Human Neutrophil Peptide: Diagnostic and Prognostic Properties in Critically Ill Patients

by

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Institute of Medical Science
University of Toronto

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Abstract

**Background:** Neutrophil activation is a hallmark of the inflammatory process and human neutrophil peptides (HNPs) are the most abundant proteins released from neutrophils. We hypothesized that HNP concentrations are associated with: (a) the presence of infection, (b) high blood neutrophil activity, (c) organ dysfunction and (d) mortality in critically ill patients.

**Methods:** Plasma samples, demographic, resuscitative and biochemical data were obtained from 100 patients admitted to the intensive care unit with acute physiology and chronic health evaluation II scores of 22–29. Nine healthy volunteers served as controls.

**Results:** Plasma HNP concentrations were higher in critically ill patients than in healthy controls. Organ dysfunction was more common in noninfected than in infected patients, specifically among cardiothoracic patients. Higher plasma HNP concentrations were associated with higher mortality.

**Summary:** The inflammatory process contributes to higher plasma HNP concentrations, which correlate with organ dysfunction and mortality in critically ill patients.
Acknowledgments

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I would also like to thank everyone at Zhang Laboratory for accommodating me as part of the team. My gratitude goes to Julie Khang and Bing Hang for their assistance and for sharing their technical expertise, which has been invaluable at numerous times during this project.

A special thanks goes to the Institute of Medical Sciences for their dedication in making our training comprehensive and valuable, and I am grateful to the faculty and students for their camaraderie, which helped make my journey a fulfilling and thought-provoking experience.

Last, but certainly not the least, a heartfelt thank you to my family and friends for being there in my times of need. I am extremely grateful for their love, enthusiasm, encouragement, and inspiration. Their unwavering support has been paramount in my success.
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Contributions

Julie Khang and Alice Luo assisted with ELISA experiments, namely plasma samples for HNP analysis.
Bing Hang helped prepare the MPO ELISA experiments.
Patricia Liaw’s group allowed the use of the DYNAMICS database for the collection of clinical data.
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<td>APACHE II</td>
<td>Acute Physiology and Chronic Health Evaluation II</td>
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<td>AUC</td>
<td>area under the ROC curve</td>
</tr>
<tr>
<td>BSI</td>
<td>bloodstream infection</td>
</tr>
<tr>
<td>CCL</td>
<td>chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CXCL13</td>
<td>C-X-C motif chemokine 13</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>FLU</td>
<td>fluorescence intensity</td>
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<tr>
<td>GNB</td>
<td>gram-negative bacterium</td>
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<tr>
<td>GPB</td>
<td>gram-positive bacterium</td>
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<td>HNP</td>
<td>human neutrophil peptide</td>
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<tr>
<td>HMGB1</td>
<td>high mobility group box B1</td>
</tr>
<tr>
<td>ICU</td>
<td>intensive care unit</td>
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<td>IgE</td>
<td>immunoglobulin-E</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>ISF</td>
<td>International Sepsis Forum</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MIP</td>
<td>macrophage inflammatory protein</td>
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<td>MODS</td>
<td>multiple organ dysfunction score</td>
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<td>MPO</td>
<td>myeloperoxidase</td>
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<td>NET</td>
<td>neutrophil extracellular trap</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PaO2/FIO2</td>
<td>ratio of arterial oxygen partial pressure to fractional inspired oxygen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operative characteristic</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>WBC</td>
<td>white blood cell</td>
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CHAPTER 1—LITERATURE REVIEW

1. SEPSIS

1.1 Background

1.1.1 Definition

The term sepsis [σηψις], which was initially used by Hippocrates (c.460–c.370 BC), originates from the Greek word sepsin, which means “to make putrid” or “decomposition of animal or vegetable organic matter in the presence of bacteria” (Geroulanos, 2006). Historically, sepsis has been a condition that is difficult to identify and diagnose. In 100 BC, the ancient Roman scholar Marcus Terentius Varro described it as follows: “small creatures, invisible to the eye, fill the atmosphere, and breathed through the nose cause dangerous diseases” (Martin, 2012). The Renaissance author Niccolo Machiavelli provided a clearer description, suggesting that hectic fever (now called sepsis) is difficult to recognize in its early stages at a time when the condition may be amenable to treatment but is more difficult to treat in its later, more obvious stages (Martin, 2012).

Sepsis is currently defined as a clinical syndrome that results from a dysregulated systemic inflammatory response [systemic inflammatory response syndrome (SIRS)] due to documented or suspected infection (Dellinger, 2013; Martin, 2012). The diagnostic criteria for sepsis are listed in Table 1.
Table 1. Diagnostic Criteria for Sepsis (Iskander, 2013) (Adapted from Journal of Physiol Rev, 2013, with permission).

However, it has also been recognized that sepsis can be present without positive blood cultures ("American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis," 1992).

Currently, according to generally accepted definitions, there are three clinical stages of sepsis: 1) sepsis; 2) severe sepsis, which includes sepsis with the dysfunction of a least one organ or organ system; and 3) septic shock, which is severe sepsis with hypotension ("American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis," 1992; Dellinger, 2013).

Figure 1. Three Clinical Stages of Sepsis. Septicemia is a spectrum ranging from sepsis to severe sepsis and septic shock (Lever, 2007) (Adapted from BMJ, 2007 with permission).
1.1.2 Epidemiology

Over the last decade, the incidence of sepsis has increased with a consequent rise in the number of hospitalizations (Kumar et al., 2011). In fact, the incidence of sepsis per 100,000 people increased from 83 in 1979 to 240 in 2000 (Martin, Mannino, Eaton, & Moss, 2003). In the face of antimicrobial resistance, a growing elderly population, and the wider use of immunosuppressive therapies, the worldwide incidence of sepsis can be expected to continue to rise (Iskander, 2013).

A nationwide cohort of 6,555,621 hospitalized adults in the United States revealed that 4.3% had been diagnosed with sepsis and 10.9% had been diagnosed or were suspected to have sepsis (i.e., infection and acute organ failure). The total in-hospital mortality rate was 21.9%. From these data, the in-hospital mortality related to sepsis was estimated to be 34.7% for diagnosed sepsis. Moreover, it was found that sepsis was involved in one of every two to three deaths in hospitals (Liu et al., 2014) (Wang, Devereaux, Yealy, Safford, & Howard, 2010) found that the national age-adjusted sepsis mortality was 65.9 deaths per 100,000 people.

In summary, sepsis remains the leading cause of death in patients hospitalized in intensive care units (ICUs), with mortality rates of 50% at 1 year (Drifte, Dunn-Siegrist, Tissieres, & Pugin, 2013) and 74% at 5 years (Weycker, Akhras, Edelsberg, Angus, & Oster, 2003; Wheeler & Bernard, 1999). A systematic review studying long-term mortality from sepsis with a follow-up ranging from 3 months to 10 years confirmed these results (Winters, 2010).
Figure 2. Mortality Levels Over Time. Data from 23 studies providing at least two mortality time points (Winters, 2010) (Crit Care Med 2010, adapted with permission).

