Neutrophils *versus* Pathogenic Fungi through the magnifying glass of nutritional immunity

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To all those who were always convinced this moment would come.
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Paper I

Restoration of anti-Aspergillus defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calprotectin-dependent.
Bianchi M* & Niemiec MJ*, Siler U, Urban CF and Reichenbach J.
Journal of Allergy and Clinical Immunology, 2011.
* equal contribution

Paper II

Trace element landscape of resting and activated human neutrophils on the sub-micrometer level.
Metallomics, 2015.

Paper III

RNA-Seq transcription profile of the neutrophil – Candida albicans in vitro interaction.
Manuscript.

Those articles will be referred to in this thesis as paper I, II, and III.
Publications not included in this thesis

Paper IV

*Candida albicans* escapes mouse neutrophils.
Ermert D* & **Niemiec MJ***, Röhm M, Glenthøj A, Borregaard N, Urban CF.
* equal contributions

Paper V

Malaria-enhanced neutrophil-dependent clearance of *S. pneumoniae* in an in vivo co-infection model.
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Abstract

Neutrophils are among the first white blood cells recruited to the site of infection once microbial pathogens enter the host organism. At site, they perform a well-orchestrated chain of processes that aims to kill the microbial invader. Most prominent, neutrophils engulf microbes to inactivate them intracellularly, a process called phagocytosis. Alternatively, neutrophils can release neutrophil extracellular traps (NETs). NETs consist of chromatin decorated with antimicrobial effector proteins – a structure that can entangle bacteria and fungi. Neutrophils are crucial during fungal infections. This is reflected in the increased risk of fungal infections resulting of neutropenia. The concept of nutritional immunity describes every infection as a battle for resources. Those are mostly metal trace elements.

While neutrophils were seen for a long time as powerful, but “mindless”, killers with a limited set of actions and no transcriptional capacity, this view is in the flux.

In the presented thesis, it was my goal to gain new insights into the interplay of neutrophils and fungi – with special attention to metal nutritional aspects.

We compared human neutrophils lacking the ability to undergo NETosis, due to a non-functional NADPH complex, and neutrophils from the same person that were “cured” by gene therapy. We investigated those NETs and found that their inhibitory activity towards the mold Aspergillus nidulans depends on calprotectin, a known zinc-chelator.

Considering the high influx of neutrophils, we wanted to unravel the neutrophils’ contribution to the metal milieu at the site of infection and trace element changes resulting from NETosis. By combining synchrotron radiation XRF and ICP-MS, we analyzed the neutrophil metallome and the spatial element distribution in activated neutrophils and NETs. Most strikingly, we found neutrophils to be exceptionally high in Fe and the process of NETosis to be reducing available Zn in the surrounding and the early phagosome, possibly by the formation of Zn-rich vesicles.
Using RNA-sequencing, we analyzed the interplay of the *Candida albicans* and neutrophils face-to-face. We dissected their transcriptional profile and revealed a complex response in neutrophils that include cytokine induction and cellular rearrangement. We further were the firsts to explore the transcriptional response of *C. albicans* to NETs. Our data indicates a distinct response compared to intact neutrophils or other known stress triggers. Metal homeostasis was affected in *Candida* in both set-ups.

In summary, this thesis provides new insights into the interaction of fungal pathogens with neutrophils and emphasizes the impact of nutritional aspects on this interplay. A deeper understanding of the nutritional immunity during fungal infection might open up new strategies to tackle fungal infections – a growing threat worldwide.
Clarifications

CAT  catalase
CD  cluster of differentiation
CGD  chronic granulomatous disease
CLR  C-type lectin receptor
CR  complement receptor
Ctr  Cu transporter
DC  dendritic cell
DEG  differentially expressed gene
ERK  extracellular signal-regulated kinases, = MAPK
ET  extracellular traps
FcγR  Fc (fragment crystallizable) γ receptor
Fet  ferrous transporter
FPR  formylated peptide receptor
Fre  ferric reductase
Ftr  Fe transporter
G-CSF  granulocyte-colony stimulating factor
GPX  glutathione peroxidase
GUT  gastrointestinal induced transition
ICAM  intracellular adhesion molecule
ICP-MS  inductively coupled plasma mass spectrometry
Ig  immunoglobulin
IL  interleukin
IRAK  interleukin-1 receptor-associated kinase
LAD  leukocyte adhesion deficiency
MAC  membrane attack complex
MBL  mannan-binding lectin
MEK  = MAPKK or MAP2K, Mitogen-activated protein kinase kinase
MMP  matrix metalloproteinase
MPO  myeloperoxidase
MR  mannose receptor
MYD88  myeloid differentiation primary response gene 88
NADPH  nicotinamide adenine dinucleotide phosphate
NETs  neutrophil extracellular traps
NLR  nod-like receptor
NLRP3  NOD-like receptor family, pyrin domain containing 3
NO  nitric oxide
p.i.  post infection
PAD  peptidylarginine deiminases
PMN  polymorphonuclear cell
PSGL-1  P-selectin glycoprotein ligand-1
RLH  RIG-like helicases
RNS  reactive nitrogen species
ROS  reactive oxygen species
SCN  severe congenital neutropenia
SOD  superoxide dismutase
SR-XRF  synchrotron radiation X-ray fluorescence
TLR  toll-like receptor
TNF  tumor necrosis factor
WHIM  Warts, Hypogammaglobulinemia, Infections, and Myelokathexis syndrome
ZnT  Zn transporter
Zrt  Zn-regulated transporter
Introduction & Background

1. Neutrophils

Neutrophils are the most abundant white blood cells. With numbers up to $10^{11}$ generated every day, they constitute 60-80 % of all nucleus-containing blood cells. Neutrophils mature in the bone marrow under the control of G-CSF and belong to the myeloid lineage of hematopoietic cells. After leaving the bone marrow to enter circulation, 95 % of mature neutrophils are post-mitotic. As professional phagocytes, they are assigned to be part of the innate immune system [1].

1.1 The innate immune system - overview

The human immune system is traditionally divided into two major branches: the adaptive and the innate immune system. As indicated by their names, the innate immune system is present from the very beginning of life and needs no further training, while the adaptive immune system can learn and, very importantly, memorize. Due to these properties, the innate immune response is fast, but rather unspecific, and the adaptive immune system is slower, but pathogen-specific. While the innate immune response can occur immediately, the adaptive immune response takes between 3-4 days to be fully engaged [2]. Both branches of the immune system communicate with each other, and new findings of recent years indicate that the strict separation of the two is not entirely correct [3]. For instance, activated innate immune cells produce cytokines that are recognized by cells from the adaptive immune system. In addition, innate immune cells present microbial antigens to T cells [2]. On top of this cross-talk between the branches, trainable aspects of the innate immune system in organisms lacking an adaptive immune system (plants, invertebrates), but also in mammals, are observed and debated [3].

The first line of defense of the human body against invaders are mechanical and chemical barriers, composed and produced by skin, hair, nail, and mu-
cosal tissues. While hair and nail, consisting of dead cells, exclusively provide mechanical resistance, skin and mucosal tissues have functions way beyond. Skin and mucosal cells as well as glands contribute to a chemical barrier. Well-known examples are the very low pH in the stomach, the enzyme lysozyme in tears or fatty acids on the skin. Epithelial cells are so-called non-professional immune cells and provide dual function. They are tightly connected, and therefore provide mechanical resistance. Most importantly, they sense microbial invaders and cell damage leading to secretion of peptides and proteins. Those weaken microbes or recruit professional immune cells, like neutrophils, to the site of infection. Finally, the normal flora on body surfaces contributes to this first hurdle for invading pathogens [2, 4].

In areas below skin or mucosa, tissue-residing macrophages patrol to fend off invading microbes. Upon encounter of an intruder, they will not only initiate the killing of the pathogen, but also release cytokines to promote inflammation and chemokines to recruit neutrophils and monocytes [2]. In addition to macrophages, tissue-residing mast cells contribute to inflammation. Upon activation, they secrete pro-inflammatory mediators that are stored ready-made in granules or synthesized on demand. Archetypical mast cell effectors are histamine, heparin, or TNF-α [2]. Inflammation is characterized by four hallmarks: pain, redness, heat, and swelling. Heat and redness are caused by an increased blood flow. Swelling and pain result from increased permeability of the blood vessels to allow white blood cells to enter the tissue – accompanied by influx of fluid and proteins. The deliberate clotting of local blood vessels, to reduce the chance of a pathogen spreading systemically, contributes to the phenotype of inflammation [2].

Other tissue-residing immune cells are dendritic cells. Dendritic cells are often assigned to be part of the innate immune system, but at the same time known to be crucial for the cross-talk with the adaptive immune system. Like macrophages, they are antigen-presenting cells.

In contrast, a number of other innate immune cells are present mostly in circulation. They are basophils, eosinophils, and NK cells. Basophils and eosinophils are closely related to neutrophils, since they all contain charac-
teristic granules and stem from the myeloid lineage in the bone marrow. NK cells originate from the lymphoid lineage and attack host cells that present markers of cancer or viral infection. Together with DCs, NK cells act at the interface of innate and adaptive immune system. Activated by certain pathogens, both cells produce cytokines that cause proliferation of CD4 T cells [2, 5].

The complement system supports the function of the cell-mediated immune system. It consists of 20-30 different proteins synthesized mostly by hepatocytes. Three complement cascades are known: the classical, the alternative pathway and the lectin pathways. While activated differently, they all have three major functions: 1) opsonization and clumping of foreign material to enhance phagocytosis; 2) induction of chemotaxis to the site of infection; 3) lysis of microbes by the membrane attack complex (MAC) [2, 4]. Due to chemotactic and phagocytosis-inducing capacities, the complement system is tightly connected to phagocytes, and therefore to neutrophil functionality.

### 1.2 Professional phagocytes

Phagocytes are classified into professional and non-professional [6]. This classification remains debatable though, as it is solely based on phagocytosis efficacy.

Uptake of microbes is a crucial weapon in the armory of the innate immune system. During internalization, microbes are engulfed and enclosed by plasma membrane until a vesicle segregates, which is then called phagosome. This phagosome fuses with the lysosomal or granular vesicles to form the phagolysosome – a very hostile intracellular compartment in which the microbe is exposed to low pH, nutrient starvation, lytic enzymes and reactive oxygen species (ROS) [7].

