclast count (N.Oc./B.Ar.), increased bone erosion (Er.Ar./B.Ar.), and increased inflamed tissue area (Infl.Ar./T.Ar.).

In this work, neutrophils infiltrated the interosseous spaces of the STA mice, especially in HSD. This likely contributed to the release of pro-inflammatory mediators leading to inflammation and arthritis aggravation. Whether this was a direct effect of NaCl, or an indirect effect mediated by increased ROS production of neutrophils was examined in a simplified in vitro system. As in the in vitro studies using human neutrophils, various NaCl conditions were tested in experiments using in vitro generated osteoclasts. Our findings indicated that H2O2 alone, increased osteoclasts’ resorative activity. Therefore, we suggest that the accumulation of neutrophils in inflamed synovium is an important source of ROS promoting osteoclast differentiation (48).

This study examined the relation between NaCl, neutrophils and osteoclasts both in vitro and in vivo. It investigated the effect of increased hypertonicity on neutrophils in more detail, and how this affects osteoclast differentiation and function in the context of RA. Our study has some limitations, and to clarify the exact relations between neutrophils and osteoclasts, further experiments need to be conducted. These include neutrophil depletion in vivo in a STA model with mice on NSD/HSD and the use of ROS scavengers such as NAC. Based on our data, we postulate that the increased osteoclast differentiation and bone erosion observed in arthritis under HSD are not a direct effect of NaCl on osteoclast differentiation and activity, but rather a consequence of the increased local ROS production by the infiltrating neutrophils in a hypertonic environment. HSD contributed to an enhanced oxidative milieu maintained by infiltrating neutrophils, which greatly aggravated arthritis by supporting bone erosion.

**Author’s note**

This manuscript will be used as part of Leticija Zlatar’s doctoral thesis.

**Data availability statement**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

**Ethics statement**

The studies involving human participants were reviewed and approved by Ethical committee of the University Hospital Erlangen (permit 243_15 B). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Local Animal Care and Use Committees of the Danylo Halytsky Lviv National Medical University (permit numbers 20191219/10 and 20201221/9).

**Author contributions**

MH, RB and JT conceived the project. JK and LM designed and supervised the study. US, MHH and GS supported the work with their expertise. LZ, AM, GB and DW performed the experiments. LZ, AM, and MM-B analyzed the data. LZ wrote the original manuscript. JK, LM and MH revised it. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

**Funding**

This work was partially supported by the Deutsche Forschungsgemeinschaft (DFG) 2886 PANDORA Project-No. B3 to MH and JT; Project-No. TP04 to US; CRC1181- 261193037 (C03) to MH; SFB/TRR 241 (B04) to MH; by the European Union H2020-FETOPEN-2018-2019-2020-01 to MH; by the European Commission 861878, “NeutroCure”; to MH; by the Volkswagen-Stiftung (Grant 97744) to MH and RB; by the National Research Foundation of Ukraine grant 2020.02.0131 to RB; ERC Synergy grant 810316 4D NanoSCOPE to US, EU/EFPIA Innovative Medicines Initiative 2 Joint Undertaking RTCure grant no. 777357 to US, ELAN Fond of the Friedrich-Alexander-Universität Erlangen-Nürnberg (P097) to US; DFG MU 4240/2-1 (Project Nr. 470134687) and International Collaborative Project of Science & Technology Department of Sichuan Province (2022YFH0023) to LM. We acknowledge financial support by Deutsche Forschungsgemeinschaft and Friedrich-Alexander-Universität Erlangen-Nürnberg within the funding programme “Open Access Publication Funding”.

**Acknowledgments**

We acknowledge excellent technical assistance from Silke Winkler, Barbara Happich and Nicole Berndt. We thank Dr. Wolfgang Baum for his skillful assistance in animal care taking.
Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.1174537/full#supplementary-material

References


Neutrophil Extracellular Traps Drive Dacryolithiasis

Leticija Zlatar 1,2,*†, Thomas Timm 3, Günter Lochnit 3, Rostyslav Bilyy 4, Tobias Bäuerle 5, Marco Munoz-Becerra 1,2, Georg Schett 1,2, Jasmin Knopf 1,2,6, Jens Heichel 7, Mohammad Javed Ali 8,9, Mirco Schapher 10,11, Friedrich Paulsen 9 and Martin Herrmann 1,2

1 Department of Internal Medicine 3—Rheumatology and Immunology, Universitätsklinikum Erlangen, Friedrich Alexander University Erlangen-Nürnberg (FAU), 91054 Erlangen, Germany; marco.munozbecerra@uk-erlangen.de (M.M.-B.); georg.schett@uk-erlangen.de (G.S.); jasmin.knopf@medma.uni-heidelberg.de (J.K.); martin.herrmann@uk-erlangen.de (M.H.)
2 Deutsches Zentrum für Immuntherapie (DZI), Universitätsklinikum Erlangen, Friedrich Alexander University Erlangen-Nürnberg (FAU), 91054 Erlangen, Germany
3 Institute of Biochemistry, Justus-Liebig University Giessen, 35392 Giessen, Germany; thomas.timm@biochemie.med.uni-giessen.de (T.T.); guenter.lochnit@biochemie.med.uni-giessen.de (G.L.)
4 Department of Histology, Cytology, Embryology, Danylo Halytsky Lviv National Medical University, 79010 Lviv, Ukraine; rbilyy@gmail.com
5 Institute of Radiology, Preclinical Imaging Platform Erlangen (PIPE), Universitätsklinikum Erlangen, Friedrich Alexander University Erlangen-Nürnberg (FAU), 91054 Erlangen, Germany; tobias.baueuerle@uk-erlangen.de
6 Department of Pediatric Surgery, University Medical Center Mannheim, University of Heidelberg, 68167 Mannheim, Germany
7 Department and Policlinic of Ophthalmology, Martin Luther University of Halle-Wittenberg, 06108 Halle, Germany; jens.heichel@uk-halle.de
8 Govindram Seksaria Institute of Dacryology, L.V. Prasad Eye Institute, Road No 2, Banjara Hills, Hyderabad 500034, India; javed@lvpei.org
9 Institute of Functional and Clinical Anatomy, Friedrich Alexander University Erlangen-Nürnberg (FAU), 91054 Erlangen, Germany; friedrich.paulsen@fau.de
10 Department of Otorhinolaryngology, Head and Neck Surgery, Universitätsklinikum Erlangen, Friedrich Alexander University Erlangen-Nürnberg (FAU), 91054 Erlangen, Germany; mirco.schapher@klinikum-nuernberg.de
11 Department of Otorhinolaryngology, Head and Neck Surgery, Paracelsus University, 90419 Nürnberg, Germany

* Correspondence: leticija.zlatar@uk-erlangen.de; Tel.: +49-9131-85-34788
† This manuscript will be used as part of Leticija Zlatar’s doctoral thesis.