Sepsis will continue to have a negative impact on the population. Early recognition and aggressive intervention are critical to reduce the associated morbidity and mortality (Dellinger, 2013; Rivers et al., 2001).
CHAPTER 2—INFLAMMATION

2. INFLAMMATION

2.1 Inflammation Definition and Types

2.1.1 Definition

The word inflammation is derived from the Latin “īnflammō,” which means “ignite; set alight.” The inflammatory process involves the removal and repair of damaged tissue and/or the neutralization of harmful agents (Fritz, Ferrero, Philpott, & Girardin, 2006; Maslinska & Gajewski, 1998). In 40 AD, the Roman doctor Celsus, in his treatise De Medicina, defined inflammation for the first time (Majno, 1975; Majno & Joris, 2004; R, 2010). He outlined that inflammation presents with five cardinal signs: rubor et tumor cum calore et dolore or redness and swelling with heat and pain. The fifth sign is functio laesa, or loss of function (Majno, 1975; Majno & Joris, 2004; R, 2010).

Inflammation is an adaptive response triggered by noxious stimuli, such as infection and tissue injury, which pose a threat to tissue homeostasis (Kumar, 2003; Majno, 2004; Nathan, 2002). The primary consequence of the inflammatory response is clearance of the insult with resolution and restoration of tissue homeostasis (Freire & Van Dyke, 2013). A controlled inflammatory response is beneficial to the host, but it can become detrimental if dysregulated. The inflammatory response to microorganisms is central to the pathogenesis of sepsis (Cohen, 2002; Drifte et al., 2013). Regardless of the cause, inflammation presumably evolved as an adaptive response for restoring homeostasis (Medzhitov, 2008).

2.1.2 Types of Inflammation

Acute inflammation is the initial response of the body to an injurious stimulus. This process is relatively short-lived, being initiated within minutes to hours after injury and lasting for a period of a few days. Neutrophils play a predominant role in acute inflammation (Maslinska & Gajewski, 1998; Medzhitov, 2008).
Conversely, chronic inflammation is prolonged, and it can last for weeks to months or even years. It is caused by a continual inflammatory stimulus and is characterized by persistent tissue destruction and repair, proliferation of blood vessels, and fibrosis. The main types of cells that migrate to areas of chronic inflammation are monocytes and lymphocytes (Fujiwara & Kobayashi, 2005). Chronic, uncontrolled inflammation is a hallmark of various human diseases, including cardiovascular and autoimmune diseases. Diseases associated with uncontrolled acute inflammation are characterized by a lack of activation of resolution programs (Freire & Van Dyke, 2013).

Figure 3. Different Types of Inflammatory Responses. The upper figure presents the events associated with the acute inflammatory response and resolution of the inflammatory response, and the lower figure illustrates the ongoing inflammatory response that persists in chronic inflammation (Serhan et al., 2007) (Journal FASEB, 2007, adapted with permission).
The focus of this thesis is acute inflammation, and as such, this process will be described in more detail.

Acute inflammation is generally triggered by infection or tissue injury (Majno & Joris, 2004), and the process involves the rapid recruitment of blood components (plasma and granulocytes: neutrophils, eosinophils, and basophils) to the site of infection or injury (Majno, 1975; Majno & Joris, 2004; Medzhitov, 2008). The migration of granulocytes to inflammatory loci is a necessary requirement for the neutralization and removal of deleterious agents (Medzhitov, 2008; Serhan et al., 2007). The relative contribution of these cell types is dependent on a number of factors, including the location of the inflammatory site, the nature of the insult, and the genetic background of the host. Resolution of inflammation is perceived to occur via the elimination of granulocytes and the eventual return of tissue mononuclear cell (i.e., macrophages and lymphocytes) numbers to basal levels (Serhan et al., 2007).

Figure 4. Cellular and Molecular Changes Caused by Inflammatory Insults. Top left figure demonstrates the infiltration of polymorphonuclear neutrophils (PMNs) into tissues. Top right figure illustrates the infiltration of monocytes/macrophages into tissues. (Serhan et al., 2007) (Adapted from FASEB journal, 2007, adapted with permission).
In acute inflammation, the early response is characterized by the extravascular accumulation of polymorphonuclear neutrophils (PMNs) and edema formation. Later, during the inflammatory response, mononuclear cells and macrophages accumulate and help prepare the tissue for resolution. The initial recognition of infection is mediated by tissue-resident macrophages and mast cells through pattern recognition receptors, namely Toll-like receptors (TLRs). This in turn leads to the production of a variety of inflammatory mediators, including chemokines, cytokines, vasoactive amines, eicosanoids, and products of proteolytic cascades (Medzhitov, 2008).

The most immediate effect of these mediators is to elicit an inflammatory exudate locally. Plasma proteins and leukocytes (mainly neutrophils) that are normally restricted to the blood vessels gain access, through the postcapillary venules, to the extravascular tissues at the site of infection or injury. The activated endothelium of the blood vessels allows selective extravasation of neutrophils while preventing the exit of erythrocytes. This selective extravasation is mediated by the interaction of endothelial cell selectins with integrins and chemokine receptors on leukocytes (Pober & Sessa, 2007; Tirouvanziam et al., 2008).
Figure 5. The Inflammatory Pathway. The image depicts the inflammatory cascade starting with inducers that induce mediators to be released, which act on target tissues (Medzhitov, 2010) (Cell, 2010, adapted with permission).

2.1.3 Resolution of Acute Inflammation

During the early phases of inflammation, tissue-resident cells sense damage and begin to release signals that promote rapid neutrophil accumulation and delayed monocyte emigration. The resolution of inflammation is critical to the restoration of normal tissue homeostasis. Resolution is initiated by an active class switch from mediators such as prostaglandins and leukotrienes to the production of immunoresolvents. Endogenous lipid mediators, including resolvins, protectins, lipoxins, and maresins, are biosynthesized during the resolution phase of acute inflammation (Banneberg, 2010). In the resolution phase, neutrophils become apoptotic, thus secreting mediators that inhibit continued neutrophil infiltration. The ingestion of apoptotic neutrophils changes the phenotype of macrophages toward a resolution-phase phenotype, which promotes a return to tissue homeostasis (Ortega-Gomez, Perretti, & Soehnlein, 2013).
2.2 Cells Involved in Acute Inflammation

2.2.1 Monocytes/Macrophages

Monocytes comprise a subset of circulating white blood cells (WBCs) in the blood, bone marrow, and spleen, constituting approximately 10% of the total leukocytes in humans. They can remain in the circulation for up to 1–2 days, after which they are removed if they have not been recruited to a tissue to help with defending from an invading...
microorganism or damage. Monocytes originate from hematopoietic stem cells in the bone marrow and develop through a series of sequential differentiation stages (Hettinger et al., 2013; Italiani & Boraschi, 2014). They can further differentiate into a range of tissue macrophages and dendritic cells (DCs) (Auffray, Sieweke, & Geissmann, 2009).