In addition to neutrophils and monocytes/macrophages, DCs and mast cells are also sometimes considered professional phagocytes. Since discussing these two cell types in detail lies beyond the scope of this thesis, the term ‘professional phagocyte’ will in the following only refer to the neutrophils and monocytes/macrophages.
Like neutrophils, monocytes are generated during myelopoiesis in the bone marrow and from there released into the blood stream. Around 10% of all white blood cells are in fact monocytes [8]. Unlike neutrophils, monocytes can leave the blood stream without an acute infection signal. Monocytes that enter the tissue mature subsequently into macrophages. Remarkably, the type of tissue and the type of stimulation determine the ‘activation phenotype’ of these macrophages. In general, macrophages can be either M1 or M2 macrophages, which corresponds to classically or alternatively activated, respectively. M1 macrophages are described as the ‘pro-inflammatory’ type, as they produce nitric oxide more effectively and secrete large amounts of pro-inflammatory cytokines and chemokines. M2 macrophages are the ‘inflammation-limiting’ or ‘wound-healing’ type. They secrete extracellular matrix proteins and express cytosolic arginase. Examples for tissue-specific macrophages are the alveolar macrophages of the lung or microglia of the brain [8].

Even though both cells types are often used interchangeably, neutrophils and macrophages show some important differences – not only in the interaction with other immune cells, but also at their core function: phagocytosis. While both cell types actively engulf microbes, the phagolysosome maturation and the processes inside show some distinct features [8, 9]. Both cell types take up foreign material, e.g. microbes, in the same two ways. Either by FcγR-mediated extension of the cell as a pseudopodium to take up IgG-opsonized particles, or by complement receptor-mediated uptake that is described as a ‘sinking into the cell’ to internalize complement-opsonized particles [10]. The mechanisms are very similar, but the speed of internalization is different. IgG-opsonized targets were described to be taken up faster by neutrophils than by macrophages [9].

In macrophages, the phagosome fuses with endosomes and lysosomes to mature gradually to the phagolysosome [11]. In neutrophils, neutrophil-specific granules rapidly fuse with the phagosome [12]. It therefore seems to be more suitable to refer to a ‘mature phagosome’ to describe the hostile, granule-infused phagosome in neutrophils, instead of calling it a phagolysosome as in macrophages. Due to their distinct formation process,
the proteins in the phagolysosome and the ‘mature phagosome’ are not the identical.
And neither is the pH. While the pH in the ‘mature phagosome’ stays more or less at around neutral pH 6-7, the phagolysosomal pH decreases down to pH 4-5 during maturation.
The last major difference of neutrophil and macrophage phagocytosis is the oxidative burst. The NADPH complex, assembled in activated neutrophils and macrophages, is present in the cytoplasmic membrane and generates superoxide towards the extracellular milieu, and accordingly in the phagosome towards the microbe inside. Superoxide (O$_2^-$) is converted to hydrogen peroxide (H$_2$O$_2$) by superoxide dismutases (SODs). Hydrogen peroxide can react with present cations via the Fenton reaction to create hydroxyl radicals (OH˙). The oxidative burst in neutrophils is overall stronger than in macrophages, because neutrophils can recruit more NADPH to the ‘mature phagosome’ than dictated by the pure membrane internalization event. Further, neutrophils possess the enzyme myeloperoxidase (MPO), which is absent in macrophages [13]. MPO converts H$_2$O$_2$ to hypochlorous acid (HOCl), one of the most reactive oxidative agents. Both professional phagocytes, neutrophils and macrophages, produce reactive nitrogen species (RNS). First, the production of nitric oxide (NO˙) is catalyzed by NO synthases that convert L-arginine to L-citrulline during the reaction. NO˙ can subsequently react with O$_2^-$ to generate harmful reactive nitric oxide species [14]. Additionally, NO˙ possesses immunomodulatory functions [8]. Even though neutrophils are capable of producing NO˙ and subsequently RNS, the role of RNS in neutrophils is less well understood than in macrophages.

1.3 Neutrophils – from bone marrow to the site of infection

Neutrophils are remarkable immune cells that were for a long time understudied. Traditionally perceived as strong, but simple, killers lacking extensive regulation schemes, an increasing body of seminal studies has considerably changed the general view of these cells.
Neutrophils mature in the bone marrow during a process called granulopoiesis to be released into the blood stream [1]. Large numbers are produced and removed every day. Neutrophil life span is still a matter of debate. Generally described to be between 6 and 8 h, recent studies have suggested that neutrophils could live up to 5-6 days [15-17]. Nevertheless, the lifetime is short, compared to other immune cells. In the body, neutrophils reside either in circulation or in so-called marginated pools. In addition to the bone marrow itself, the liver, the spleen, and the lung were described as locations to contain mature neutrophils that can be quickly released, if so required [1].

Neutrophils, hence potentially harmful, do not leave the blood stream and do not migrate into the surrounding tissue randomly. Upon microbial invasion or tissue damage, the affected epithelial or tissue cells, as well as residing immune cells, will release cytokines like TNF-α or IL-1β that induce the expression of P-selectin, E-selectin or integrins on the luminal surface of the blood vessel. Constitutively expressed ligands for those on the neutrophil, namely PSGL-1 and L-selectin, will bind to P- and E-selectin. Hence, the neutrophil is captured and rolls on the endothelial surface. Firm adhesion is mediated by neutrophil integrins and ICAM-1 and ICAM-2 on endothelial cells. A complex chain of events then allows the paracellular or even transcellular passaging of the neutrophils through the endothelial lining, a process known as extravasation.

In the tissue underneath, the neutrophil is confronted with a number of new stimuli. Chemokines or chemotactic factors are sensed by neutrophils, which are able to migrate towards the infection site along a concentration gradient. Typical chemotactically active factors are IL-8, bacterial formylated peptides, or Candida albicans β-glucan [1, 15, 18]. Local G-CSF triggers cytokine production, while pro-inflammatory cytokines and pathogen-associated molecular patterns (PAMPs) can induce ‘priming’. Neutrophils present only few pattern recognition receptors (PRRs) on their surface per se, although in tissue, ‘primed’ neutrophils increase these numbers. This conversion is crucial for precise microbial location in infected tissue [19].
Once at the site of infection, neutrophils recognize and distinguish the microbial invader by the binding of PAMPs to their PRRs. Neutrophil-attached PPRs include toll-like receptors (TLR), C-type lectin receptors (CLR), NOD-like receptors (NLR), RIG-like helicases (RLH) as well as formylated peptide receptors (FPR) and the yet unclassified TREM1[20]. Neutrophils also produce secreted PRRs. From all known fungal-sensing PRRs, approximately half are present on neutrophils (Tab. 1). These are the surface TLRs TLR2 and TLR4, the intracellular TLR9, the CLR Dectin-1, Mincle and Galectin-3, the NOD-like receptor NLRP3, the FcγR and the complement receptor 3 [21-29].

The recognition of a microbe immediately causes a chain of events aimed at killing the pathogen. Most frequently, neutrophils will attempt to engulf the invader and inactivate it intracellularly by toxication in the ‘mature phagosome’ (see 1.2). Executing effectors are primarily ROS and antimicrobial peptides and proteins previously stored in granules; reduction of the pH seems to be less important in neutrophils [7].

Granules are filled with hundreds of different proteins and are traditionally subdivided into 3+1 classes. They vary in size, content and even origin [30, 31]. Instead of understanding granules as clearly distinguishable, “granules can be viewed as a continuum where subsets are defined based on a selection of marker proteins” [19]. The three archetypical granule classes are primary/azurophil, secondary/specific, and tertiary/gelatinase granules. Their marker proteins are MPO, lactoferrin, and gelatinase MMP9, respectively. Only during granulocyte differentiation in the bone marrow, granules are generated and filled – a process referred to as the ‘first transcriptional burst’. The granule content varies according to the ‘targeting-by-timing’ model, since not all granules accrue at the same time. Interestingly, the granules formed last, the tertiary granules, are the ones to be mobilized first [18, 19]. Degranulation is directed towards the phagosome, but also to the extracellular surrounding.
Table 1: PRRs involved in fungal immune recognition
PRRs involved in the recognition of fungal pathogens are listed together with their ligands and related to their presence in human neutrophils [20-29].

<table>
<thead>
<tr>
<th>PRR class</th>
<th>PRR</th>
<th>PAMP</th>
<th>Expressed / functional in neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR</td>
<td>TLR2*</td>
<td>Phospholipomannan</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>TLR3</td>
<td>RNA (Aspergillus)</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>Mannan</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>TLR7</td>
<td>RNA (Candida ssp.)</td>
<td>not in humans</td>
</tr>
<tr>
<td></td>
<td>TLR9</td>
<td>CpG-oligodeoxy-nucleotides</td>
<td>yes</td>
</tr>
<tr>
<td>CLR</td>
<td>MR</td>
<td>N-linked-mannan</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Dectin-1 (CLEC7A)</td>
<td>β-1,3-Glucan</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Dectin-2* (CLEC6A)</td>
<td>High-mannan structures + α-mannan</td>
<td>unclear</td>
</tr>
<tr>
<td></td>
<td>DC-SIGN/SIGNR1</td>
<td>Mannan</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Mincle** (CLEC4E)</td>
<td>Unknown</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Galectin-3 (soluble)</td>
<td>β-mannosides (α?)</td>
<td>yes, in humans</td>
</tr>
<tr>
<td></td>
<td>CR3</td>
<td>β-glucan</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>MBL (soluble)</td>
<td>Mannan</td>
<td>no</td>
</tr>
<tr>
<td>Scavenger receptor</td>
<td>CD36</td>
<td>Unknown</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>SCARF</td>
<td>Unknown</td>
<td>no</td>
</tr>
<tr>
<td>NLR</td>
<td>NLRP3</td>
<td>unknown</td>
<td>yes</td>
</tr>
</tbody>
</table>

* With or without TLR1 or TLR6, ** with FcγR

Secretory vesicles are classified as granules, too. In simple terms, secretory vesicles are subcellular compartments storing a number of receptors that are quickly translocated to the plasma membrane during ‘priming’ [19]. Largely underestimated, neutrophils participate in shaping the inflammatory response at the site of infection and prepare the ground for immune cells that are recruited at later time points – from both the innate and adaptive immune system.
**Figure 1: Major antimicrobial processes performed by neutrophils during innate immune defense**

Neutrophils enter the site of infection following a chemokine gradient. Upon microbial encounter, they engulf and intracellularly toxify the microbe, secrete antimicrobial proteins into the surrounding, or release cytokines to recruit other immune cells. Upper left: chemotaxis, upper right: phagocytosis, lower left: degranulation, lower right: cytokine secretion.

Interaction occurs with macrophages, DCs, NK cells, and T cells via the release of cytokines and chemokines, which are expressed on demand, not stored like most antimicrobial effectors [1]. This protein expression event is sometimes referred to as the ‘second transcriptional burst’ – with the first burst being the one in the bone marrow and no transcriptional activity in circulation [15].

During intraphagosomal toxification of a microbe, bacterial or fungal, ROS are generated that will induce neutrophil cell death eventually. Most prominently, ROS induce apoptosis, but it is also proposed that very high levels of ROS promote necrosis [32]. ROS-dependence of NETosis was demonstrated with different stimuli [33, 34]. Apoptosis is in many respects the cell death route to prefer, since it prevents release of harmful neutrophil content into the surrounding. Apoptotic neutrophils are removed by tissue-residing macrophages in liver, spleen, and bone marrow during normal homeostasis. Dur-
ing infection, they are taken up by macrophages, but also DCs, at the respective site [32]. Remarkably, this so-called ‘efferocytosis’ dampens a cascade that induces G-CSF production – thus, high numbers of apoptotic neutrophils in tissue reduce the G-CSF controlled release of neutrophils into circulation [18].