Abstract: Mucopeptide concretions, previously called dacryoliths, are macroscopic stones that commonly obstruct the lacrimal sac. The mechanism behind dacryolithiasis remains unclear; however, the involvement of various immune cells, including neutrophils, has been confirmed. These findings remain limited, and no information on neutrophil extracellular traps (NETs), essentially involved in the pathogenesis of other lithiases, is available yet. Here, we employ microcomputed tomography, magnetic resonance tomography, histochemistry, mass spectrometry, and enzyme activity analyses to investigate the role of neutrophils and NETs in dacryolithiasis. We classify mucopeptide concretions into three types, with respect to the quantity of cellular and acellular material, polysaccharides, and mucosubstances. We propose the role of neutrophils and NETs within the existing model of gradual formation and growth of mucopeptide concretions, with neutrophils contributing to the initial stages of dacryolithiasis, as they localized on the inner (older) parts of the tissue. As NETs localized on the outer (newer) parts of the tissue, we link their role to the late stages of dacryolithiasis, presumably maintaining the proinflammatory environment and preventing efficient clearance. An abundance of IgG on the surface indicates the involvement of the adaptive immune system later as well. These findings bring new perspectives on dacryolithiasis, in which the innate and adaptive immune system are essentially involved.

Keywords: mucopeptide concretions; dacryoliths; dacryolithiasis; lacrimal sac; neutrophils; neutrophil extracellular traps
1. Introduction

Mucoprotein concretions or dacryoliths (MPC-D), formerly also referred as “mucoliths”, are macroscopic stones that are formed in the lacrimal sac and the nasolacrinal ducts [1,2]. Unlike other ‘liths’ in several parts of the human body, MPC-Ds are mostly composed of organic material, and their pathophysiology remains unclear [3]. However, some predisposing factors that contribute to their formation have so far been described. These include smoking, primary acquired nasolacrimal duct obstruction (PANDO) [3,4], and previous chronic dacryocystitis [3]. The effects of age and gender remain elusive [2], and some even claim that the age, duration of epiphora, history of acute dacryocystitis, or previous use of medications have no effect on dacryolithiasis [4]. An alteration in the production of mucins and trefoil factors TFF1 and TFF3, as well as the induction of TFF2, have been established as major risk factors for dacryolithiasis [1,2]. Under physiological conditions, trefoil factors protect the mucous epithelium post injury by supporting the trapping of viruses with the help of immunoglobulins, fostering cell migration to the place of injury, or displaying generally antiapoptotic effects [5–7]. Under pathological conditions, such as during dacryolithiasis, the production of trefoil factors and cosecretion of mucins is significantly increased. Two major types of lacrimal system concretions exist; mucoprotein and bacterial. They differ in their location and histopathologic composition. A third category of intermediate concretions, with characteristics of both of these types, has also been described [8]. The formation of bacterial concretions has been classically attributed to *Actinomyces* species. Recently, *Streptococcus* and *Staphylococcus* species have also been reported as major genera involved in their formation. Other bacteria, and also fungi, were previously isolated from bacterial concretions [9]. Not surprisingly, epithelial cells, neutrophils, T and B lymphocytes, as well as macrophages, have been found in MPC-Ds as well. [1,10]. Ali and Paulsen performed several electron microscopic, immunohistochemistry, and cinematic rendering techniques to determine that the initial pathogenic event is a microscopic trauma within the lacrimal drainage and blood clotting in the nidus which initiates the dacryolithiasis in a predisposed individual [11–13]. Subsequently, the local mucopolypeptides and those in the tears sequentially lay mucoproteins around this nidus.

Neutrophils are immune cells involved in phagocytosis, degranulation, reactive oxygen species (ROS) and cytokine production, and in neutrophil extracellular trap (NET) formation. They can be found in blood in high quantities, and migrate to the site of infection to initiate the immune response [14]. A well-known mechanism of bacterial killing by lysozyme highlights the importance of neutrophil antimicrobial proteins in host defense against pathogens [15]. Within cytoplasmic granules, neutrophils contain various serine proteases, including neutrophil elastase (NE), cathepsin G, and proteinase 3 (PR3) [16,17]. Neutrophil granules also contain azurocidin, an antimicrobial protein similar in structure to the three aforementioned serine proteases, with important roles in the host defense and inflammation [18]. Within secondary granules, neutrophils contain lactoferrin, the first line of defense against microbial infections [19]. S100A8 and S100A9 proteins are constitutively expressed in neutrophils in high levels, and comprise up to 45% of all cytoplasmic proteins. They act as calcium binding proteins under physiological conditions; however, during inflammation, their upregulation modulates cytokine secretion [20,21]. Another proinflammatory molecule expressed by neutrophils is resistin; it also has antimicrobial function [22]. In addition, defensins, cationic antimicrobial peptides, are produced by neutrophils [23].