Monocytes have different roles during homeostasis, immune defense/inflammation, and tissue repair in terms of their capacity to become activated and secrete inflammatory cytokines in response to different stimuli (Italiani & Boraschi, 2014). They have the capacity to mediate the host antimicrobial defense (Serbina, Jia, Hohl, & Pamer, 2008). In fact, monocytes are key players during inflammation and pathogen challenge, and they are also implicated in many inflammatory diseases, including atherosclerosis (S, 2011).

Monocyte numbers increase substantially in inflammatory conditions. During inflammation, circulating monocytes leave the bloodstream and migrate into tissues to the site of inflammation (Cotran & Mayadas-Norton, 1998; Issekutz, 1995; Shi C. & Pamer, 2011). Under inflammatory states, they are conditioned by local growth factors, pro-inflammatory cytokines, microbial products, and granulocyte-macrophage colony stimulating factor, which enables them to differentiate into macrophages or DCs (Burgess & Metcalf, 1980; Gasson, 1991; Shi C. & Pamer, 2011). Monocyte infiltration into the inflammatory site is highly regulated by gradients of different chemotactic factors, including growth factors, pro-inflammatory cytokines such as interleukin-8 (IL-8), and chemokines such as macrophage inflammatory protein (MIP)-1a and the regulated on activation normal T cell expressed and secreted protein) (Italiani & Boraschi, 2014; Ortega-Gomez et al., 2013; Wetzler, Kampfer, Stallmeyer, Pfeilschifter, & Frank, 2000).

Macrophages possess a striking functional and phenotypic plasticity that becomes apparent during the resolution phase of inflammation. Upon apoptotic cell efferocytosis, macrophages deactivate the production of pro-inflammatory cytokines and lipid mediators and launch an anti-inflammatory transcriptional program characterized by the release of IL-10 and transforming growth factor-beta (TGF-β) (Fadok et al., 1998; Ortega-Gomez et al., 2013, Figure 2D). Resolution-phase macrophages are rich in molecules important for antigen processing and presentation, and they secrete T and B
cell chemoattractants [chemokine (c-motif) ligand, chemokine (C-C motif) ligand (CCL) 5, and C-X-C motif chemokine 13 (CXCL13)]. In addition, resolution-phase macrophages express T cell immunoglobulin mucin protein 4 and TGF-β, key molecules in the clearance of inflammatory cells and the return to tissue homeostasis. The functional characterization of resolving macrophages revealed lower levels of cluster of differentiation molecule 11b, an enhanced capacity to engulf dead neutrophils, and a reduced responsiveness to TLR4 ligands, possibly leading to a “satiated” state with their ultimate departure through the lymph (Schif-Zuck et al., 2011).

A chemokine scavenging-independent role for the chemokine receptor D6 in promoting macrophage-mediated resolution has also been studied. The D6 chemokine receptor controls macrophages and cytokine secretion during the resolution of inflammation. It was found that D6-deficient macrophages display a defect in conversion to resolution-phase macrophages. Moreover, D6-deficient macrophages engulfed higher numbers of apoptotic PMNs in vivo (1.6-fold increase) and secreted higher amounts of TNF-α, CCL3, and CCL5 ex vivo than their wild-type counterparts. In addition, D6 was found to be expressed on apoptotic neutrophils from healthy humans and rodents (Pashover-Schallinger et al., 2012). Moreover, the immune silencing of lipopolysaccharide (LPS)-stimulated macrophages following their incubation with senescent PMNs ex vivo (in terms of TNF-a, IL-1b, and CCL5 secretion) was diminished by 50–65% when D6−/− PMNs were present. Accordingly, the adhesive responses induced by macrophage interactions with senescent PMNs were reduced in D6-deficient PMNs (Pashover-Schallinger et al., 2012).

The resolution of acute inflammation is accompanied by the apoptotic death of inflammatory PMNs, followed by their clearance by macrophages. In turn, resolution-phase macrophages exhibit reduced pro-inflammatory cytokine production, termed immune silencing.
Figure 7. The Activation and Interaction of Neutrophils with Macrophages. Interactions shown are at the site of infection or inflammation (Kumar, 2010) (Adapted from International Immunopharmacology, 2010, with permission).

2.2.2 Mast Cells

Mast cells were first observed in the 19th century by von Recklinghausen, and they are best known for their involvement in immunoglobulin-E (IgE)-mediated allergic reactions.
However, they are also involved in the maintenance of homeostasis and regulation of the immune response. In fact, the roles of mast cells in regulating immune responses include both the innate and acquired immune systems (Frenzel & Hermine, 2013; Moon, 2010).

Mast cell proliferation is regulated via stem cell factor by several cytokines and chemokines (Frenzel & Hermine, 2013). Overactivation of this regulatory mechanism can result in abnormal mast cell accumulation and activation in various tissues. Generally, mast cells are found in vascularized tissues such as the mucous membranes and epithelia, being particularly abundant in the nervous systems and at sites exposed to the environment, including the skin and the respiratory and gastrointestinal tracts (Frenzel & Hermine, 2013). Mast cells influence cell-mediated responses either directly by interacting with T cells or indirectly via the production of cytokines such as CCLs, CXCLs, and certain pro-inflammatory cytokines. The produced IgE activates the mast cells, and this in turn contributes to the activation, proliferation, and migration of DCs (the main antigen-presenting cells), thus regulating the immune response (Abraham, 2010; Frenzel & Hermine, 2013; Galli, 2005). Moreover, MIP-1a and MIP-1b activate monocytes, macrophages, natural killer (NK) cells, and immature DCs. Interferon-gamma released from neutrophils also promotes the activation of macrophages. Myeloperoxidase (MPO) released from neutrophils binds to macrophage mannose receptors expressed on macrophages, leading to the activation of the pro-inflammatory functions of macrophages, which in turn increases neutrophil survival (Kumar, 2010).