1.4 NETosis – neutrophil functionality post mortem

Since their discovery about a decade ago, neutrophil extracellular traps have been the subject and inspiration for many studies. The list of pathogens described to be either inducing NET formation and/or sensitive to NETs has become long, and includes organisms as different as the bacteria *Mycobacterium tuberculosis* and *Shigella flexneri*, the fungi *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, but also parasites like *Plasmodium falciparum* [35-40]. NETs might even play a role during viral infections [41]. Since NETs fibers have a DNA backbone, it is not surprising that many bacterial pathogens possess extracellular DNAses to escape entrapment by NETs [42, 43].

Phagocytes of many organisms produce NETs or ETs: from mammals, birds, fish, but also invertebrates, such as acoelomates, oysters or shrimp [36, 44-48]. In humans, not only neutrophils, but also mast cells and eosinophils release DNA traps [49, 50].

Although several NETosis models were proposed in the last years, only one will be described and referred to in this thesis: the ROS-dependent NET formation involving DNA from the nucleus [34, 51]. According to current understanding, this is most likely the NETosis mechanism induced by fungal pathogens [52, 53].

In this model (Fig. 2), an external stimulus triggers the induction of the proteinase kinase C (PKC), which leads to the formation and activation of the NADPH complex. The produced ROS induce, over yet not fully understood routes, the loss of integrity of nuclear and granular membranes. At this point, the plasma membrane is still intact, allowing intracellular components to get in contact and to mix – even though originating from formally separated compartments. Granular proteins promote chromatin decondensation.
Eventually, the plasma membrane ruptures and the neutrophil DNA is released “decorated” with a number of antimicrobial proteins [34, 38, 54]. Remarkably, these proteins are not randomly NET-associated, but defined, and reflect the NETosis process well: the NETome includes indeed proteins from the nucleus, from granules, and from the cytoplasm.

**Figure 2: ROS-dependent NETosis**
Neutrophils undergo NET formation upon external stimulation. Four stages are characteristic in this process (from left to right): 1) Neutrophils sense an activating stimulus. The NADPH oxidase complex is formed and superoxide is generated. 2) Membranes of nucleus and granules disintegrate. 3) Content of nucleus, granules and cytoplasm mix. 4) Plasma membrane ruptures, NETs are released [51]. [Re-used with permission of the Nature Publishing Group, License 3601390788934]

NETosis is distinct from apoptosis and necrosis, and accordingly considered an additional cell death mechanism [34, 55].

NET induction and the responsible intracellular events are not fully understood, yet. Several pathways are supposedly involved in NETosis: the Raf-MEK-ERK pathway, PAD4-mediated citrullination of histones, elastase-mediated chromatin decondensation, and autophagy [54, 56-58]. Numerous stimuli, biotic and abiotic, induce NETs. The most prominent abiotic stimulus used to trigger NET formation very effectively *in vitro* is phorphol 12-myristate 13-acetate [36].

When considering the versatile armory of neutrophils, it is obvious that one of the most intriguing questions is, how neutrophils “decide” to undergo NETosis instead of other antimicrobial defense mechanisms. While the one trigger and the one pathway are not identified yet, neutrophil heterogeneity might hold some clues. A recent study demonstrated a possible link between NETosis and an olfactomedin 4-expressing neutrophil subtype [59]. In addition, a preferential release of NETs towards bulky pathogens was proposed [60].
It should be mentioned here as well that NETs do not only play a role during microbial infections, but also during sterile inflammation, e.g. in autoimmune diseases [1].
The presented thesis’ focus lies on the antimicrobial mode of action of NETs, especially towards fungal pathogens.

1.5 Neutrophil disorders

Neutrophil disorders can be divided in inherited genetic defects and non-inherited disturbances of neutrophil homeostasis. Neutropenia, the reduced count of neutrophils, is in some cases the result of accelerated neutrophil death and is amongst others caused by chemotherapy or bone marrow transplantation. Neutrophilia, the pathologically increased number of neutrophils, can be the result of repressed neutrophil death, and occurs e.g. in infections and auto-immune diseases [32]. A number of genetic neutrophil defects are known today – in all of them, the patient is facing an increased risk of microbial infection.

Severe congenital neutropenia (SCN) is a disease caused by the blockage of full bone marrow maturation of neutrophils. Life-long G-CSF treatment is necessary to counteract the increased risk of bacterial and fungal infections. In leukocyte adhesion deficiencies (LADs), neutrophils are not able to leave the blood stream and enter the site of infection, because the adhesion cascade is corrupt. In patients suffering from the WHIM syndrome, neutrophils are less prone to leave the bone marrow, reducing the number of neutrophils in circulation [1].

In addition to diseases mostly affecting neutrophil homeostasis, direct neutrophil antimicrobial efficacy can be abrogated by genetic defects, too. Chronic granulomatous disease (GGD) is characterized by a non-functional NADPH complex that restrains the neutrophil’s superoxide production, impairing its ability to kill microbial pathogens effectively. This in turn affects the patient’s life expectancy and requests life-long surveillance [1]. In addition, CGD patients suffer from sterile inflammation causing predominantly kidney damage. The mechanisms of the hyper-inflammatory state are less
well understood. Most likely, macrophage activation and regulation of neutrophil apoptosis play a role [61, 62].

Mutations of down-stream mediators of TLR signaling, namely MYD88 and IRAK4, increase the risk of infection predominantly in young children. Interestingly, IRAK4-deficient neutrophils are capable of killing *S. aureus*, *E. coli*, and *C. albicans* [63]. Subtotal or total MPO-deficiency increases the risk of life-threatening infections. Still, an inherited MPO deficiency can also be asymptomatic [64]. Similarly, a lack of specific granules, affecting antimicrobial proteins from secondary and tertiary granules, subsequently, leads to recurrent microbial infections [1].
2. Human fungal pathogens

Human fungal pathogens account for high number of superficial and systemic infections worldwide [65]. Nevertheless, they lead a shadowy existence compared to bacterial or viral infectious agents. Not only in basic research, but also in training of medical professionals, fungi are often undervalued. Initiatives like LIFE (Leading International Fungal Education, life-worldwide.org), the Fungal Infection Trust, or the ‘Mycotic Diseases Branch’ of the CDC aim to change perception worldwide.

2.1 The fungal threat

Even though the exact numbers vary from source to source, it is clear that only a small group of all fungal species can cause disease in humans. A dozen of those account for 90 % of all mycoses [4]. A group of 4 genera is responsible for 90 % of all fungal-related deaths: Candida, Aspergillus, Cryptococcus and Pneumocystis [65].

According to LIFE, fungal diseases can be assigned to five different classes: invasive fungal infections, chronic lung and deep tissue infections, allergic fungal disease, mucosal infections, and skin, hair, and nail infections. The last two groups are often paired and as such called ‘superficial infections’. With approximately 25 % of all humans worldwide affected, these infections lead to an immense physical burden [65]. Invasive fungal infections are scarce in comparison, but accompanied by relatively high mortality. Recent estimations suggest 1.5 million deaths per year. Individual mortality rates vary due to fungus and location. Extremes are 95 % mortality in A. fumigatus infections, 75 % in C. albicans infections, 70 % in C. neoformans infections, or 80 % for Pneumocystis jirovecii [65]. While transmission from human to human does not play a major role for fungal infections in general, treatment of fungal pathogens is challenging. Limited choices of antifungals, drug resistances, and severe side effects complicate successful therapy [4].
2.2 The yeast Candida albicans and other Candida ssp.

*Candida* ssp. are the most important cause of opportunistic fungal infections worldwide. They are the fourth most common nosocomial blood stream isolates in the U.S. In Europe, *Candida* causes systemic infections just as often as the prominent bacteria *E. coli* or *S. aureus* [66, 67]. Like most fungi, *Candida* ssp. are opportunistic, meaning they are pathogenic only under certain circumstances, namely if the host immune defense is impaired. Noteworthy, the number of immuno-compromised has increased over the past decades [68, 69]. The reasons for immunodeficiency are as versatile as its extent. A healthy human life withholds passages of weaker immune defense, since the extremes of age and pregnancy decrease the system’s force. In addition, many diseases and disorders impair the immune defense. Risk factors are e.g. organ transplantation, gastrointestinal surgery, autoimmune diseases, treatment with corticosteroids, blood cancers, and HIV. Further, the protective microflora can be out of order due to long-term antibiotic treatment. Fungi are normally outcompeted by commensal bacteria colonizing the respective niche in the body. If the equilibrium is destroyed, resistant bacteria and non-bacterial invaders are at chance [4]. Therefore, it seems fair to claim that advanced medical care paradoxically favors fungal infections to some extend - not only *Candida*.

As an archetypical opportunist, *C. albicans* causes the majority of all invasive fungal infections in humans, while simultaneously being carried by approximately every other human without doing any harm [70, 71].

When *C. albicans* infects the immuno-compromised, the spectrum of tissues and organs targeted is broad. Mucosal tissue in mouth and gut, lung, and nails can be infected. In systemic candidiasis, *Candida* smites kidneys, heart, and even the central nervous system [4]. Severe candidiasis obtained in a medical care unit has a 30-50% chance of being fatal [67, 72].

The most remarkable feature of *C. albicans* is to grow as round budding yeasts or as long filamentous hyphae. Both morphotypes are essential for virulence: The yeast is highly proliferative while hyphae are considered important for invasion and destruction of tissues [73-75]. Additionally,
C. albicans grows as pseudo-hyphae of which the contribution to pathogenicity is largely unknown. Primary patient samples contain usually a mixture of yeasts and filamentous fungal cells [4]. Not only amongst all fungal pathogens, but also amongst the Candida ssp., C. albicans is the dominating species [71]. Nevertheless, other Candida ssp. are on the rise – mostly due to their natural resistance to common antifungals. They are e.g. C. glabrata, C. kruzei or C. parapsilosis.

2.3 The mold Aspergillus nidulans and other Aspergillus ssp.

Unlike Candida ssp., Aspergillus ssp. are no natural colonizers of the human body. Nevertheless, contact with Aspergillus is inevitable, since spores are omnipresent in the environment worldwide. Aspergilli are molds, growing primarily on rotting biological material and spread as small and light spore forms via airborne routes. Spores, or conidia in Aspergillus, are dormant until certain environmental conditions (temperature, moisture) allow them to revive. Conidia swell, germinate to germlings, which grow further into somatic hyphae, and those eventually give rise to conidiophores that produce new conidia [4, 76]. The infectious capacity of Aspergillus ssp. is often described as an evolutionary accident [77]. The live style in rotting biological material with its occasional high temperatures might allow this mold to tolerate 37° C body temperature better than other pathogens [78]. On the contrary, Aspergilli favor carbon-rich substrates.