Neutrophils are well-known for forming NETs, whose primary function is to trap and kill various pathogens. To detect NETs, antibodies against granular proteins, such as NE and myeloperoxidase (MPO), as well as citrullinated, decondensed chromatin, using citrullinated histone H3 (citH3) are commonly used in combination with various DNA intercalating dyes, such as DAPI, Sytox Green, Hoechst 33342, and PI [24,25]. In the context of the immune system, NETs are “double edged swords”, meaning they can act pro- or anti-inflammatory [26], depending on the density of neutrophil infiltration [27]. Eventually, serum DNases degrade NETs, and phagocytes engulf them. However, an imbalance in NET formation and clearance leads to pathogenesis of various diseases, and this balance
needs to be strictly maintained [28]. Importantly, the involvement of NETs in the pathology of other stone diseases has already been confirmed. Examples include cholelithiasis or sialadenitis, in which gallstone formation or salivary gland stone formation, respectively, occur. In such pathologies, NETs act as the “initiators” for the development and growth of sialoliths or gallstones, respectively [29,30].

In this study, we investigate the link between neutrophils and NETs and human MPC-Ds. Even though heterogenous in shape and size, all samples studied here obtained the shape of the lacrimal sac in which they were formed. They displayed a stratified structure and contained only small amounts of electron dense inclusions. We classified them into three different types based on the amount of cellular and acellular material, polysaccharides, and mucosubstances. Using immune fluorescence (IF) for various neutrophil and NET associated markers (citH3, MPO, and NE), we examined the role of neutrophils and NETs in dacryolithiasis in the context of their localization within the tissue. We found neutrophil- and NET-associated markers at different quantities in all samples, and also detected high NE activity in those samples with a high expression of NE. Importantly, NETs localized on the surface of the samples, linking NET formation to the later stages of disease pathogenesis. We propose that NETs are rather involved in later stages and might contribute to their aberrant clearance via a yet-undescribed mechanism. We performed stainings of various other components of the innate and adaptive immune system such as hemoglobin or IgG, respectively. In general, we found all antigens at various expression levels, however, at higher quantities on the surface of the samples. Taken together, data presented here indicate that processes related to neutrophils, NETs, and the adaptive immune response occur during the formation, growth, or maintenance of MPC-Ds.

2. Materials and Methods

2.1. Ethical Statement

Investigations on human material were performed in accordance with the Declaration of Helsinki and with the approval by the ethical committee of the University Hospital Erlangen (permit number 243_15 B). Informed written consent about the use of tissue samples was given by each patient.

2.2. Human Tissue Samples

Seven mucopeptide concretions (MPC-Ds) were removed via dacryocystorhinostomy (DCR) and processed for histological analyses. Paraffin-embedded tissue sections (6 µm) were prepared at the Institute of Functional and Clinical Anatomy, Erlangen, Germany and sent to the Department of Medicine 3—Institute for Rheumatology and Immunology, Erlangen, Germany, for further evaluation.

2.3. Magnetic Resonance Tomography (MRT)

MRT Imaging of MPC-Ds was performed at the Institute of Radiology at the Preclinical Imaging Platform (PIPE), Erlangen, Germany.

2.4. Microcomputed Tomography (µCT)

µCT analysis of the MPC-Ds was performed at the University Hospital Erlangen, Department of Medicine 3—Institute for Rheumatology and Immunology, Erlangen, Germany as described elsewhere [30].

2.5. Mass Spectrometry (LC-ESI-MS)

We deparaffinized 10 µm thick sections of MPC-Ds. Using a scalpel and a light microscope, we scraped the inner part (core) of the tissue from the glass slide into a sample tube. Two sections per sample were prepared in this way, pooled together, and sent for complete proteome analysis (MALDI) in dry form to the Institute of Biochemistry, Justus-Liebig University Giessen, Giessen, Germany as described below:
Tryptic digestion of proteins—samples were dissolved in lysis buffer (6 M urea (Sigma, Taufkirchen, Germany), 2 M thiourea (Sigma), 4% 3-3'-(Cholamidopropyl)-3,3-dimethylammoniumpropylsulfat (CHAPS; Roth, Karlsruhe, Germany), 30 mM dithiothreitol (DTT; Fluka, Seelze, Germany), 2% IPG-buffer pH 3–10 (GE Healthcare, Freiburg, Germany), and digested following the FASP protocol [31]. Tryptic peptides were acidified using 1% TFA and purified using a C18-ZipTip (Millipore, Burlington, MA, USA), dried under vacuum, and finally dissolved in 10 µL of 0.1% TFA.

Liquid-Chromatography Electrospray-Ionization Mass Spectrometry (LC-ESI-MS)—for analysis, 1 µg of the sample was loaded onto a 50 cm µPACTM C18 column (Pharma Fluidics, Gent, Belgium) in 0.1% formic acid (Fluka) at 35 °C. Peptides were eluted with a linear gradient of acetonitrile from 3% to 44% over 240 min followed by a wash with 72% acetonitrile at a constant flow rate of 300 nL/min (ThermoScientific™UltiMate™3000RSLCnano) and infused via an Advion TriVersa NanoMate (Advion Biosciences, Inc., New York, NY, USA) into an Orbitrap Eclipse Tribrid mass spectrometer (ThermoScientific). The mass spectrometer was operating in positive-ionization mode with a spray voltage of the NanoMate system set to 1.5 kV and source temperature at 275 °C. Using the data-dependent acquisition mode, the instrument performed full MS scans every 3 s over a mass range of m/z 375–1500, with the resolution of the Orbitrap set to 120,000. The RF lens was set to 30%; auto gain control (AGC) was set to standard with a maximum injection time of 50 ms. In each cycle the most intense ions (charge state 2–7) above a threshold ion count of 50,000 were selected with an isolation window of 1.6 m/z for HCD-fragmentation at normalized collision energy of 30%. Fragment ion spectra were acquired in the linear IT with a scan rate set to rapid and mass range to normal and a maximum injection time of 100 ms. After fragmentation, the selected precursor ions were excluded for 15 s for further fragmentation.

Data acquisition and analysis—data were acquired using Xcalibur 4.3.73.11. (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed using Proteome Discoverer 2.5.0.400 (Thermo Fisher Scientific). The Mascot search engine 2.8.2 (Matrix Science) was used to search against the Swissprot_humandatabase (v. 2022_04, sequences = 568,363, residues = 205,318,884, homo sapiens sequences = 20,402). A precursor ion mass tolerance of 10 ppm was used, and one missed cleavage was allowed. Carbamidomethylation on cysteines was defined as a static modification with optional oxidation of methionine. The fragment ion mass tolerance was set to 0.8 Da for the linear IT MS2 detection. The FDR for peptide identification was limited to 0.01 by using a decoy database.