TLRs and the dectin-1 receptor on the mast cell surface directly recognize a number of pathogens, inducing bacterial, viral, and parasitic peptides such as pathogen-associated molecular patterns, leading to the production of pro-inflammatory cytokines such as TNF-alpha and IL-6 (Marshall, 2004). This mechanism perpetuates the inflammatory response, most notably by activating neutrophils and mast cells. When mast cells become activated, they release various cytokines contained in the mast cell granules in a process known as degranulation. As illustrated in Figure 9, when mast cells become activated, they release cytokines, further perpetuating inflammatory and immune responses. The main molecules released are histamine, serotonin, proteases (tryptase), lipid mediators
(prostaglandins), cytokines, chemokines, and reactive oxygen species (ROS). These molecules act at various sites; specifically, they increase the permeability of epithelia and endothelia and stimulate angiogenesis, induce smooth muscle contractions, increase the neuronal production of neuropeptides, and stimulate fibroblasts to produce collagen (Frenzel & Hermine, 2013). This mechanism perpetuates the inflammatory response, most notably by activating neutrophils.

Figure 8. Schematic Outline of the Dual Roles of Mast Cells. The dual roles of mast cells are to maintain homeostasis and regulate the inflammatory response (Frenzel & Hermine, 2013) (Adapted from Joint Bone Spine, 2013, with permission).

2.2.3 Neutrophils

Neutrophils, or PMNs, are the “front-line” defenders during acute inflammation (Kolaczkowska & Kubes, 2013; Kumar, 2010; Mantovni, 2011; Serhan et al., 2007). Neutrophils are continuously generated in the bone marrow from myeloid precursors. In a normal adult, their daily production can reach $2 \times 10^{11}$ cells, as controlled by granulocyte...
colony stimulating factor (G-CSF). G-CSF is a glycoprotein that influences the survival, proliferation, differentiation, and function of mature neutrophil granulocytes and their precursors (Lieschke et al., 1994). During infection, G-CSF becomes essential for tuning the production of neutrophils to meet increased demand. However, the overall production of neutrophils is largely regulated by the rate of apoptosis of neutrophils in tissues. During inflammation, the number of neutrophils in tissues increases, and over time, these cells die via apoptosis followed by their removal by macrophages and DCs (Borregaard, 2010).

In humans, 50–70% of circulating leukocytes are neutrophils (Kolaczkowska & Kubes, 2013). They are the main cells that are recruited to the site of injury during inflammation and are indispensable for defense against injurious stimuli, including intruding microorganisms (Borregaard, 2010; Fujiwara & Kobayashi, 2005; Serhan et al., 2007; Smith, 1994). In the circulation, mature neutrophils have an average diameter of 7–10 μm, their nuclei are segmented, and their cytoplasm is enriched with granules and secretory vesicles (Borregaard, 2010).

Different types of neutrophil granules are formed consecutively during their maturation, and they are filled with pro-inflammatory proteins (Borregaard, 2010; Galli, Borregaard, & Wynn, 2011; Hager, Cowland, & Borregaard, 2010; Kumar, 2010). The protein constituents of different granules are defined by the timing of their biosynthesis during neutrophil differentiation. Importantly, granule contents are also released according to a hierarchy, with secretory granules being the most readily exocytosed and azurophilic granules only undergoing partial exocytosis, as illustrated in Figure 9 (Pham, 2006). There are three main types of neutrophil granules. Azurophilic (primary) granules contain MPO, defensins, and proteinases. Specific (secondary) granules include proteins such as lactoferrin and lysozyme, and gelatinase (tertiary) granules contain leukolysin and matrix metalloproteinase 9 (also known as gelatinase B). Azurophilic (peroxidase-positive) and specific (peroxidase-negative) granules can be further subdivided. In humans, azurophilic granules can be differentiated into defensin-high and defensin-low subtypes (Faurschou, Sorensen, Johnsen, Askaa, & Borregaard, 2002).
Figure 9. Neutrophil Granule Content. Each neutrophil granule contains different enzymes and proteins (Pham, 2006) (Adapted from Nat Rev Immunol., 2006, with permission).

The various granule subtypes of human neutrophils are formed sequentially during myeloid cell differentiation, and they differ in their propensity for exocytosis. As a rule, granules formed at late stages of myelopoiesis have higher secretory potential than those formed in more immature myeloid cells (Faurschou et al., 2002). Azurophilic (peroxidase-positive) granules are the first to appear, and they are traditionally defined by their MPO content. They are formed at the promyelocyte stage of neutrophil development. Neutrophils contain four closely related alpha-defensins, which are stored in a subset of azurophilic granules. These defensin-rich azurophilic granules are formed later than defensin-poor azurophilic granules, near the time of the promyelocyte-myelocyte transition (Bainton, 1971; Borregaard, Sehested, Nielsen, Sengelov, & Kjeldsen, 1995).

Peroxidase-negative granules are formed in myelocytes, metamyelocytes, band cells, and segmented neutrophils (Bainton, Ullyot, & Farquhar, 1971; Borregaard et al., 1995), and they can be divided into two subsets that occur successively: specific granules, identified by a high content of lactoferrin, and gelatinase granules, identified by the presence of gelatinase (Kjeldsen, Bainton, Sengelov, & Borregaard, 1993).
Figure 10. The Stages of Granulopoiesis for Neutrophils. The figure illustrates the time of synthesis of selected proteins, transcription factors, and microRNAs known to regulate granulopoiesis. The myeloblast (MB) matures into the promyelocyte (PM), which is transformed into a myelocyte (MC) and then matures into a metamyelocyte (MM), band cell (BC), and finally into a segmented neutrophil (SC) (LF: lactoferrin) (Hager et al., 2010) (Adapted from J of Intern Med, 2010, with permission).

Neutrophils can eliminate pathogens by multiple means, both intracellular and extracellular. When neutrophils encounter microorganisms, they phagocytose them. After they are encapsulated in phagosomes, the pathogens are killed using NADPH oxygenase-dependent mechanisms (ROS) or antibacterial proteins (cathepsins, defensins, lactoferrin, and lysozyme) (Borregaard, 2010; Hager et al., 2010). These antibacterial proteins are released from neutrophil granules into either phagosomes or the extracellular milieu, thus acting on intracellular or extracellular pathogens, respectively.

Neutrophils may extend their antimicrobial activity beyond the life of the neutrophil. The formation of neutrophil extracellular traps (NETs) is an alternative to death via necrosis or apoptosis. Highly activated neutrophils can eliminate extracellular microorganisms by releasing NETs, which are composed of decondensed DNA and proteins from the
cytosol, from granules (that disintegrated at the same time that the nuclei dissolve) and chromatin (histones) (Brinkmann et al., 2004). NETs immobilize pathogens, thus preventing them from spreading but also facilitating subsequent phagocytosis. They are also thought to directly kill pathogens via antimicrobial histones and proteases (Papayannopoulos & Zychlinsky, 2009; Phillipson & Kubes, 2011).