The primary infectious route of Aspergillus, due to airborne spread, is the respiratory tract, but conidia can enter the body also through wounds. Resulting diseases are allergic bronchopulmonary aspergillosis, invasive aspergillosis, and aspergilloma [79]. Invasive aspergillosis can be either chronical, e.g. in cystic fibrosis patients, or acute, which occurs e.g. due to neutropenia. Some Aspergillus ssp. produce mycotoxins, which are taken up in contaminated food and resist cooking and extremes in pH [4]. A. fumigatus is the most common causative agent of aspergillosis. Other pathogenic species are A. flavus, A. niger, and A. nidulans.
Even though *A. nidulans* is not remarkably important in other immunocompromised risk groups, including neutropenic patients, it is highly relevant for CGD patients. In these patients, following *A. fumigatus*, *A. nidulans* is the second most encountered fungal species. Interestingly, virulence of *A. nidulans* in CGD patients exceeds that of *A. fumigatus* [80]. Because comparative studies of both molds and their interplay with the CGD host are rare, it is not yet clearly understood how this shift of power occurs.

### 2.4 Fungal defense strategies against neutrophil killing

Microbes avoid neutrophil killing by directly interfering with neutrophil functionality on different levels: recognition and contact, phagocytosis, intracellular toxication, host cell survival, and NETosis [81]. Neutrophils enter the site of infection by following gradients of chemokines. There they sense pathogens by their PRRs (see 1.3). Therefore interfering with neutrophil recruitment will diminish the risk of being attacked by neutrophils. *Cryptococcus neoformans* secretes glucuroxylomannan, a component of its capsule, that reduces chemotaxis of neutrophils, even though microglia produce neutrophil-attracting IL-8 [82]. Capsule polysaccharides, like the secreted mannoprotein MP-4 of *C. neoformans* are also capable to trigger the shedding of L-selectin and the loss of the TNF-α receptor on neutrophils, rendering them less able to leave circulation and desensitized for this pro-inflammatory cytokine, respectively [83, 84]. The surface protein RodA, or hydrophobin, of *A. fumigatus* has a rather indirect effect on neutrophils: RodA forms the outer layer of conidia, and masks them for recognition by DCs or alveolar macrophages [76]. This leads to reduced recruitment of neutrophils *in vivo* [85]. *C. albicans* circumvents its detection by masking its β-glucan with mannoproteins [86].

The major strategy of most fungi to avoid phagocytosis is most probably the sheer size of some morphotypes, such as for instance long, filamentous hyphae (see 2.5) or titan cells [87]. In addition, fungi manipulate the function of the complement system. Host complement regulator Factor H is bound by the *C. albicans* protein Pra1p, a process normally used by the host to protect
itself from the complement system [88]. Similar observations were made for *A. fumigatus* conidia [89].

Once fully taken up into a phagocyte, fungi induce intracellular survival mechanisms. The knowledge about intracellular persistence strategies of fungi in neutrophils is limited – most research in the past focused on macrophages. Even though neutrophils and macrophages share similarities, differences predominate. Therefore, conclusions from macrophages should be applied to neutrophils with caution (see 1.2).

According to transcription studies conducted with *C. albicans*, carbon starvation and the subsequent induction of the glyoxylate metabolism seem to occur in both phagocytes, while amino acid starvation was only observed in neutrophils [90-93]. Then again, a strong response to oxidative stress was observed in both neutrophils and macrophages [91, 92, 94]. In neutrophils, the *Candida* response included the up-regulation of ROS-detoxifying superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidases (GPXs). Interestingly, a direct comparison of intracellular and extracellular stress response of neutrophil-encountering *C. albicans* demonstrated that the ROS stress response occurs inside and outside of the neutrophil. The RNS stress response on the contrary, was only observed in internalized *Candida* cells [93]. Additionally, there are species-specific responses from different fungal pathogens towards the attack of phagocytes. The transcriptional response of *C. neoformans* in macrophages, for instance, is characterized by a carbon starvation response and additionally by expression of extracellular lipases. However, it lacks the induction of the glyoxylate cycle [95]. An alternative strategy to endure starvation upon internalization is employed by *C. neoformans* and *C. glabrata*. They activate autophagy within the phagolysosome [96, 97]. Conidia of *A. fumigatus* exposed to human neutrophils reprogram their metabolism in a similar fashion as *C. albicans*. Conidia induce genes from the glyoxylate cycle, but also from fatty acid metabolism. Both, conidia and hyphae induce an oxidative stress response upon neutrophil encounter [98].
Interestingly, the cell surface protein RodA, present on *A. fumigatus* conidia, but absent on hyphae, serves to diminish a general immune recognition, rendering conidia nearly immunologically inert [99].

With all this said, it should be mentioned that most pathogenic fungi are indeed susceptible to neutrophil killing. Of all three fungi discussed above, only *C. neoformans* is able to resist neutrophil killing [81]. The escape of *C. albicans* from neutrophils via hyphal growth has only been observed in mouse, but not in human neutrophils [100].

### 2.5 Impact of fungal morphology during pathogenesis

Fungal morphology is very versatile. *C. albicans* can grow as budding yeast, as pseudohypha, or as a true hypha. The life cycle of *A. nidulans* includes conidia, hyphae, and even conidiophores [101]. The exact mechanisms responsible for these many “faces” are complex and a detailed discussion goes beyond the scope of this thesis. Nevertheless, different morphologies influence virulence greatly, and will therefore be shortly introduced here – with special emphasis on filamentous growth.

Yeasts are the harmless and proliferative, hyphae the harmful and invasive growth form of *C. albicans*. While tempting, this simplification needs to be expanded by a number of observations: First, not the one-way switch from yeast to hyphae, but the transition between all growth forms is necessary for full virulence. More than one *C. albicans* morphotype is usually isolated from primary patient samples or infected animals. Biofilms consist of heterogeneous *C. albicans* communities. Second, the host immune status affects the in vivo morphology profiles of *C. albicans*, so the proportions of the individual growth form. Third, *C. albicans* mutants unable to form hyphae in vitro might still form hyphae in vivo. Forth, the act of hyphal formation itself is not the only crucial factor for host damage and immune recognition. More important are the specific properties of the hypha, like hypha-specific adhesins or invasions, e.g. Hwp1p and Als3p, respectively, as well as the lack of β-glucan exposure on hyphae. Finally, a recent study proposed an addi-
tional gastro-intestinal *C. albicans* phenotype optimized for this particular niche, the GUT cell [4, 22, 101-104].

While *C. albicans* yeast and hyphae are easily “produced” *in vitro*, the generation of uniform pseudohyphae without buds or true hyphae is still challenging [101]. Consequently, very little is known about cell wall properties and immune recognition of this morphotype [22].

The life cycle of *Aspergillus* ssp. is not tightly bound to the human host. Still, the moist and warm environment of the body can wake up conidia that will then form hyphae. Even more than *C. albicans* yeasts, *Aspergillus* conidia stay under the radar of the immune system. Shielded by immunologically inert hydrophobin RodA, resting conidia are hardly detected by tissue-residing DCs or macrophages in the lung [76]. Only upon germination, fungal PRRs are eventually exposed and the germlings and hyphae are recognized and attacked [85, 99].

![Figure 3: Neutrophils engulfing and enwrapping of *C. albicans*](image)

Neutrophils engulf *C. albicans* yeasts successfully (left), but fail to do so with hyphae. Hyphae are enwrapped (right), preferentially beginning from the hypha end. Mother cells often stick out.

Fungal hyphae easily reach lengths that exceed the dimensions of phagocytes (Fig. 3). Even though neutrophils can stretch considerably, large hyphae cannot be fully engulfed. Interestingly, NETosis toward *A. fumigatus* was demonstrated to be more common towards hyphae than to resting and swollen conidia [99]. In line with this notion, a recent study including *C. albicans* suggested that NET formation occurs predominantly towards large pathogenic structures [60].

Due to their very different dimensions and the aforementioned differential surface composition, comparative studies of fungal morphotypes must be interpreted cautiously. A recently introduced dry mass adjustment for
C. albicans yeasts and hyphae proposes to infect not with the same number of fungal cells, but with the same dry mass – which eventually matches interacting fungal surface areas [105].
3. Nutritional immunity

The term ‘nutritional immunity’ was introduced in 1975 by Eugene D. Weinberg as he reviewed host mechanisms to withhold Fe from microbes during infection [106]. Since then, numerous studies have been conducted on this topic. In recent years, the field gained increasing interest. Searching the NCBI library for ‘nutritional immunity’ in spring 2015 results in little over 60 articles since 1975, out of which more than 65 % were younger than 5 years. The renaissance of nutritional immunity (and not just the term) is likely the result of improved detection methods, especially for metal elements, and the urgent need for new, non-antibiotic, strategies to fight microbial infections.

3.1 The third branch of the human immune system – major concepts

Originally, nutritional immunity denoted host-depletion of nutrients from invading pathogens. Nowadays, the perception has widened to the two-sided battle for valuable resources. Nutritional immunity refers to the transition metals Zn, Fe, Cu, and Mn. Metals are crucial, as they act as co-factors in enzymes and stabilize three-dimensional protein structures [107, 108]. Nutritional immunological actions occur in parallel to the innate and adaptive immune response, more tightly connected to the innate immune system. Consequently, nutritional immunity can be considered either part of the innate immune system, or alternatively as a third branch.

Every aspect of nutritional immunity, from the host or pathogen perspective, exemplifies the co-evolutionary nature of infections in a remarkable manner. Best studied is the fight for Fe, but most concepts apply for all transition metals in eukaryotes.[106, 109] In the host, Fe is mainly kept intracellular - free Fe is essentially unavailable in blood or other extracellular liquids. It is bound to transferrin in plasma and lactoferrin in external secretions. However, in order to grow within the host, microbial pathogens cope with these limitations. In case of direct contact between the host Fe source and the pathogen, host Fe-retaining proteins are modified to release their Fe load into the extracellular environment for uptake. Secondly, many bacteria and
fungi secrete siderophores, small molecules with exceptionally high Fe affinity that free Fe from host proteins followed by resorption into the pathogen’s cell. In response, mammals produce lipocalin-2, to intercept siderophores. This can in turn be counteracted by many pathogens which disguise their siderophores by glycosylation [109]. Since metal limitation is a dominant antimicrobial strategy, it is not surprising that some pathogens were found to be able to sequester and store higher levels of specific essential metal than their non-pathogenic relatives [110].