2.6. Neutrophil Elastase (NE) Activity Measurement

We deparaffinized sections of MPC-Ds, scratched each tissue from the glass slide into an Eppendorf tube, and added 600 µL Dulbecco’s Phosphate Buffered Saline (DPBS, Thermo Fisher Scientific). We thoroughly resuspended all samples, and pipetted 180 µL triplicates into a 96-well plate. A total of 5 UN/mL elastase from human leukocytes (Sigma-Aldrich, Burlington, MA, USA, E8140) was used as a positive control. We added 20 µL (100 µM) of the fluorogenic substrate MeOSuc-AAPV-AMC (Santa Cruz Biotechnology, Dallas, TX, USA, sc-201163) to all wells, and measured the neutrophil elastase activity at 37 °C for 12 h at a 10 min interval by employing the TECAN Infinite 200 Pro fluorescence plate reader (Tecan, Männedorf, Switzerland) with a filter set of excitation at 360 nm and emission at 465 nm.

2.7. Histochemistry/Immune Fluorescence (IF)

We processed the histological sections by melting and washing off paraffin, then performed hematoxylin and eosin (HE) or Periodic acid-Schiff (PAS) staining, or fluorescence stainings employing the following primary antibodies (all rabbit, antihuman): neutrophil elastase (NE, Abcam, Cambridge, UK, ab68672, 1:100), myeloperoxidase (MPO, Abcam, ab9535, 1:200), citrullinated histone H3 (citH3, Abcam, ab5103, 1:300), protein arginine deiminase 4 (PAD4, Sigma, P4749, 1:50), Aquaporin 9 (AQP-9, Abcam, ab84828, 1:100), hemoglobin (α subunit, Abcam, ab92492, 1:100), immunoglobulin G (IgG, Southern Biotech,
Birmingham, AL, USA, 6140-01, 1:100), Fetuin A (ThermoFisher Scientific, PA5-51594, 1:100), and fibrinogen (Agilent Technologies, Santa Clara, CA, USA, A0080, 1:500). We used IgG goat antirabbit Cy5 (Jackson Lab., Bar Harbor, ME, USA, 111-175-144, 1:400) as a secondary antibody. We further stained mucins with primary antibodies against Mucin 5AC (MUC5AC, Sigma-Aldrich, MAB2011, 1:1000) or Mucin 5B (MUC5B, Sigma-Aldrich, MABT899, 1:50), both mouse antihuman. As a secondary antibody, we used IgG goat antimouse Cy5 (Jackson Lab., 115-175-146, 1:400). We stained various cytokeratins with rabbit antihuman polyclonal antibodies: Cytokeratin 1 (CK-1, ThermoFisher Scientific, PA5-119070, 1:100), Cytokeratin 2 (CK-2, Bio-Techne, Minneapolis, MN, USA, NBP1-31423, 1:100), Cytokeratin 9 (CK-9, ThermoFisher Scientific, PA5-24783, 1:100), and Cytokeratin 10 (CK-10, Bio-Techne, NBP1-85604, 1:1000). We employed IgG goat antirabbit Cy5 (Jackson Lab., 111-175-144, 1:400) as a secondary antibody. In addition, we performed immunostaining of glycosylated proteins Galactose-β-2,6-Sialic acid (SNA) or Galactose-β-1,3-GalNAc (GalNAc), using directly labeled lectins: Sambucus nigra lectin from Elderberry Bark conjugated with Cy5 (Vector Laboratories, Newark, CA, USA, CL-1305, 1:50) or Ricinus communis agglutinin I conjugated with Rhodamine (Vector Laboratories, Newark, CA, USA, CI-1305, 1:50) or Ricinus communis agglutinin I conjugated with Rhodamine (Vector Laboratories, Newark, CA, USA, CI-1305, 1:50).

As a counter-stain, various DNA dyes were used: Hoechst 33,342 (Molecular Probes, 0.2 µg/mL), 4′,6-Diamidin-2-phenylindol (DAPI, 0.2 µg/mL), propidium iodide (PI, Sigma-Aldrich, 2 µg/mL), or Sytox Green (SG, Thermofisher, 1:10 000). DNA was additionally stained with primary IgM antibody raised in mice (α-DNA, Merck Millipore, Darmstadt, Germany, CBL186, 1:100) and secondary IgM antibody goat antimouse TRITC (Jackson Lab., 115-025-020, 1:400). Controls were stained with DNA stain and a corresponding secondary antibody only. All stainings were embedded in DAKO fluorescence mounting medium (Agilent, Santa Clara, CA, USA, S3023).

Thioflavin T (ThT) staining was performed as follows: 3 mM ThT dissolved in 30:70 Ethanol; DPBS (ThermoFisher Scientific) was added to the deparaffinized tissue sections for 20 min at room temperature. The sections were then washed in 70% Ethanol, followed by washing in DPBS (ThermoFisher Scientific), and finally mounted using the DAKO fluorescence mounting medium (Agilent, S3023) and dried overnight.

Images obtained from PAS staining or various DNA stainings were analyzed using morphometry in Photoshop CC 2018 (Adobe, Munich, Germany). The mean fluorescence intensity (MFI) was extracted and exported in an R data table object. The Fit-SNE dimensionality reduction was performed using the function ‘run.fitSNE()’ from the Spectre R package (10.1002/cyto.a.24350), with instructions and the source code provided at https://github.com/ImmuneDynamics/spectre, accessed on 11 November 2022. Finally, we performed the image conversion to Rainbow RGB in Fiji.

2.8. Macroscopy and Fluorescence Microscopy

We took macroscopic images using a Nikon 700 camera (Nikon, Tokyo, Japan) with a CMOS sensor in FX format (36.0 × 23.9 mm and 12.87 million pixels) and performed fluorescence microscopy employing the fluorescence scanner (Aperio Versa 8, Leica Biosystems). We processed the obtained images in Photoshop CC 2018 (Adobe, Munich, Germany).