2.2.3.1 Neutrophils in Inflammation

Neutrophils are well recognized as major players during acute inflammation (Kolaczkowska & Kubes, 2013). They are the first non-resident cells recruited to the site of injury, arriving in less than 1 h. Following infection, the localization of neutrophils to
the site of inflammation is crucial for clearance of the infection.

Neutrophils are efficient phagocytes that engulf and degrade microorganisms using a combination of oxidative and non-oxidative mechanisms. Once inside the neutrophils, microorganisms are sequestered in a specialized compartment called the phagolysosome (Pham, 2006). The phagocytosis of microorganisms activates the membrane-bound NADPH oxidase system. This system generates large quantities of ROS that are released into the phagolysosome, which constitutes the oxidative arm of the microbicidal action of neutrophils (Pham, 2006). Traditionally, ROS are considered responsible for the direct killing of microorganisms. Sequestration of microorganisms intracellularly also induces the fusion of neutrophil granules with the phagolysosome and the release of antimicrobial peptides and proteases from the granules into the phagolysosome. The action of these antimicrobial peptides and proteases constitutes the non-oxidative arm of the microbicidal action of neutrophils. Therefore, neutrophils contain an array of cytotoxic agents that are ready for mobilization in response to invading microorganisms (Pham, 2006).

Neutrophils contain granules and carry easily mobilized secretory vesicles that can rapidly transport their content to the cell surface, where the vesicle proteins are incorporated into the surface membrane (Borregaard, Christensen, Bejerrum, Birgens, & Clemmensen, 1990). Thus, upon neutrophil activation, secretory vesicles first transport molecules that are required for cell adhesion (for example, beta-2 integrins) to the neutrophil surface. Then, gelatinase granules deliver proteases that can digest the basement membrane and/or extracellular matrix, perhaps allowing for neutrophil transmigration (Hager et al., 2010).
2.2.3.2 Killing Mechanisms

Neutrophils employ both intracellular and extracellular mechanisms using phagocytosis when encountered by pathogens. After they are encapsulated in phagosomes, neutrophils kill the pathogens using NADPH oxygenase-dependent mechanisms, ROS, or antibacterial proteins (cathepsins, defensins, lactoferrin, and lysozyme) (Borregaard, 2010; Pham, 2006). As discussed previously, antibacterial proteins are released from neutrophil granules into either phagosomes or extracellular milieu, thus acting on either intracellular or extracellular pathogens, respectively. Highly activated neutrophils can eliminate extracellular microorganisms by releasing NETs. NETs immobilize pathogens, thus preventing them from spreading but also facilitating the subsequent phagocytosis of trapped microorganisms. They are also thought to directly kill pathogens by means of antimicrobial histones and proteases (Papayannopoulos & Zychlinsky, 2009).

Neutrophils also contain a large number of agents with the capacity to injure tissue (Haslett, Savill, & Meagher, 1989) and promote inflammation by degrading matrix proteins into chemotactic fragments (Vartio, Seppa, & Vaheri, 1981). Once activated, neutrophils are stimulated to produce large amounts of cytotoxic molecules such as ROS and reactive nitrogen species (RNS) (Cohen, 2002). The principle role of these molecules is to destroy invading pathogens; however, in a hyper-inflammatory response, the amount of these cytotoxic molecules released can also cause damage to surrounding tissues, and if sufficient damage occurs, organ failure and death may ensue (Kobayashi et al., 2003; Savill, 1997).

2.2.3.3 Turnover of Neutrophils

The production of neutrophils is extensive in the steady state, with $1 \times 10^{11} - 2 \times 10^{11}$ cells generated per day in a normal adult (Borregaard, 2010). It has been estimated that $1 \times 10^9 - 1 \times 10^{11}$ neutrophils are released into the circulation from the bone marrow on a daily basis (Hong et al., 2012). To maintain homeostasis, an equivalent number of senescent neutrophils must be removed from the circulation. Apoptotic or aged...
neutrophils are cleared primarily by resident tissue macrophages in the liver, spleen, and bone marrow (Furze & Rankin, 2008; Shi J., Gilbert, Kokubo, & Ohashi, 2001). Normal neutrophil turnover in humans is mediated by apoptosis (Whyte, Meagher, MacDermot, & Haslett, 1993), a process that presumably downregulates pro-inflammatory capacity and microbicidal function and prepares these cells for removal from tissues by macrophages (Savill, 1997). Removal of neutrophils by apoptosis is an essential phase in the normal resolution of the inflammatory response, as it prevents damage to healthy tissues that would otherwise occur following necrotic cell lysis. In addition to normal turnover, phagocytosis initiates a molecular cascade of events that results in the accelerated induction of apoptosis in human PMNs (Kobayashi & DeLeo, 2003). Thus, apoptosis likely represents the terminal stage of inflammation initiated by neutrophil activation.

Neutrophils have shorter life spans than do macrophages and mast cells, and unlike macrophages and mast cells, neutrophils are released into the blood as mature or nearly mature cells devoid of proliferative potential (Galli et al., 2011). In healthy individuals, neutrophils have a short half-life, which usually does not exceed 12 h and normally ranges 1.5–8 h in the circulation (approximately 1.5 h in mice and 8 h in humans) (Basu, Hodgson, Katz, & Dunn, 2002; Pillay et al., 2010; Suratt et al., 2001).

However, under inflammatory conditions, neutrophils become activated, and their longevity increases by several fold, which ensures the presence of primed neutrophils at the site of inflammation (Summers et al., 2010). The estimated time that neutrophils spend in the circulation increases by 10-fold from 5–10 h to 5.4 days (Pillay et al., 2010).

When encountering an inflammatory stimulus, apoptosis is avoided for 24 h or longer (Lee, Whyte, & Haslett, 1993). It is thought that a longer lifespan may allow neutrophils to perform more complex activities, including resolution of inflammation or shaping adaptive immune responses, but their persistence in tissues may lead to bystander cell injury (Kolaczkowska & Kubes, 2013). Thus, in instances in which there is an excess of inflammatory stimuli, prolongation of the neutrophil lifespan can contribute greatly to the morbidity (and possible mortality) associated with inflammation (Haslett et al., 1989;
Savill et al., 1989; Savill, 1997). A prospective multicenter observational study found that the percentage of neutrophil apoptosis was significantly decreased at 24 h, 5 days, and 12 days after the diagnosis of septic shock (14.8 ± 13.4, 13.4 ± 8.4, and 15.4 ± 12.8%, respectively; all P < 0.0001) compared with the control group (37.6 ± 12.8%) (Tamayo et al., 2012). Apoptosis provides a mechanism for the clearance of unwanted cells in a variety of situations in which programmed or physiological cell death occurs (Lee et al., 1993). It is through this mechanism that neutrophils can maintain a homeostatic balance.