3.2 Zinc during fungal infections

Zn is a special element. Unlike other transition metals, Zn is environmentally very abundant, water-soluble at +II, and redox-stable. It is the strongest Lewis acid amongst the +II cations. Consequently, Zn is a co-factor for many catalytic reactions. Of all metal-containing enzymes, 9 % use Zn – so Zn is the second most abundant metal cofactor after Mg. Zn enzymes catalyze reactions from all classes of enzymes (EC 1-6), but the most abundant is EC 3, the hydrolases [107]. Further, Zn stabilizes Zn-finger domains, a function that is especially important in eukaryotic organisms. Bioinformatic approaches comparing genomes predict that approximately 7 % of all proteins in eukaryotes are Zn proteins, which is in the same range as in prokaryotes. Differences appear in the usage of Zn-finger proteins. In prokaryotes, only 0.2-0.4 % of the proteome are Zn-fingers. In eukaryotes, this increases to an average of 3 % [111]. Considering the connection of Zn availability and gene regulation, it is not surprising that a lack of Zn is potentially harmful, and leads to apoptosis – as demonstrated for mammalian cells [112]. Several studies have demonstrated the importance of Zn in pathogenic and non-pathogenic fungi. C. albicans is very sensitive to Zn starvation. Zn availability does not only affect morphogenesis, it can actually be the limiting factor if all other nutrients are available [113, 114]. A role in virulence is indicated by the relevance of Zn in biofilm formation, but also tolerance to intrinsic oxidative stress by the cytoplasmic Sod1p [115]. Sod1p is a Cu/Zn superoxide dismutase. Notably, the cell surface superoxide dismutases Sod4p and Sod6p
that are crucial to survive host-derived ROS, have Cu/Zn as their active site [116].
Zinc acquisition in fungal pathogens is mediated by a small number of proteins and is conserved in *C. albicans* and *A. fumigatus*. Zn enters the cytoplasm via Zrt plasma membrane transporter (CaZrt1p/AfZrfC, CaZrt2p/AfZrfB). From there, Zn can be transported further into the vacuole through ZnT transporters (Zrc1p/Cot1p). In mammalian cells, ZnT transporters are responsible for cellular Zn efflux. Zn can be mobilized from the vacuolar pool by Zrt exporters (CaZrt3p). Interestingly, no cellular Zn exporter has been described in fungi, yet. The homeostasis of Zn is regulated on two levels: the expression of Zrt importers, controlled by Zap1p in yeasts and Zaf1p in Aspergilli, and Zn import and export from subcellular organelles [117]. Vacuolar storage might be especially important during infection, since it allows proliferation even under Zn restricting conditions [118]. In addition, *C. albicans* uses a zincophore system – a secreted protein that sequesters Zn from host cells and re-associates with CaZrt1p to deliver Zn to the fungal cell: Pra1p [119]. The expression of Pra1p is repressed under acidic conditions, but induced under zinc starvation or at neutral/alkaline pH [119]. A similar zincophore system might exists in *A. fumigatus* [120]. Remarkably, Pra1p is not only beneficial for the fungus, it is actually immunogenic and immune-modulatory [120]. Amongst many functions, Pra1p can serve as a ligand to neutrophil integrin receptor αMβ2 and promote neutrophil killing [121]. Probably due to this “side effect”, Pra1p in not conserved in all pathogenic fungi.
As for other transition metals, the host restricts also Zn availability tightly. Zn concentrations are kept low in the extracellular milieu, the cytosol and the phagosome [117, 122]. Remarkably, zinc poisoning has been observed in the phagosome of *Mycobacterium*-infected macrophages [123].
There have now been attempts to disturb Zn homeostasis as an antifungal treatment [124].
3.3 Iron, copper, and manganese during fungal infections

The transition metals Fe, Cu, and Mn also act as co-factors in metalloproteins. The respective metal enzymes are less abundant than Zn enzymes, but still exceed those with elements like Co and Ni. Of all enzymes, 8 % contain Fe, 6 % contain Mn, and 1 % contains Cu. The dominant catalytic classes are oxidoreductases (EC 1) for Fe and Cu, and transferases for Mn (EC 2)[107]. Nevertheless, the general concepts of nutritional immunity apply to Fe, Cu, and Mn.

Fe uptake in pathogenic fungi is quite similar in C. albicans, C. neoformans, and A. fumigatus and occurs in two ways, reductive and the non-reductive [107, 125]. Briefly summarized, the high affinity reductive uptake system functions by the interplay of three enzymes. A reductase (Fre) converts ferric \( \text{Fe}^{3+} \) to ferrous \( \text{Fe}^{2+} \). The actual uptake is mediated by a permease (Ftr) and a multicopper ferroxidase (Fet). Probably due to gene expansion (and functional redundancy) of the reductases and multicopper oxidases, only the permease Ftr1 has been shown to be crucial in blood stream infections of C. albicans. Of note, the pigment melanin can reduce ferrous iron. In C. neoformans, melanin biosynthesis and Fe uptake are closely connected. In C. albicans, the retrieval of Fe from both host proteins, ferritin and transferrin, is dependent on the reductive pathway [126, 127].

Non-reductive Fe uptake is based on the import of Fe-containing proteins, namely hemoglobin and siderophores. C. albicans has receptors for heme (e.g. Rbt5p), but also for ferritin (reductive uptake). The ferritin receptor Als3p is expressed only by hyphae [127]. Siderophores are not produced by C. albicans, but they can be utilized as an Fe source [125]. The production, secretion, and uptake of siderophores plays an important role in A. fumigatus, and the reductive Ftr-based uptake system is less important for A. fumigatus virulence. A. fumigatus is also not able to retrieve Fe from heme [128]. The gut is probably the only site in the body where microbes encounter high Fe levels. Indeed, C. albicans is, as a colonizer of this niche, able to resist Fe toxicity. The transcriptional regulator Sfu1p represses Fe uptake, and represses the Fe-uptake and virulence regulator Hap43p. Sfu1
homologues exist in *C. neoformans* and *A. fumigatus* [128]. Finally, the vacuole was demonstrated to play a role in Fe homeostasis in *Saccharomyces cerevisiae*, which could indicate a similar system in pathogenic yeasts [128].

Fe uptake is amongst those processes that are modulated in the recently identified GUT morphology, a *C. albicans* phenotype specialized in gastrointestinal persistence [103]. One of the most important direct functions of Fe during host-pathogen interactions is likely the detoxification of H$_2$O$_2$ by the iron-dependent heme-enzyme catalase.

Uptake of Fe and Cu are very tightly connected and conserved in fungi. Most obviously, the ferroxidase (Fet) is actually a Cu-dependent enzyme, and the Fe reductases (Fre) are indeed ferric/cupric reductases that reduce Cu$^{2+}$ to Cu$^{+}$ through the oxidation of Fe$^{2+}$ to Fe$^{3+}$, and *vice-a-versa* [129]. Reduced Cu$^{+}$ is taken up by Cu transporters (Ctr). Ctr1p and Ctr3p are plasma membrane-localized, Ctr2p is vacuolar – and all three transport towards the cytosol. Interestingly, the number of *CTR* copies varies between fungal species and might affect virulence due to an effect on e.g. hyphal growth. Melanin formation in *C. neoformans* is Cu-dependent. The export of Cu from the cytosol occurs actively through the Golgi-located ATPase Ccc2p. A *C. neoformans* mutant lacking CCC2 was shown to be attenuated in virulence, likely due to the impaired melanin formation in this mutant [128]. The same mutation had no effect in *C. albicans*, a fungal species for which melanin production has not been reported. Ccc2 receives Cu from the Cu chaperone Atx1 [129]. Homologues of the Ccc2/Atx1 system exist in *A. fumigatus* [128]. During an infection, pathogens face Cu starvation as well as Cu intoxication. Cu “poisoning” was described as a mode of action against intraphagosomal *Mycobacteria* in macrophages [130]. Because of this, Cu export and Cu detoxifying metallothioneins are very important.

Metallothioneins are conserved in prokaryotes and eukaryotes. A potential role in virulence was demonstrated in *C. neoformans*, a known macrophage persister, but not yet in *C. albicans* and *A. fumigatus*. However, *C. albicans* is known to export excess Cu, indicating that fungi are primed to resist potential Cu poisoning *in vivo* [128]. At the same time, Cu is essential for pathogenic survival. Most fungal SODs, crucial to withstand superoxide
stress, have Cu in their active site. In *C. albicans*, SOD1, SOD4, and SOD6 are Cu/Zn-dependent, SOD5 was recently described to be a Cu-only SOD [131]. *C. albicans* extracellular SOD4 and SOD5 were demonstrated to detoxify superoxides produced during interactions with macrophages. Interestingly, secreted SODs from *A. fumigatus* are recognized by the human immune system [128].

Manganese plays a decisive in photosynthesis. In non-plant eukaryotes, its function is restricted to mitochondrial enzymes, including SOD2 and SOD3. Nevertheless, the human host restricts access to Mn by releasing calprotectin that is capable of binding Zn and Mn [132, 133]. The macrophage phagosome is actively depleted of Fe and Mn upon infection. Phagosomal Mn intoxication, as demonstrated for Cu and Zn, has not been observed [134]. Mn restriction is important during bacterial infections, especially since some bacterial pathogens have substituted Fe metalloproteins by Mn metalloproteins, but its role during fungal pathogenesis requires more investigation [132, 135, 136].
Methodological remarks

In the following, major remarks regarding the methods used in paper I, II, and III are briefly discussed.

Neutrophil isolation

In paper I, II and III presented in this thesis, neutrophils were isolated from human blood by Histopaque/Percoll purification. The resulting cell suspension of this protocol contains > 90 % neutrophils. Contaminating cells are granulocytes, mostly eosinophils, and T-cells. Monocytes and B-cells are consistently present with an abundancy < 0.1 %. The later is especially important for the expression profiling in paper III. Other white blood cells are very potent cytokine producing cells, and especially the discrimination of monocyte and neutrophil functionality crucial – due to their close relationship and yet striking differences.

Fungal strains

The A. nidulans strains used for the in vitro experiments presented in paper I is the original isolate derived from the CGD patient. The C. albicans strain SC5314 was used in the experiments of paper III. Originally a patient isolate in the 1970s, it is a widely used strain for in vitro and in vivo research. No virulence defects are known today [71].

Metal consistency

Amongst the trace elements, Zn is the environmentally most abundant. Due to this, special precautions were taken for quantitative analyses. Water was always taken directly from a Millipore water suspensor and stored in plastic vessels. Purchased media and buffers were used consistently from one batch throughout all experiments. Salts, acids, and bases were purchased as clean as possible (e.g. “TraceSELECT”). No glass ware was used – only plastic materials got in contact with the solutions. Finally, no autoclaved goods were
used, due to possible contamination via metal-enriched steam during this process.