3. Results

We first analyzed the morphological features of human MPC-Ds via macrophotography (Figure 1a), magnetic resonance tomography (MRT, Figure 1b), and microcomputed tomography (µCT, Figure 1c). We observed differences between MPC-Ds in both shape and size, but they commonly confirmed to the shape of the lacrimal sac in which they were formed. They were stiff and their color varied from yellow to orange (Figure 1a). MRT imaging and 3D surface reconstruction revealed a stratified structure (Figure 1b). Employing the µCT analysis, we observed only a few electron dense inclusions spread across the whole body of the MPC-D (Figure 1c). This indicates limited calcifications.
3. Results

We first analyzed the morphological features of human MPC-Ds via macrophotography (Figure 1a), magnetic resonance tomography (MRT, Figure 1b), and microcomputed tomography (µCT, Figure 1c). We observed differences between MPC-Ds in both shape and size, but they commonly confirmed to the shape of the lacrimal sac in which they were formed. They were stiff and their color varied from yellow to orange (Figure 1a). MRT imaging and 3D surface reconstruction revealed a stratified structure (Figure 1b). Employing the µCT analysis, we observed only a few electron dense inclusions spread across the whole body of the MPC-D (Figure 1c). This indicates limited calcifications.

Figure 1. Macroscopic images, MRT, and µCT data of human MPC-Ds: (a) Macroscopic photograph of human MPC-Ds (n = 6); (b) Magnetic resonance tomography (MRT) and 3D surface reconstruction (grey) of a human MPC-D, t1: T−1 weighted, t2: T−2 weighted; (c) Microcomputed tomography (µCT) images of human MPC-Ds; electron dense inclusions are shown in green. Note human MPC-Ds are stiff, beige, yellow to orange in color, and appear like the inner cast of the lacrimal sac. MPC-Ds display like a layered structure and harbor only small calcified electron dense inclusions. In (a) and (c), the regions of interest are surrounded by automatically assigned white lines. Abbreviations: i.p. res: in-plane resolution.

Seven MPC-Ds obtained from both female (29%) and male (71%) patients with an average age of 63 (46–85) years, were analyzed via histochemistry. We employed hematoxylin and eosin (HE) staining to differentiate between cellular and acellular regions (Figure 2a). HE staining revealed morphological differences between the seven MPC-Ds tested. Accordingly, we divided them into three groups: type I, characterized by intense hematoxylin staining of chromatin/cells’ nuclei (blue to purple); type II, characterized by predominant eosin staining of acellular material (pink); and type III, characterized by an equal staining of hematoxylin and eosin, derived from a mixture of chromatin and non-nuclear material (purple to pink). Nonetheless, we observed regions containing cells in all samples. Macrophotographs of paraffin-embedded tissue sections are displayed in Figure 2b. We further employed Periodic acid-Schiff (PAS) staining to detect polysaccharides and mucosubstances. Figure 2c displays a Flt-SNE plot based on the PAS staining. Different clusters can be observed: (I) P93, (II) P94 and P102, (III) P95 and P99, (IV) P96 and P101, (V) P97, and (VI) P98 and P100.
Cells 2023, 12, x FOR PEER REVIEW 7 of 16

characterized by an equal staining of hematoxylin and eosin, derived from a mixture of chromatin and non-nuclear material (purple to pink). Nonetheless, we observed regions containing cells in all samples. Macrophotographs of paraaffin-embedded tissue sections are displayed in Figure 2b. We further employed Periodic acid-Schiff (PAS) staining to detect polysaccharides and mucosubstances. Figure 2c displays a FIt-SNE plot based on the PAS staining. Different clusters can be observed: (I) P93, (II) P94 and P102, (III) P95 and P99, (IV) P96 and P101, (V) P97, and (VI) P98 and P100.

Figure 2. MPC-Ds are heterogenous but can be sorted into three major groups with respect to their morphology. (a) Hematoxylin and eosin (HE) staining of MPC-Ds (n = 7): P94, P99, P95, P96 (first row), P102, P98, and P97 (second row); vertically grouped based on staining: type I (left), type II (middle), and type III (right). Type I is characterized by a strong hematoxylin signal from chromatin or nuclei (blue), type II by a strong eosin signal from acellular material (pink), and type III consists of intermediate hematoxylin and eosin staining, from both chromatin/nuclei (blue) and non-nuclear material (pink). (b) Macrophotographs of paraffin-embedded MPC-Ds sections. Pictures were taken using the fluorescence scanner (Aperio Versa 8, Leica Biosystems). Samples were resized for easier comparison; all bars represent 2 mm. (c) FIt-SNE plot of PAS staining for all samples. Note the high pleomorphism of the MPC-Ds.

We next performed complete proteomic analysis of human MPC-Ds, employing the LC-ESI-MS technique (Figure 3a). To examine the composition of MPC-Ds in the early stages of their formation, only the inner (core) parts were analyzed. The analysis revealed an abundance of neutrophil-associated markers in all samples tested: S100A9, cathepsin G (CTSG), lactoferrin (LTF), lysozyme (LYZ), resistin (RETN), neutrophil defensin 1 (DEFA1), and myeloperoxidase (MPO). The samples also contained average to high amounts of S100A8, neutrophil elastase (ELANE), and azurocidin (AZU1). Some proteins were found in either low quantities, or not at all: eosinophil cationic protein (RNASE3), lipocalin-1 (LCN1), zymogen granule protein 16 homolog B (ZG16B), defensin alpha 4 (DEFA4), neutrophil-gelatinase-associated lipocalin (NGAL), S100A12, cathelicidin antimicrobial peptide (CAMP), grancalcin (GCA), bactericidal permeability-increasing protein (BPI), S100P, and matrix-metalloproteinase 8 (MMP8).