2.2.3.4 The Death of Neutrophils

In physiological conditions, it is thought that neutrophils are mainly cleared from the circulation in the liver, spleen, and bone marrow (Hong et al., 2012; Shi J. et al., 2001). Increased CXC-chemokine receptor 4 expression is observed in aged neutrophils, and this is believed to help direct them back to the bone marrow, where they are then eliminated (Kolaczkowska & Kubes, 2013). Neutrophils can also die in the vasculature, after which they are removed by Kupffer cells (liver-resident macrophages) that live immobilized in the liver vasculature. This applies to both senescent neutrophils and neutrophils that die after fighting infection (Shi J. et al., 2001). Finally, a recent aspect of neutrophil death has described neutrophils as having the ability to degrade their nuclear contents and release them as NETs (Brinkmann et al., 2004; Yipp et al., 2012).

2.3 Defensins/Human Neutrophil Peptides (HNPs)

2.3.1 Definition and Characteristics

Defensins comprise a family of small, cysteine-rich antimicrobial peptides found in vertebrae, plants, fungi, myxobacteria, and invertebrates (Belarmino, Capriles, Crovella, Dardene, & Benko-Iseppon, 2010; Gerdol, De Moro, Manfrin, Venier, & Pallavicini, 2012; Isogai et al., 2011; Lehrer & Lu, 2012; Mygind et al., 2005; Zhu, 2008). These cationic and amphipathic peptides contain 18–45 amino acid residues and have a characteristic beta-sheet-rich fold and a framework of six disulfide-linked peptides. They comprise three subfamilies, termed alpha-, beta-, and delta-defensins. Each subfamily has
a conserved motif that includes six cysteine residues that form three intramolecular disulfide bonds with a characteristic pattern of pairing (Ganz & Lehrer, 1995; Lehrer & Lu, 2012).

These peptides are abundant in phagocytes in the small intestinal mucosa of humans and other mammals and in the hemolymph of insects (Lehrer & Lu, 2012). They contribute to host defense against microbes, and they may participate in tissue inflammation and endocrine regulation during infection (Ganz & Lehrer, 1995; Lehrer & Lu, 2012). They are known to be part of the innate immune system, the role of which is to directly neutralize invading microbes (Hazlett & Wu, 2011).

2.3.2 Types and Functions of HNPs

HNP-1, HNP-2, HNP-3, and HNP-4, also known as alpha-defensins, are constitutively produced by myeloid precursor cells, and their production can be induced by activated CD8 T cells (Oppenheim, 2003; Yang, Biragyn, Hoover, Lubkowski, & Oppenheim, 2004; Zhang L. et al., 2002). When induced to degranulate, neutrophils release these alpha-defensins locally (Oppenheim, 2003). Alpha-defensins are stored in neutrophil (PMN) azurophilic granules and to a lesser extent in macrophages. They comprise 30–50% of the protein content in primary granules and 5–7% of the total protein content in PMNs (Ganz & Lehrer, 1994; Ganz et al., 1985; Ihi, Nakazato, Mukae, & Matsukura, 1997; Rice et al., 1987; Selsted, 1985). The highest concentrations of defensins (>10 mg/mL) are found in granules, specifically in the storage organelles of leukocytes (Ganz et al., 1985; Ganz, 1987).

Alpha-defensins play an important role in innate immunity for host defense (Selsted, 1985), as they exert broad antimicrobial activities (Ganz, 2004; Selsted, 1985). HNPs are found predominantly in cells and tissues involved in host defense against microbial infections, and as such, they can be released into the extracellular milieu following PMN activation as a consequence of degranulation, leakage, cell death, and lysis during inflammation (Ganz, 1987).
Figure 12. Synthesis and Packaging of Alpha-defensins. Once matured in the bone marrow, neutrophils cease granule synthesis and release alpha-defensins in the blood, after which they enter tissues. During phagocytosis (the target microorganism is represented as a black sphere), defensin-rich primary granules (red) fuse with phagocytic vacuoles, in which they generate high concentrations of defensins (Ganz, 2003) (Adapted from Nature Reviews, Immunology, 2003, with permission).

2.3.3 Interactions between Neutrophils and HNPs

Neutrophils are the predominant phagocytes in blood. Neutrophils move to infectious sites or respond to chemotactic substances that emanate from inflammatory sites. The first alpha-defensins were purified from human PMNs (Selsted, 1985). Chemotactic substances are generated by invading bacteria (for example, formylmethionyl peptides) (Lehrer, Ganz, Selsted, Babior, & Curnutte, 1988). Macrophages or neutrophils at the site of invasion also generate chemotactic substances. Neutrophils can detect minute differences in the occupancy of chemotactic receptors on different parts of its cell surface, integrate this information, and translate it into directional movement (Lehrer, Ganz, Selsted, et al., 1988). In fact, chemokine gradients and adhesion molecules expressed on neutrophils play central roles in regulating neutrophil release from the bone marrow (Lerman & Kim, 2015).

HNPs are found in azurophilic granules, and they represent 5–7% of the total cellular protein in human neutrophils. HNPs are constituents of granule extracts that were lysosomal cationic proteins (Lehrer, Ganz, Selsted, et al., 1988).
2.3.4 Interactions between Inflammatory Diseases and HNP Concentrations

The normal plasma levels of HNPs range from undetectable to 50–100 ng/mL. At the onset of infection, mean HNP levels are 2–4-fold greater than those in healthy volunteers (Ihi et al., 1997). Similarly, it has been found that during sepsis, HNP levels in the plasma of patients ranges 900–170,000 ng/mL (Panyutich, Panyutich, Krapivin, Baturevich, & Ganz, 1993). Moreover, an excellent correlation was found between the concentration of HNPs and the number of PMNs in the blood of patients with inflammatory diseases (Panyutich et al., 1993). The elevated levels of HNPs in inflammatory diseases suggest that HNPs play a critical role in the leukocyte-dominant pro-inflammatory responses that may contribute to several inflammatory disorders, including cardiovascular disorders (Nassar et al., 2007; Panyutich et al., 1993).

Examining the distribution of HNP in inflammatory processes more closely, it has been found that HNPs are abundant in and around intimal and medial smooth muscle cells within human atherosclerotic carotid and coronary arteries (Higazi et al., 1997). Furthermore, a significant correlation was revealed between HNP skin deposition and the severity of coronary artery disease, as evaluated by the number of blood vessels associated with focal lesions and stenosis (Nassar et al., 2007). HNPs released from activated PMNs in the circulation may reflect the acute inflammatory phase, whereas the tissue deposition of HNPs as a biomarker may indicate an accumulative inflammatory contribution to atherosclerosis.