**Synchrotron radiation X-ray fluorescence**

Synchrotron radiation X-ray fluorescence (SR-XRF) is a method originally utilized in material science. Nowadays, its applications are very versatile. Very simplified, a strong beam is pointed at a sample. In every atom, the electrons are excited to an extent where ionization occurs, so an electron is abstracted. This causes instability, which is compensated by other electrons “falling” on lower energy levels. As a result of this “fall”, energy is emitted. If the beam is strong enough, even the lowest orbitals are affected. Every element will emit energy specific for its electron profile. The beam strength and focusing, which is, in technical terms, by far the most challenging factor for the methodology, correlate with sensitivity. As of today, investigations of biological samples are only possible at synchrotron facilities. For the measurements of paper II, we used the Po6 beamline at DESY (Germany) and the ID22NI beamline at the ESRF (France). When conducting such an experiment, three essentials must be considered. Firstly, it is not possible yet to analyze living cells or wet samples. Attempts are made to allow the analyses of frozen samples, or cells in a liquid drop, but those are not technically matured enough. At the moment, the analysis of single cells with subcellular resolution is only possible in fixed and dehydrated samples. Secondly, the element integrity of the samples needs to be preserved as much as possible. This can be challenging when using biological samples. Finally, the number of samples is limited due to long measurement times (3-8 h per scan) and limited access to SR-XRF facilities (usually maximum one week at a time). Nevertheless, SR-XRF is the only method as of today that allows element mapping of single cells at this resolution.

**In vitro infections for RNA-sequencing**

The aim of the study presented in paper III was to elucidate the influence of *C. albicans* morphotypes on the interaction with neutrophils and NETs. To
do so, we complemented the assessment of pathogen amounts infecting host cells. We utilized the well-known multiplicity of infection (MOI) and additionally a dry mass correlation set up [105]. Using this correlation of dry mass and metabolic activity of *Candida*, we infected the neutrophils with MOI 1 of yeasts, and the same dry mass of hyphae. By doing so, the infectious load like e.g. the interacting surface, should be more comparable in the yeast and hypha infection.
**Aims**

Neutrophils are potent killers of fungal pathogens. Much is known about their antimicrobial weapon arsenal and the survival strategies of the fungal counterparts. At the same time, relatively little is known about the neutrophils’ potential to contribute to the events of ‘nutritional immunity’.

The aim of this thesis was therefore to investigate mechanisms of neutrophils to interfere with fungal metal homeostasis.

**Paper I**

Is the Zn chelator calprotectin, whose release from neutrophils is dependent on NETosis, a universal antifungal effector?

**Paper II**

Do neutrophils indeed have the potential to starve fungal pathogens from crucial trace element?

**Paper III**

Does the fight for trace element resources reflect in the transcription profile of *C. albicans* and neutrophils during infection? Do NETs actually affect metal homeostasis in *C. albicans*?
Results & Discussion

Paper I

**NET calprotectin is an effector during *A. nidulans* inhibition**

Infections with *A. nidulans* occur almost exclusively in CGD patients. As a very last course of treatment, the defective gene of the NADPH complex can be temporarily substituted with a functioning gene through gene therapy. Besides lacking the ability to produce sufficient superoxide, CGD neutrophils cannot undergo NETosis. After gene therapy, both functions were restored in the cured gp91 phox+ neutrophils. *A. nidulans* was sensitive to NETs derived from gp91 phox+ neutrophils [53].

![Figure 4: NET-calprotectin-induced growth inhibition of *A. nidulans*](image)

A+B: NETosis was triggered neutrophils isolated from peripheral blood by stimulation with PMA. To create NETs extracts, NETs were digested with MNase/DNAse and concentrated. NETs extracts were supplemented with an α-S100A9 antibody or an isotype control. Extracts were infected with *A. nidulans* conidia (A) or pre-grown hyphae (B). Fungal growth was scored by metabolic activity using XTT. C: Neutrophils from WT or S100A9−/− mice were triggered with PMA to undergo NET formation. NETs were infected with *A. nidulans* conidia or pre-grown hyphae. Fungal growth was scored by metabolic activity using XTT. [Re-produced from [137] with permission from Elsevier]
Earlier studies demonstrated that calprotectin is the dominant NET protein to inhibit *C. albicans* [38]. The sensitivity of *C. albicans* to calprotectin is long known [138]. We therefore aimed to analyze whether calprotectin was the key effector in NET inhibition of *A. nidulans* when NETs were derived from gp91 phox+ neutrophils.

And indeed, both growth forms of *A. nidulans*, conidia and hyphae, were inhibited in NET extracts and not affected if NET extracts were treated with an α-S100A9 antibody masking the protein (Fig. 4A + 4B). Further, conidia and hyphae were inhibited by NETs from C57BL/6 mouse neutrophils, but not or only fairly, by NETs from S100A9-/- mice (Fig. 4C).

**NET inhibition is reversible by Zn supplementation**

Calprotectin chelates Zn and this function is acknowledged as the major antifungal mode of action. *C. albicans* could be rescued from NET inhibition by the addition of Zn^{2+}[38]. Similarly, *A. nidulans* conidia and hyphae were not growth inhibited by gp91 phox+ NETs, if the infection was supplemented with additional Zn^{2+} (Fig. 5A + 5B). It should be noted that gp91 phox+ NETs were as efficient as control NETs from healthy donors throughout all experiments, while gp91 phox- NETs failed to inhibit *A. nidulans*. Considering the incapability of gp91 phox- the undergo NETosis, those “NETs” should be probably better referred to as long-term PMA-stimulated neutrophils. In spite of this, long-term PMA-stimulated gp91 phox- neutrophils did not have an inhibitory power towards *A. nidulans* either.

In conclusion, these *in vitro* experiments demonstrate the functionality of NET-associated calprotectin as an inhibitor of *A. nidulans* – by the chelation of Zn. This indicates a universal importance of calprotectin in NET-mediated inhibition of pathogenic fungi. Of note, NET formation was demonstrated *in vivo* towards *C. albicans* and *ex vivo* towards *A. fumigatus* [38, 99]. In an *Aspergillus* lung infection model, NET formation was also dependent on the function of the NADPH complex [62].
Figure 5: Rescue of fungal growth from NET inhibition by Zn

NETosis was triggered in neutrophils isolated from peripheral blood by stimulation with PMA. NETs were infected with *A. nidulans* conidia (A) or pre-grown hyphae (B) and supplemented with one µM Zn^{2+}. Fungal growth was scored by metabolic activity using XTT. [Re-produced from [137] with permission from Elsevier].
**Paper II**

*NETosis reduces Zn availability in the surrounding*

NETs inhibit fungal growth in a calprotectin-dependent manner and this is reversible by Zn supplementation. Calprotectin release occurs in neutrophils only during NETosis and significant proportions of calprotectin stay NET-bound [38]. At the same time, NET formation releases the entire intracellular content. This event is not necessarily favorable, since this includes also metal ions carefully kept inside the immune cell. It was therefore important to determine whether the process of NET formation actually reduces Zn availability for the fungal pathogen. To answer this, NETosis was induced in cell culture medium supplemented with increasing concentrations of Zn\(^{2+}\) and the resulting Zn concentration was measured before and after NETosis by ICP-MS. Indeed, NET release affected Zn in the NET supernatant (Fig. 6).

![Figure 6: NETosis-mediated reduction of zinc availability.](image)

NET formation was induced in neutrophils with PMA for 4 h in the presence of different Zn\(^{2+}\) concentrations. The remaining zinc concentration in protein-filtered supernatants was quantified by ICP-MS. Zinc-binding is saturated at higher concentrations at 62 % (grey dash line). Zinc reduction is stronger at concentrations below ca. 4-5 µM indicating specific binding (grey dotted line).

[Re-produced from [139] with permission from The Royal Society of Chemistry]
The strongest reduction was detected if the Zn concentration was below 3-6 µM. Here, only 0-55 % of unbound Zn remained. At higher concentration, the Zn reduction capacity reached saturation, but the remaining Zn never exceeded 62 % of the original concentration.

This indicates that available Zn can be diminished through NET formation by binding specifically to Zn-binding proteins, like calprotectin, but can also be loosely associated to negatively charged biomolecules, like e.g. DNA.

**NETs contain Fe, but not Zn**

NET formation is characterized by a stage during which granular, cytoplasmic and nuclear contents are allowed to mix. Since neutrophils are not *per se* Zn free, the low Zn content of NETs upon release was put to the test. NETosis was induced in low Zn cell culture medium and the resulting NETs were analyzed by SR-XRF (Fig. 7). In this *in vitro* set-up, NETs contained only Fe clearly co-localizing with their filamentous structure, while no Zn, Cu, or Mn were detected. Occasional hotspots of these four elements were most likely due to cell debris accumulations. Cell residues lining the circular area of the former cell are typical. They are visible in simple light microscopy, but also in the element maps.

**Neutrophils are exceptionally high in Fe**

At the site of infection, neutrophils that have phagocytosed a pathogen will eventually undergo apoptosis and be removed by other immune cells [18, 32]. While no neutrophil content is released in this process, NET formation, but also necrosis are characterized by the loss of plasma membrane integrity. Considering the high influx of neutrophils to the infection site, their presence could easily affect the local metal milieu. To avoid providing trace element nutrients to microbial pathogens, neutrophils might be exceptionally low in these elements. We analyzed neutrophil lysates and compared their metallome, full metal content, to other cells widely used in *in vitro* experiments: macrophage-like J774 cells and HeLa cells (Fig. 8).
Figure 7: Element distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of NETs Neutrophils were seeded on a Si$_3$N$_4$ membrane, stimulated with PMA for 4 h and subsequently freeze-dried. Image size: 100 μm x 100 μm, step size: 1 μm and 3 s scanning time/point. The RGB image represents a colored overlay of the elemental intensities corresponding to Ca (red), Zn (green) and Fe (blue). An optical image was taken at the beamline microscope (2500x magnification). Beamline: P06, DESY, Hamburg. [Re-produced from [139] with permission from The Royal Society of Chemistry]

Figure 8: Metallome comparison of neutrophils with J774 and HeLa cells Cultured J774 and HeLa cells were alkaline-lyzed and the resulting lysates were quantified by ICP-MS. The average content was related to the average content of neutrophils, n = 3, and normalized by cell volume. [Re-produced from [139] with permission from The Royal Society of Chemistry]
Our analyses revealed that J774 cells contain more Zn, Cu, and Mn, but less Fe than neutrophils. HeLa cells displayed higher intracellular abundance of Cu and Mn, and lower abundance of Fe. The Zn content was very similar to that of neutrophils. In other word, neutrophils were consistently lower in Mn, but higher in Fe. The Zn and Cu content was very similar to the other cells, indicating that neutrophils are not purposely low in Zn. Similar tendencies were observed when our results were compared to a comparable analysis conducted with numerous cell lines [140]. While the low Mn content can likely be explained by the low mitochondria count in neutrophils, containing e.g. Mn-SODs, the high Fe content is probably resulting from the enzyme MPO, which contains Fe-binding heme [13, 141].