As we found many proteins characteristic to neutrophils, we further analyzed the obtained tissues sections for NE activity (Figure 3b). NE activity was detected in all MPC-Ds tested, at different levels. We observed the highest NE activity for samples P102 and P96, robust activity for samples P99 and P95, and lower, but nevertheless considerable,
NE activity for samples P94, P97, and P98. Of note, NE in the positive control (5 mU NE enzyme) had different kinetics than the NE in the MPC-Ds.

**Figure 3.** MPC-Ds are abundant in neutrophil-associated markers. (a) Proteomic analysis (LC-ESI-MS) of human MPC-Ds. Heat map displays MASCOT scores; (b) NE activity, normalized to sizes of tissues (MFI/cm²). NE (5 mU) served as positive control; (c) Representative immune fluorescence images of MPC-D (P99). All antigens are displayed in green, DAPI in red. The control was stained with DAPI and secondary antibody only (wo1st); (d) Representative image of mucopeptide concretion P98 stained with Thioflavin T (ThT). Upper row: ThT, lower row: control; bars represent 2 mm, and 4 mm, respectively. Note, neutrophil markers appear in all samples. NETs, characterized by the colocalization of citH3, MPO, or NE with DNA, preferentially appear on the surfaces. Abbreviations: QP-9: aquaporin 9, AZU1: azurocidin, BPI: bactericidal permeability-inhibitory protein, MPO: myeloperoxidase, NE: neutrophil elastase.

We then performed fluorescence stainings of various proteins, some of which were also found via proteomic analysis, to determine their localization within the tissue. A part of the tissue from one representative stone (P99) is shown in Figure 3c. Fluorescence stainings revealed a high signal for PAD4, IgG, GalNAc, SNA, hemoglobin, and CK-1. Table 1 contains mean values for each antigen in all MPC-Ds, obtained via the visual estimation of the fluorescence stainings. Some antigens were found in less than half of the samples (NE, citH3), and the rest were found in the majority (MUC5B, Fetuin A, MPO, MUC5AC, CK-1, fibrinogen, AQP-9, CK-9) or all of the samples (hemoglobin, CK-2, SNA, IgG, PAD4 and GalNAc). All antigens were predominantly found on the surface of the MPC-Ds, except for hemoglobin which was found in higher quantities in the inner (older) parts of MPC-Ds. The expression levels of different antigens varied considerably among the seven MPC-Ds tested (Table S1). Interestingly, in those samples in which little to no neutrophil markers were detected by IF, but found by LC-ESI-MS (P94, P96, P97, and P98), we detected a positive ThT signal (Figure 3d). This indicated an abundance of amyloid fibrils, i.e., filamentous protein aggregates that sterically hinder the accessibility of the antigens for detection by antibodies.

Table 1. Immune fluorescence of various antigens in MPC-Ds (average of all samples)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Core</th>
<th>Surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC5B (57%)</td>
<td>0.14</td>
<td>0.29</td>
</tr>
<tr>
<td>Fetuin A (57%)</td>
<td>0.43</td>
<td>0.57</td>
</tr>
<tr>
<td>citH3 (43%)</td>
<td>0.14</td>
<td>0.71</td>
</tr>
<tr>
<td>CK-10 (10%)</td>
<td>0.71</td>
<td>1.00</td>
</tr>
<tr>
<td>NE (43%)</td>
<td>0.86</td>
<td>1.00</td>
</tr>
<tr>
<td>MPO (71%)</td>
<td>0.29</td>
<td>1.14</td>
</tr>
<tr>
<td>MUC5AC (86%)</td>
<td>0.57</td>
<td>1.14</td>
</tr>
<tr>
<td>CK-1 (86%)</td>
<td>0.57</td>
<td>2.00</td>
</tr>
<tr>
<td>Fibrinogen (86%)</td>
<td>0.86</td>
<td>1.86</td>
</tr>
<tr>
<td>AQP-9 (86%)</td>
<td>1.00</td>
<td>1.86</td>
</tr>
<tr>
<td>CK-9 (86%)</td>
<td>1.57</td>
<td>1.71</td>
</tr>
<tr>
<td>Hemoglobin (100%)</td>
<td>1.86</td>
<td>1.71</td>
</tr>
<tr>
<td>CK-2 (100%)</td>
<td>1.43</td>
<td>2.14</td>
</tr>
<tr>
<td>SNA (100%)</td>
<td>1.57</td>
<td>2.57</td>
</tr>
<tr>
<td>IgG (100%)</td>
<td>2.00</td>
<td>2.57</td>
</tr>
<tr>
<td>PAD4 (100%)</td>
<td>2.14</td>
<td>2.71</td>
</tr>
<tr>
<td>GalNAc (100%)</td>
<td>2.43</td>
<td>3.00</td>
</tr>
</tbody>
</table>

1 The intensity of various IF stainings was visually estimated for each MPC-D using a 0–3 point scale (no to high antigen abundance). Mean values from all samples ($n = 7$) were taken to construct the table, and the antigens were sorted according to their abundance. Parentheses display the percentage of samples in which certain antigens were detected.
Lastly, various DNA dyes, including propidium iodide (PI), Hoechst33342, and DAPI, as well as a DNA antibody (anti-DNA), were used to detect DNA in MPC-Ds (Figure 4). Here, different tissue fragments, displayed as single dots, similar to each other in terms of DNA staining, clustered (group) together. The anti-DNA staining differed from all other DNA stainings, and preferentially stained the scattered DNA (Figure 4b), marked as region I in the schematic representation of the sample P94 (Figure 4a); such DNA can be found in NETs. FIt-SNE plots obtained by plotting the fluorescence intensity of separate DNA stainings are depicted in Figure 4d (sample P94) and Figure 4f (fluorescence intensity of all DNA stainings for all samples). No differences in the density of the DNA fluorescence was observed (Figure 4c). The DNA staining of MPC-Ds from different patients was generally heterogenous (Figure 4e).

Since we discovered an abundance of neutrophil-associated markers, we examined the tissue for neutrophils and NETs via fluorescence microscopy. A representative sample, abundant in both NE and MPO in the MALDI analysis and detected by IF, is shown in Figure 4g. We also observed an abundance of NE in the following MPC-Ds: P95, P99, and P102 as confirmed by robust NE activity (Figure 3b), high NE fluorescence signal (Table S1), and high MASCOT scores obtained in proteomic analysis (Figure 3a). The colocalization of NE and DNA appeared exclusively on the surface of the sample, indicating NET formation.