2.3.5 Effects of Vasopressors on Leukocyte Activation/Degranulation

The use of catecholamine affects leukocyte activity. Several neurotransmitters have distinct and varying functions on different leukocyte subsets. Different studies have indicated that norepinephrine and other adrenergic agonists can modulate many aspects of the immune response (initiative, proliferative and effector phases), altering the production of and cellular responses to cytokines, lymphocyte proliferation, antibody secretion, and inflammatory gene expression (Feinstein et al., 2002; Madden, Sanders, & Felten, 1995; Szelenyi, 2001). Studies have illustrated that catecholamines predominantly affect NK cell and granulocyte circulation. Characteristically, two phases are recognized
after catecholamine administration: the rapid (approximately 30 min) mobilization of lymphocytes, followed by an increase in granulocyte numbers with decreasing lymphocyte numbers. Norepinephrine is locally released from sympathetic nerve cells and disseminated systemically after release from the adrenal gland (Strell et al., 2009). Studies uncovered that norepinephrine administration further increases circulating catecholamine levels, often to a degree that far exceeds physiologic levels (Beloeil, Mazoit, Benhamou, & Duranteau, 2005).

Exogenous catecholamines modulate the phagocytosis process in that they enhance peripheral blood neutrophil phagocytic function (Ortega, Marchena, Garcia, Barriga, & Rodriguez, 2005), and exogenous norepinephrine suppresses wound neutrophil phagocytic function. Moreover, alterations in neutrophil phagocytosis following sympathectomy (Campagnolo, Bartlett, Keller, Sanchez, & Oza, 1997; Derevenco et al., 1992; Zhang P., Summer, Bagby, & Nelson, 2000. In all cases, the phagocytic function of peripheral blood neutrophils was assessed and compared between sympathectomized subjects and untreated controls, and a decrease in phagocytosis by neutrophils in the sympathectomy group was observed.

2.3.6 Effects of Vasopressors on Plasma Lactate

Lactate is a three-carbon compound formed from pyruvate by pyruvate dehydrogenase in the cytosol of cells (Grip et al., 2015). Under stress, its metabolic importance increases, as it has been identified as the main substrate used for gluconeogenesis (Meyer et al., 2003). Plasma lactate is frequently used as a biomarker in various clinical settings, and elevated plasma levels are associated with poor prognosis (Grip et al., 2015).

During normal physiological conditions, lactate production occurs at an estimated rate of 1500 mmol per day. Its production during critical illness is extremely variable, with an estimated lactate production of approximately 1 mmol/min during low-dose adrenaline infusion (Gjedsted et al., 2011). According to the literature, plasma lactate concentrations increase in response to vasopressor use. In fact, a study by Grip et al. revealed that plasma lactate concentrations increased approximately 4-fold in response to the
adrenaline challenge. It is thought that adrenaline generates hyperlactatemia (Laurent, Petersen, Russell, Cline, & Shulman, 1998), possibly via the stimulation of muscle lactate release.

Hyperlactatemia has important clinical implications, as a high lactate level is a strong predictor of mortality in the critically ill (Khosravani, Shahpori, Stelfox, Kirkpatrick, & Laupland, 2009).

2.4 Biomarkers

2.4.1 Definition of a Biomarker

A “biomarker” or “biological marker” is an objective biochemical or physiologic variable that can be measured accurately and reproducibly (Strimbu & Tavel, 2010). The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Pletcher & Pignone, 2011).

Figure 13. Capabilities of Biomarkers. Biomarkers have various capabilities, as outlined in the figure (Mayeux, 2004) (Adapted from NeuroRx, 2004, with permission).
Biomarkers can inform clinical practice by providing quantitative data regarding a pathophysiological mechanism that can be used for the early diagnosis of a specific disease, to monitor and guide treatment, or to predict the risk of death or other adverse events (Balmelli, Drexler, & Mueller, 2011). Research has illustrated that the utility of biomarkers requires accounting for their performance and also estimating the downstream health consequences of having the biomarker information (Aronson, 2005; Pletcher & Pignone, 2011). Whether a biomarker is adopted as a useful tool is influenced by its ability to lead to an alteration in the course of a disease (Aronson, 2005), its ability to allow intervention before the onset of symptoms, or its ability to detect early or prodromal disease states (Mayeux, 2004). The clinical utility of a biomarker increased with increasing strength of the link between the information provided by the biomarker and the immediate clinical course of action that physicians take in response (Balmelli et al., 2011).

![Disease Pathway](image)

**Figure 14. Disease Pathway and Potential Impact of Biomarkers.** Biomarkers can be used to identify risk factors, screen and diagnose patients, and influence prognosis (Mayeux, 2004) (Adapted from NeuroRx, 2004, with permission).
2.4.2 Types of Biomarkers

Biomarkers can be classified into two main categories: diagnostic and prognostic. A diagnostic (screening) biomarker is used to detect and identify a given disease in an individual. These types of markers are expected to have high sensitivity and specificity. For example, an elevated troponin level in serum provides information useful for diagnosing acute myocardial infarction. These types of biomarkers have the ability to direct therapies. A prognostic biomarker is a marker that predicts the course of action of a disease and therefore potentially influences therapy. An ideal biomarker is one that is easily and reliably measured and is readily available with high sensitivity and specificity.

2.4.3 Biomarkers for Sepsis

To be clinically useful, a sepsis biomarker needs to provide information additional to that already available from established clinical assessments (e.g., history and examination) and investigations. For example, it must accurately differentiate bacterial and other causes of SIRS and be available in a timely and cost-effective manner. The utility of a biomarker is further enhanced if it can indicate the severity of infection and can guide effective therapeutic intervention (Kibe, Adams, & Barlow, 2011). A biomarker of sepsis might be used to diagnose infection, but it might also be used to identify some aspect of the host response that is amenable to intervention.

Current biomarkers used in the diagnosis of sepsis include biomarkers such as procalcitonin [PCT] and High Mobility Group Box 1 [HMGB1]. PCT has been studied in critical care patients, both as a diagnostic and prognostic biomarker, and for its ability to aid antibiotic stewardship by safely shortening the length of an antibiotic course (Kibe et al., 2011). In fact, it is used in the early detection of sepsis and effective treatment. Similarly, HMGB1 levels are elevated in most patients with severe sepsis (Sunden-Cullberg et al., 2005). However, correlations between HMGB1 levels and levels of organ dysfunction have been inconsistent (Karlsson et al., 2008). For this reason, it is believed that HMGB1 levels do not offer any helpful prognostic information regarding survival (Karlsson et al., 2008). An effective diagnostic and prognostic biomarker for patients with sepsis has yet to become available.
CHAPTER 3—RATIONALE AND OBJECTIVES

3.1 Rationale and Hypotheses

Rationale: All critically ill patients have some degree of systemic inflammatory response. Given that there are key players involved in the activation and promotion of the inflammatory response which in turn can impact on its course, it would be helpful to find a molecule which could change overall clinical outcome. This prompted us to examine whether there are steps in which the inflammatory response can be modified or inhibited? Moreover specifically we wanted to examine whether there was a biomarker that can help direct clinicians in modifying the inflammatory response and overall prognosis?