**Neutrophil nucleus, granules and void vacuoles show characteristic element profiles**

SR-XRF allows the mapping of elements at subcellular level. When analyzing resting and activated neutrophils, typical morphological changes characteristic of NETosis were reflected in the element maps of these cells. In resting neutrophils, two major features stood out. The lobulated nucleus contained higher P, Ca, Zn, and Fe than the cytoplasm, while it contained lower density of S and Cu. Especially in the Fe map, subnuclear structures resembling potential nucleoli as well as eu- and heterochromatin were clearly recognizable. In the cytoplasm, Fe- and Zn-rich speckles were visible. Due to their size and partial co-localization of both elements, those speckles most likely represent granules. Expectedly, stimulation with PMA considerably affected neutrophil morphology (Fig. 9). Among the most striking features were appearing ‘void vacuoles’ – intracellular compartments that contained lower concentrations of Ca, Zn, Fe, Cu, and potentially Mn. General low abundance of the latter two elements impairs certainty though. Those ‘void vacuoles’ result from pinocytotic events and are therefore likely to resemble features of an early stage phagosome of engulfing neutrophils [142]. Morphological remodeling of the neutrophil nucleus – loss of lobules and changing size - could be observed in the element maps of activated neutrophils, even up to two-three h
post stimulation. Granular Fe/Zn speckles were detectable one h post infection, but nearly undetectable in later stages of NETosis, indicating the loss of granular membrane integrity. Since SR-XRF detects elements independent of their chemical status, we investigated the fate of labile Zn, also known as loosely-bound or associated Zn, by FluoZin-staining neutrophils and following them after PMA stimulation by life cell microscopy until they release NETs (ca. three h)*. Again, the nucleus and cytoplasm were distinguishable. Void vacuoles, low in labile Zn, appeared as well. Most interestingly, we observed Zn-rich small and round structures appearing that increased in number and intensity (i.e. concentration of Zn) over time. They stayed partially associated with the cell debris, even after NET release. Therefore might therefore contribute to the typical shape of cell residue after NET formation. NETs themselves were not detectable by FluoZin. Like in the SR-XRF analysis of NETs, NETosis was induced in a low-Zn cell culture medium, so NETs were likely not loaded with Zn upon release. Altogether, this data indicates that neutrophils aim to starve microbial pathogens, intra- and extracellularly. Further, the Zn-rich vesicles appearing in later stages of NETosis might contribute to an active removal in Zn from the cytoplasm, before neutrophils open up to release NETs.

* [www.rsc.org/suppdata/mt/c4/c4mt00346b/c4mt00346b1.avi](http://www.rsc.org/suppdata/mt/c4/c4mt00346b/c4mt00346b1.avi)
Figure 9: Element distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of two activated human neutrophils
Neutrophils were stimulated with PMA for 1 h, high pressure frozen, cryosubstitued in resin and sliced in 2 μm thin sections before deposition onto a Si₃N₄ wafer. Image area is 15 μm (hor.) x 11.2 μm (vert.); step size is 50 nm and 400 ms dwell time/point. The RGB image represents a colored overlay of the elemental intensities corresponding to Ca (red), Zn (green) and Fe (blue). The additional image is a corresponding TEM image from a similar but not exact same area of the grid (2500x magnification, lower right). Beamline: ID22NI, ESRF, Grenoble. [Reproduced from [139] with permission from The Royal Society of Chemistry]
Neutrophils are transcriptionally more active than widely believed. Their life cycle rather contains a low-transcription phase in circulation. In the tissue, stimulated miscellaneously, these phagocytes are capable of producing a number of cytokines - the ‘second transcriptional burst’. Effector proteins are produced during granulopoesis in the bone marrow [1, 18].

On these grounds, it was no surprise that most archetypical neutrophil effector genes were unaffected in an in vitro infection with C. albicans. Having a closer look, some effector genes were actually transcribed to remarkable extent, even in uninfected neutrophils. Most dominantly, the genes coding for the heterodimer calprotectin were very strongly transcribed (Tab. 3). Together, the transcription of only S100A8 and S100A9 corresponds to > 6% of the entire transcription (sum of rpkm). And indeed, calprotectin is known as the most abundant cytosolic protein in neutrophils – it constitutes 60% of total cytosolic protein. Other genes with high transcription rates are coding for components of the NADPH complex (NCF2, NCF4 and RAC2), extracellular proteases (LYZ and MMP9), and another S100 protein (S100A12). In the group with medium transcription, we detected genes for proteins of the NADPH complex (NCF1 and CYBA) and the heme-containing catalase (CAT). Genes transcribed with a low rate were coding for additional components of the NADPH complex (RAC1 and CYBB), the antimicrobial peptide cathelicidin (CAMP) and the alarmin amphoterin (HMGB1). No, or nearly no transcripts were detected for most granular proteins. Amongst those are e.g. genes for elastase, myeloperoxidase, lactotransferrin, defensins, or collagenase. In other words, the only granular proteins that are continuously transcribed in circulating neutrophils were lysozyme C and gelatinase B.
Table 3: Consistency in neutrophil effector protein transcription

Neutrophils were infected with *C. albicans* yeasts and hyphae. The transcription rate of typical neutrophil effector genes, reflected by the average rpkm of resting and infected cells, is displayed and ranked: ++++/dark grey = very high, +++/grey = high, ++/pale grey = normal, +/-light grey = low, -/white = not detected or irrelevant.

<table>
<thead>
<tr>
<th>gene</th>
<th>effector protein</th>
<th>average rpkm</th>
<th>rated</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A9</td>
<td>S100A9</td>
<td>17890</td>
<td>++++</td>
</tr>
<tr>
<td>S100A8</td>
<td>S100A8</td>
<td>4721</td>
<td>++++</td>
</tr>
<tr>
<td>NCF2</td>
<td>NADPH oxidase, p67</td>
<td>669</td>
<td>+++</td>
</tr>
<tr>
<td>LYZ</td>
<td>lysozyme C</td>
<td>435</td>
<td>+++</td>
</tr>
<tr>
<td>S100A12</td>
<td>S100A12</td>
<td>372</td>
<td>+++</td>
</tr>
<tr>
<td>NCF4</td>
<td>NADPH oxidase, p40</td>
<td>293</td>
<td>+++</td>
</tr>
<tr>
<td>MMP9</td>
<td>gelatinase B</td>
<td>258</td>
<td>+++</td>
</tr>
<tr>
<td>RAC2</td>
<td>NADPH oxidase, Rac2</td>
<td>236</td>
<td>+++</td>
</tr>
<tr>
<td>CYBA</td>
<td>NADPH oxidase, p22-PHOX</td>
<td>115</td>
<td>++</td>
</tr>
<tr>
<td>NCF1</td>
<td>NADPH oxidase, p47</td>
<td>62</td>
<td>++</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
<td>51</td>
<td>++</td>
</tr>
<tr>
<td>RAC1</td>
<td>NADPH oxidase, Rac1</td>
<td>25</td>
<td>+</td>
</tr>
<tr>
<td>CAMP</td>
<td>cathelicidin, LL-37</td>
<td>25</td>
<td>+</td>
</tr>
<tr>
<td>CYBB</td>
<td>NADPH oxidase, gp91-PHOX</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high-mobility group protein 1/amphoterin</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>TCN1</td>
<td>transcobalamin-1</td>
<td>4</td>
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<tr>
<td>CRISP3</td>
<td>cysteine-rich secretory protein-3</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>LTF</td>
<td>lactotransferrin</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>ELANE</td>
<td>neutrophil elastase</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>DEFA1B</td>
<td>defensin 1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>DEFA1</td>
<td>neutrophil defensin 1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>MMP8</td>
<td>neutrophil collagenase</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>CTSG</td>
<td>cathepsin G</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>PRTN3</td>
<td>proteinase 3</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>BPI</td>
<td>bactericidal/permeability-increasing protein</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>DEFA3</td>
<td>neutrophil defensin 3</td>
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<tr>
<td>DEFA4</td>
<td>neutrophil defensin 4</td>
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<tr>
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<td>defensin 5</td>
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</tr>
<tr>
<td>DEFA6</td>
<td>defensin 6</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
This observation correlates with the fact that gelatinase B is expressed very late in granulopoiesis, at the band cell stage and in the segmented neutrophil. MPO, elastase, defensins and lactotransferrin are produced at earlier stages [143].

**Neutrophil transcriptional response to C. albicans contains few metal retaining genes**

In paper III, we investigated the impact of the *C. albicans* morphotype on the interaction with neutrophils and NETs. In neutrophils infected with *Candida*, we identified a total of 318 DEGs throughout the course of infection. The majority was regulated similarly in response to yeasts or hyphae. Still, a group of 36 genes was identified to be induced or repressed stronger in the presence of *Candida* hyphae. We dissected the neutrophil transcription response with respect to genes that play a role in nutritional immunity. Since oxidative stress is very tightly connected to metal homeostasis, genes from both groups were considered. As described in paper III, the gene function was assigned using the online databases GeneCards and NCBI.

As stated earlier (see page 42), genes for antimicrobial effector proteins were either not transcribed or constitutively expressed at high levels. Remarkably, we identified a confined number of DEGs in *Candida*-infected neutrophils that are functionally related to nutritional immunity (Tab 4). Four induced DEGs are involved in the detoxification of ROS. They are namely the oxidative stress response 1 gene *OXSR1*, the glutathione peroxidase 1 gene *GPX1*, the oxLDL receptor gene *OLR1*, and the gene for the iron-sulfur cluster assembly 1 *ISCA1*. Especially the protein coded by *OLR1* is a very versatile protein. One of its functions is to bind oxidizes lipids resulting from intracellular ROS and mediate a cell response, but is also described to have a role in cell adhesion. The hemoglobin delta gene *HBD* was repressed in neutrophils infected with *C. albicans* hyphae. The coded protein is part of hemoglobin A2, which is the rarer form of hemoglobin A in adult humans. Other hemoglobin genes (*HBA1, HBA2, and HBB*) were transcribed at high levels, but not differentially expressed (data not shown). Interestingly, we found a Zn transporter gene to be induced in the response of neutrophils to *C. albicans*.
hyphae: *SLC30A6*. The gene product, the solute carrier family 30 member 6, transports Zn from the cytosol into the trans-Golgi network and vesicular compartments. The up-regulation of *SLC30A6* is especially interesting considering the findings from paper I and II. We showed that cytosolic calprotectin, which is released during NETosis, is the key effector protein during NET-mediated inhibition of *A. nidulans* confirming our earlier study with *C. albicans*.

**Table 4: Nutritional immunity DEGs in Candida-infected neutrophils**

Neutrophils were infected with *C. albicans* yeasts and hyphae. The fold change of transcription (log2) related to unstimulated neutrophils is displayed. Genes with a fold change above the threshold of 2 are highlighted with a grey background (DEGs). Fold changes excluded due to statistical uncertainties (cpm threshold) are not considered: n.c.