**Figure 4.** Neutrophils contribute to the formation and growth of MPC-Ds. (a) Overview of MPC-D P94, divided into 5 distinct regions (I–V); (b) Immune fluorescence images of MPC-D P94 stained with various DNA dyes (the signal was transformed into Rainbow RGB using Fiji); (c) FIt-SNE density plot indicating the automatic cluster assignments for all samples; (d) FIt-SNE plots of various DNA stainings for sample P99; (e) FIt-SNE plots as in (c), colored by patient ID and clustering DNA stainings from all patients (n = 7). Note, the high variability of individual samples; (f) FIt-SNE plots of each staining for all samples; bar represents 2 mm; (g) P102 stained for NE (green) and DNA (DAPI, red), or for extracellular DNA (anti-DNA, green). Images wo1st IgG, wo1st IgM served as controls for NE and anti-DNA, respectively. “+”: NET formation, “−”: no NET formation; bar represents 3 mm. Note NETs, preferentially stained by anti-NE and anti-DNA, can be found on the surfaces of MPC-Ds, and may possibly contribute to their inappropriate clearance.
We detected NE in the core of the sample, however, without DNA colocalization. The staining in which antibody against DNA was used (anti-DNA), shows a different staining than DAPI, confirming that extracellular DNA from NETs can only be found on the surface of the sample.

4. Discussion

Mucopeptide concretions (MPC-Ds) are known to take the shape of the lacrimal sac and nasolacrimal duct, in which they are formed [12]. Findings presented here are in line with previous publications, which reported the heterogeneity of MPC-Ds [2]. All samples we analyzed differed in size and shape. They obtained the form of the lacrimal sac and contained very few electron dense inclusions, indicating low number of calcifications. This confirmed that the MPC-Ds contain low amounts of inorganic material in the form of minor calcifications and are mostly composed of organic material and biological components [3]. Their layered, onion-like structure points towards gradual formation and growth [13].

In 2006, Paulsen et al. performed an extensive study on the composition of dacryoliths. None of the stones examined revealed any calcification under X-ray examination. HE staining, as well as PAS staining, were then performed, revealing a structure made of lobes and lobules, built on amorphous core material with scant cellular material. The amorphous material had either the appearance of debris or was organized in different layers [1]. Here, we define this as only one type of the MPC-Ds, considering there are other types, semirich or rich in cellular material, as well. Therefore, type I, II, and III MPC-Ds are described, based on sample morphology. The amount of cellular infiltration could indicate different extents of inflammation within the lacrimal sac obstructed by MPC-Ds. Even though stainings for neutrophils were performed at that time, only neutrophil antimicrobial substances (defensins) were investigated. Neutrophils were present in impressive amounts in most, but not all, of the investigated dacryoliths [1]. In line with these findings, we found defensins, secretory products of neutrophils, as well. In addition, we employed stainings for NE, citH3, and MPO together with DNA to differentiate between neutrophils and NETs within MPC-Ds. A recent publication by Wang et al. reports the superiority of the anti-DNA-IgM antibody in detecting loose, decondensed DNA, such as that in the patches of NETs [25]; we used this novel method to detect extracellular DNA in dacryoliths. We found neutrophils on the inside of the tissue and NETs on the rim of the tissue, i.e., NE colocalizing with the DNA. This indicated that, whereas neutrophils are involved in the early stages of the formation and growth of MPC-Ds, NETs play a role in disease pathogenesis later on. By localizing on the surface of the MPC-Ds, they might prevent their clearance and maintain the proinflammatory environment.

In 2018, Ali et al. showed via electron microscopy that the inside (core) of the MPC-Ds consist of extensive fibrillary network, rich in red blood cells. The presence of granulocytes and epithelial cells has been described as occasional here [12]. Accordingly, when we analyzed MPC-Ds, we investigated the core separately from the surface. Using IF, we detected hemoglobin in the core of the dacryolith as well, the only antigen studied found in higher quantities in the core of the sample, rather than the surface. This also supports the current hypothesis, where blood leaking is considered as the first step in dacryolithiasis, preceded only by a trauma of mechanical or chemical type [12]. Interestingly, we also observed hemoglobin on the surface of the samples, pointing toward possible blood leaking in later stages of dacryolithiasis as well. In line with these findings, we observed the occasional presence of granulocytes in the core of the concretions using IF, but an abundance of granulocyte-derived peptides and protein fragments using mass spectrometry.

We initially hypothesized that the reason for this was the inability of the antibody to physically reach to the antigen due to a dense/compact and, therefore, impenetrable network of cytokeratins. Therefore, IF stainings for four different cytokeratins (CK-1, CK-2, CK-9, and CK-10) were performed. We detected cytokeratins in almost all samples, which confirmed the presence of epithelial cells within MPC-Ds [32]. We mainly found cytokeratin 2 (CK-2) and cytokeratin 9 (CK-9). CK-1 localized mostly on the rim of the
tissue; CK-2 and CK-9 were found in other (inner) parts of the tissue. CK-10 was the least abundant cytokeratin. Mucins and TFF peptides have been described as major components of MPC-Ds; however, reactivity against MUC5AC and MUC5B was observed in most cases, but not all [1]. No reactivity for mucins in some cases was attributed to a lack of cells in the section. Accordingly, we did not detect mucins in high quantities and in all samples tested; their distribution was rather inhomogeneous. We, therefore, stained the tissue for glycosylated proteins (GalNAc and SNA), as mucins are often glycosylated and TFF activity depends upon their glycosylation state [33]. The discrepancies between low mucins signal and high glycosylation signal indicated that mucins might be present but could not be detected by immune fluorescence. Considering that all MCP-Ds in our case contained at least some cellular regions, we contribute this to the aggregation of proteins into amyloid-like structures, which form a dense network and cause the impermeability of the tissue for antibody binding. We confirmed this by the use of Thioflavin T (ThT), a cationic benzothiazole dye which increases in fluorescence upon binding to amyloid fibrils (filamentous protein aggregates) [34,35], commonly used in histology and for protein characterization. With ThT staining, one can detect the conformationally altered, folded intermediates, and aggregated or fibrillated proteins, which can still be immunogenic [36]. We detected a strong ThT signal only for those samples in which the antigens could not be detected by IF (P94, P96, P97, and P98). In addition, the correlation between the HE staining and NE activity is not a direct one. A reason for this is that not all infiltrating cells seen in HE staining correspond to neutrophils, rather other immune cells as well. Considering, however, the immense heterogeneity of the samples analyzed, our classification based on HE and PAS stainings appeared most appropriate. Further studies with larger numbers of patient samples might bring some clarity to the pattern of heterogeneity, such as age or gender.

The first proteomic analysis of MPC-Ds (dacryoliths) in the context of rebamipide treatment was conducted in 2018 as well, identifying the proteins present in MPC-Ds [37]. Here, we link these findings to the localization of various antigens in the tissue, employing IF. By performing proteomic analysis, we observed an abundance of MPO in all MPC-Ds, with the highest expression in tissues P96 and P102. In line with this, we also found MPO in all samples when we performed IF stainings. Accordingly, MPO expression was highest in sample P102. However, we observed a discrepancy between the two methods for sample P96. MPO was found in high quantities in proteomic studies; however, it was not present in IF staining at all. Employing MALDI, we detected NE in all MPC-Ds, with the lowest expression in P94 and P97, in line with the IF stainings. Two other samples, P96 and P98, were poor in NE in IF stainings. We contribute these discrepancies to the aforementioned dense network of amyloid fibers, confirmed by the ThT staining. In line with previous findings, we found abundant S100A9, S100A8, lysozyme C, neutrophil defensin 1, cathepsin G, azurocidin, neutrophil elastase, eosinophil cationic protein, myeloperoxidase, and lactotransferrin. In addition, we found an abundance of resistin.

Finally, in 2019, immunohistochemical analysis of the lacrimal sac MPC-Ds was performed and strong immunoreactivity for lysozyme was described. Few peripheral areas of concretions were positive for S100-A9, and all samples were negative for IgG [11]. Here, we show by the use of IF that IgG is, in fact, one of the most abundant antigens found. It was present in all samples studied, and predominantly localized on the surface, however, with high abundance in the core.

5. Conclusions

Taken together, here we examined the involvement of neutrophils and NETs in the formation and growth of human MPC-Ds. Considering the relatively low incidence of lacrimal sac obstruction with MPC-Ds, this study was constrained by a limited sample size. Despite the small size of our patient cohort, we ensured diversity by including participants from both genders and various age groups, thus providing a broader representation. We performed complete proteomic analysis by employing LC-ESI-MS and found neutrophil
and NET markers in high quantities. We observed considerable NE activity in all tissues. By performing various stainings of all MPC-Ds, we determined the (co)localization of neutrophil markers (NE, citH3, and MPO), citrullination (citH3 and PAD4), immunoglobulins (IgG), and other types of leukocytes (hemoglobin for red blood cells and fibrinogen for thrombocytes). Through a multidisciplinary approach, we propose a classification system for mucopeptide concretions, with respect to the quantity of cellular and acellular material, polysaccharides, and mucosubstances. We describe the role of neutrophils as pivotal in the initial stages of dacryolithiasis, whereas NETs contribute to the later stages of disease pathogenesis, presumably maintaining the proinflammatory environment and impeding efficient clearance. These findings provide valuable insights into dacryolithiasis, highlighting the integral roles of both the innate and adaptive immune systems.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cells12141857/s1, Table S1. Immune fluorescence of various antigens in MPC-Ds (all samples).

**Author Contributions:** Conceptualization, M.S., F.P., G.S. and M.H.; Data curation, T.B., M.M.-B. and G.S.; Formal analysis, L.Z., T.T., G.L., R.B., T.B., M.M.-B., J.H., M.J.A. and F.P.; Funding acquisition, R.B., F.P. and M.H.; Investigation, L.Z., T.T., G.L. and R.B.; Methodology, M.S., G.S., F.P. and M.H.; Project administration, J.K.; Resources, J.H., F.P., M.H. and G.S.; Software, T.B. and M.M.-B.; Supervision, T.B., M.M.-B., G.S., J.K., J.H., M.J.A. and M.S.; Validation, L.Z.; Visualization, L.Z.; Writing—original draft, L.Z.; Writing—review and editing, T.T., R.B., J.K., M.J.A., G.S., F.P. and M.H. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was partially supported by the Deutsche Forschungsgemeinschaft (DFG) 2886 PANDORA Project-No. B3 to MH, CRC1181-261193037 (C03) to MH, SFB/TRR 241 (B04) to MH, PA738/15-1 to FP, by the European Union H2020-FETOPEN-2018-2019-2020-01 to MH, by the European Commission 861878 “NeutroCure” to MH, by the Volkswagen Foundation (Grant 97744) to MH and RB, and by the National Research Foundation of Ukraine grant 2020.02.0131 to RB. We acknowledge financial support from Deutsche Forschungsgemeinschaft and Friedrich-Alexander-Universität Erlangen-Nürnberg within the funding programme “Open Access Publication Funding”. The study was conducted in accordance with the Declaration of Helsinki, and approved by the ethical committee of the University Hospital Erlangen (permit 243_15 B).

**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, and approved by the ethical committee of the University Hospital Erlangen (permit 243_15 B).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Data generated during the study are within the article or Supplementary Materials.

**Acknowledgments:** The authors gratefully acknowledge the valuable support provided by Hong Nguyen (Institute of Functional and Clinical Anatomy, FAU Erlangen-Nürnberg).

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

6. Hoffmann, W. Trefoil Factor Family (TFF) Peptides and Their Links to Inflammation: A Re-evaluation and New Medical Perspectives. *Int. J. Mol. Sci.* 2021, 22, 4909. [CrossRef]


Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.