<table>
<thead>
<tr>
<th>Gene</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
<th>Me/Ox</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBD</td>
<td>0.06</td>
<td>-0.12</td>
<td>-0.07</td>
<td>-1.88</td>
<td>-2.58</td>
<td>-2.30</td>
<td>Fe</td>
</tr>
<tr>
<td>GPX1</td>
<td>0.03</td>
<td>0.37</td>
<td>1.59</td>
<td>-0.11</td>
<td>0.54</td>
<td>n.c.</td>
<td>Ox</td>
</tr>
<tr>
<td>ISCA1</td>
<td>-0.73</td>
<td>0.29</td>
<td>1.90</td>
<td>-0.74</td>
<td>0.47</td>
<td>1.83</td>
<td>Ox</td>
</tr>
<tr>
<td>OLR1</td>
<td>n.c.</td>
<td>n.c.</td>
<td>5.45</td>
<td>n.c.</td>
<td>n.c.</td>
<td>5.20</td>
<td>Ox</td>
</tr>
<tr>
<td>OXSR1</td>
<td>0.23</td>
<td>0.25</td>
<td>1.76</td>
<td>0.18</td>
<td>0.20</td>
<td>1.73</td>
<td>Ox</td>
</tr>
<tr>
<td>SLC30A6</td>
<td>n.c.</td>
<td>0.61</td>
<td>0.51</td>
<td>3.29</td>
<td>3.45</td>
<td>2.94</td>
<td>Zn</td>
</tr>
</tbody>
</table>

Both fungi were rescued from NET inhibition by supplementation of Zn [38, 137]. In paper II, the Zn landscape of resting and activated neutrophils was analyzed by XRF, ICP-MS and FluoZin-based life cell imaging. Interestingly, we identified vesicular compartments containing labile Zn in neutrophils upon stimulation with PMA. Further, we demonstrated the actual reduction of available Zn in the supernatant of NETs for the first time. Considering that the up-regulation of *SLC30A6* is a specific response towards hyphae of *C. albicans* and that NETs are preferentially released towards hyphae, this data suggests the following: Upon NET-inducing stimulation, neutrophils remove labile Zn from their cytosol via Zn transporters like the product of *SLC30A6* and store it in vesicular compartments, possibly zincosomes, to reduce Zn release during NETosis cell burst. In resting cells, free Zn is very limited. Yet during the major intracellular rearrangements that accompany NETosis, precaution might be necessary. For full functionality, it is crucial that calprotectin remains unsaturated with Zn before NET release. Only
then, the extracellular calprotectin can chelate Zn from the surroundings and possibly from cell residues, rendering it less available for microbial pathogens [60, 99, 137, 139, 144-146].

**C. albicans response to neutrophils and NETs includes metal acquisition genes**

The transcriptional response of *C. albicans* yeasts and hyphae was analyzed for genes whose products are likely to be involved in aspects of nutritional immunity (NI) - relevant during the interaction of the pathogen and the phagocyte. In paper III, we only considered genes with a fold change > 4 as DEGs. In the following, genes with a fold change > 2 will be discussed, too.

It is evident, that most of the NI genes affected are only differentially expressed in yeasts (Tab. 5). Among those, the genes of the *C. albicans* zincophore system were induced in yeasts very early in the infection of neutrophils (*PRA1, ZRT1*), but also when challenged with NETs. The orf19.6555 was only induced in neutrophil-encountering yeasts. Orthologues of this gene code for mitochondrial Zn transporters. In addition to Zn, Cu homeostasis was clearly affected in neutrophil-encountering yeasts. Ferric reductase genes, catalyzing the initial step of Cu uptake, were induced in yeasts (*FRE3, CFL11, orf19.7077*). *ATX1*, the gene for the intracellular Cu metallochaperone, is strongly repressed in yeasts. In the secretory Cu pathway of eukaryotic cells, Atx1p delivers Cu to ATPases of the trans-Golgi network to mediate export from the cytosol [129]. Two such ATPase genes were affected in neutrophil-challenged *Candida* yeasts: *CCC2* and *CRP1* were induced and repressed, respectively. Ccc2p is described as an ATPase pumping Cu into the Golgi network, while Crp1p is considered to pump Cu out of the cell. The metallothionein gene *CRD2* was induced in yeast, but repressed in hyphae. In summary, although Cu-related gene regulation is not an explicit reflection of a high or low Cu concentration, it certainly documents a disturbance in Cu homeostasis and oxidative stress, the latter being indicated by the presence of three different ferric reductases (personal correspondence with Prof. Simon Labbé, Université de Sherbrooke, Canada; and [147]).
A third important metal homeostasis is concerning Fe. Four related genes were differentially expressed in the infection. Three of those genes code for proteins involved in heme uptake and metabolism; they either were only induced in hyphae (PGA7) or repressed in yeasts (orf19.1034, HEM15). Interestingly, FTH1, the gene for a putative vacuolar Fe transporter, was induced only in hyphae, but not in yeasts. Finally, a number of oxidative stress detoxification genes were also identified. Most of them were induced in yeasts and hyphae (SOD3, SOD4, SOD5, GCS1, IFR1, and OYE32). Of note, IFR1 and OYE32 are described to be induced upon nitric oxide stress. TRP99 is the only potential ROS detoxifier gene repressed in neutrophil-challenged Candida yeasts.

Table 5: Nutritional immunity DEGs in neutrophil-challenged C. albicans

Neutrophils were infected with C. albicans yeasts and hyphae. The fold change of transcription (log2) related to Candida incubated in the same medium for the same time w/o neutrophils is displayed. Genes with a fold change above the threshold of 2^2 are highlighted with grey background (DEGs); genes with a fold change > 2^2 are highlighted in light grey.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change in yeasts (log2)</th>
<th>Fold change in hyphae (log2)</th>
<th>Me/Ox</th>
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<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>ATX1</td>
<td>-5.46</td>
<td>-5.56</td>
<td>-5.82</td>
</tr>
<tr>
<td>CCC2</td>
<td>2.68</td>
<td>2.74</td>
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<tr>
<td>CRD2</td>
<td>1.42</td>
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<td>2.85</td>
</tr>
<tr>
<td>CRP1</td>
<td>-4.76</td>
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<td>-5.54</td>
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<td>SOD5</td>
<td>2.25</td>
<td>2.61</td>
<td>1.84</td>
</tr>
<tr>
<td>CFL1</td>
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<td>4.70</td>
<td>5.87</td>
</tr>
<tr>
<td>FET3</td>
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<td>-1.19</td>
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<tr>
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<td>2.40</td>
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<td>1.12</td>
<td>2.53</td>
</tr>
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<td>2.53</td>
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<td>FTH1</td>
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<td>orf19.1034</td>
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<td>-2.66</td>
<td>-2.77</td>
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<tr>
<td>PGA7</td>
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<td>0.35</td>
<td>1.04</td>
</tr>
<tr>
<td>SOD3</td>
<td>1.75</td>
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</tr>
<tr>
<td>TRP99</td>
<td>-0.99</td>
<td>-1.51</td>
<td>-2.03</td>
</tr>
<tr>
<td>GCS1</td>
<td>0.81</td>
<td>1.36</td>
<td>2.09</td>
</tr>
<tr>
<td>IFR1</td>
<td>2.12</td>
<td>2.36</td>
<td>2.46</td>
</tr>
<tr>
<td>OYE32</td>
<td>2.56</td>
<td>2.06</td>
<td>1.69</td>
</tr>
<tr>
<td>orf19.6555</td>
<td>2.92</td>
<td>3.03</td>
<td>2.30</td>
</tr>
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<td>ZRT1</td>
<td>2.01</td>
<td>-0.04</td>
<td>0.34</td>
</tr>
</tbody>
</table>

neutrophils NETs neutrophils NETs
Of all genes presented, only a small group was differentially expressed in *Candida* challenged by NETs – none of those beyond the DEG threshold used in paper III (4-fold). Weakly induced in yeasts were oxidative stress response genes *IFR1* and *OYE32*, the zincophore genes *PRA1* and *ZRT1*, and the metallothionein gene *CRD2*. Slightly repressed in yeasts were the Golgi-ATPase gene *CCC2* and the glutathione synthesis gene *GCS1*. Of those, *IFR1* and *OYE32* were slightly induced in NETs-challenged hyphae. The heme acquisition gene *PGA7* was found to be slightly induced in NETs-triggered hyphae.

In summary, this transcription profile indicates that *C. albicans* encountering neutrophils and NETs faces not only oxidative stress, but also disturbances in metal homeostasis. The urge to acquire Zn is evident; the Cu and Fe homeostases need to be restored. Interestingly, NETs seem to trigger Zn acquisition and an oxidative stress response in *C. albicans* yeasts.
Conclusions & Outlook

Neutrophils can effectively kill *C. albicans* yeasts and hyphae, by either direct interaction or post mortem as NETs. Calprotectin was identified as the key effector protein in NETs, and Zn chelation is most probably the main mode of action [38, 81]. This indicates that neutrophils and in particular NETs control fungi by applying the armory of nutritional immunity. In the present thesis, the interaction of *C. albicans* with neutrophils and NETs was investigated by RNA-sequencing with respect to metal acquisition and transport. The trace element landscape of resting and activated neutrophils and NETs was analyzed by combining SR-XRF, ICP-MS, and life cell imaging. Finally, the universal antifungal potential of NET-associated calprotectin was confirmed for *A. nidulans*. In conclusion, the results of paper I, II, and III point to an important role of nutritional immunity aspects of the *Candida*-neutrophil interplay. Neutrophils show potential to starve fungi for metals in two ways: intracellularly after phagocytosis and extracellularly by NETosis (Fig. 10). Further, hints were collected indicating the formation and packing of intracellular zinc-retaining vesicles, possibly with the involvement of the zinc transporter SLC30A6, that could drain the cytoplasm of labile Zn before cells open up to release NETs. These vesicles might even stay intact after NETosis and keep Zn separated from microbes after the neutrophil lost integrity.

As of today, there are still many open questions that need to be addressed in order to support our model. What exactly are the Zn-rich vesicles appearing in stimulated neutrophils? Do they contain the transporter SLC30A6? Do the same events occur in neutrophils infected with *C. albicans* yeasts and hyphae? Do these vesicles appear during phagocytosis of fungi, or only during NETosis? Can phagocytosis and NETosis be separated, or do cells release NETs even after fungi engulfment of fungal cells?

The importance of Zn during neutrophil interaction with pathogenic fungi is supported by the findings of the papers included in this thesis. They shed
new light on the impact of ‘nutritional immunity’ during fungal infection, an emerging field of research.

**Figure 10: Model of intra- and extracellular Zn starvation by neutrophils**
During infection, neutrophils encounter *C. albicans* yeasts and hyphae (upper left, upper right). Yeast cell are preferentially engulfed into a low-Zn phagosome. Upon contact with hyphae, neutrophils can induce NETosis. During this, Zn-containing vesicles appear that potentially drain the cytoplasm from labile Zn. After NET release, Zn might be retained in vesicle-withholding cell debris, while NETs are decorated with calprotectin to sequester Zn from invading microbes.
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