3.1.1 Hypothesis: We hypothesized that high plasma HNP concentrations are associated (1) with the presence of infection, (2) with plasma neutrophil activity, (3) with perfusion and organ dysfunction, and (4) with mortality.

3.1.1.1 Objective 1: To determine whether plasma HNP concentrations are associated with the presence of infection

3.1.2.1 Objective 2: To determine whether plasma HNP concentrations are associated with plasma neutrophil activity

3.1.3.1 Objective 3: To determine whether plasma HNP concentrations are associated with perfusion and organ dysfunction

3.1.4.1 Objective 4: To determine whether plasma HNP concentrations are associated with mortality
CHAPTER 4—STUDY METHODS

4.1 Patient Recruitment and Data Collection

4.1.1 Patient Recruitment in the DYNAMICS Study

The DYNAMICS study was a multicenter, prospective, observational study of critically ill septic and nonseptic patients (Fox-Robichaud, 2012). A description of the inclusion and exclusion criteria as well as the primary and secondary outcomes is provided as follows.

A. Inclusion criteria (Appendix A): The nonseptic group included patients who were critically ill and who had been admitted to the ICU with one of the following diagnoses: trauma, cardiogenic shock, neurosurgical-related diagnosis (aneurysms, subarachnoid, intracranial, or subdural hemorrhage), or other causes of shock (e.g., pulmonary embolism).

B. The primary outcome: ICU mortality.

C. The secondary outcomes: (1) the temporal relationships between plasma DNA levels and other markers of inflammation and blood clotting, and (2) the temporal relationships between plasma DNA levels and clinical parameters [e.g., sequential organ failure assessment and multiple organ dysfunction score (MODS), interventions, use of blood products and plasma expanders].

D. Nine different ICUs participated in the study. These included Hamilton Health Sciences, St. Joseph’s Health Centre in Hamilton, London Health Sciences Centre in London, Ontario, Laval Hospital in Quebec, Ottawa General Hospital, Ottawa Civic Hospital, St. Paul’s Hospital in Vancouver, Calgary Foothills Hospital, and St. Michael’s Hospital in Toronto.

E. Dedicated research coordinators from each site were responsible for daily screening of patients admitted to the adult ICUs. These research coordinators were responsible for obtaining consent from patients or substitute decision makers, collecting clinical data, and processing the blood samples.

F. The case report forms from the study contained information such as patient demographics, confirmed or suspected site of infection, comorbidities, acute physiology
and chronic health evaluation (APACHE) II score, MODS, fluid resuscitation data, antimicrobial and pharmacological co-interventions (e.g., steroid therapy), and outcomes (e.g., discharge status or primary cause of death).

G. Exclusion criteria: <18 years of age, pregnancy, or breastfeeding.

Once patients were recruited, blood samples were drawn for the first 7 days. If the patient expired or if the patient was transferred out of the ICU, the collection of blood was discontinued.

4.1.2 Study Population and Sample Size

Initially, a pilot study was performed using 10 samples classified as septic and 10 samples classified as nonseptic obtained at baseline (baseline was defined as within 24 hours of meeting the inclusion criteria) from the DYNAMICS study patient pool. HNP values are summarized in Tables 2 for the presence of absence of infection.

<table>
<thead>
<tr>
<th>Infection</th>
<th>N Obs</th>
<th>N</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>no</td>
<td>5</td>
<td>5</td>
<td>9325.6</td>
<td>3379.8</td>
<td>3571.5</td>
<td>11943.2</td>
</tr>
<tr>
<td>yes</td>
<td>15</td>
<td>15</td>
<td>4284.7</td>
<td>3266.6</td>
<td>528.3</td>
<td>10478.2</td>
</tr>
</tbody>
</table>

Table 2. Summary of HNP by Infection Group.

Logistic regression and ROC analysis was performed on the pilot data to get an estimate of AUC value.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNP values (unit=1000)</td>
<td>0.631 (0.409, 0.974)</td>
<td>0.867</td>
</tr>
</tbody>
</table>

Note: Odds Ratio was estimated using HNP increasing by 1000.

Table 3. Univariate logistic regression results for HNP predicting infection=yes.
4.1.3 Sample Size Calculation

With an AUC estimate of 0.87, sample size was calculated to identify a CI (confidence interval) width of 0.1. A random sample of 200 (100 infected + 100 non-infected) produced a two-sided 95% confidence interval with a width of 0.1 when the sample AUC is 0.87. A random sample of 100 (50 infected + 50 non-infected) produced a two-sided 95% confidence interval with a width of 0.143 when the sample AUC is 0.87.

We therefore chose 100 patients as the study’s main population.

4.1.4 Selection of Healthy Controls

All healthy subjects were recruited by Li Ka Shing laboratory staff. Ethics approval was obtained from the St. Michael’s Hospital research ethics board (see Appendix B: Blood Collection from Healthy Controls). Both healthy male and female volunteers aged 18 years or older were screened. For the pilot study, healthy subjects were not age-matched, but for the main study, efforts were made and preference was given to age-matched healthy subjects who met the inclusion and exclusion criteria for the main study. Subjects needed to be fasting for a minimum of 6 hours and abstain from the consumption of caffeine for at least 6 hours prior to blood collection. Non-eligible candidates included active smokers, subjects who were coagulopathic, those who had an active medical condition, and those who had received chemotherapy or immunosuppressive therapy within the last 4 weeks. Subjects were also deemed ineligible if they had the following on both arms: rashes, gauze dressings, casts, edema, paralysis, open sores or wounds, sclerosed or occluded veins, allergies to cleansing reagents, and burned or scarred tissue.

4.2 Data Collection and Study Design

This study was an observational retrospective study. Data were obtained from the DYNAMICS study database. The DYNAMICS study had a total of 784 patients, including 404 patients with sepsis and 380 nonseptic patients. Patients in the DYNAMICS were recruited according to whether they had or were suspected of having
sepsis, severe sepsis, septic shock, multiple trauma, cardiogenic shock, neurosurgery, aneurysms, or other causes of shock. Patients 18 years or younger as well as pregnant or breastfeeding women were excluded from the study.

In total, 100 of the total 784 patients in the DYNAMICS study were recruited into our study. Fifty patients identified as septic and 50 patients identified as nonseptic (outlined in Appendix A) were randomly selected according to APACHE II scores ranging from 22 to 29. For each patient, we collected APACHE II scores, age, gender, admission data, study recruitment data, microbiological information, resuscitative data, blood work, and patient outcome.
Figure 15. Study Algorithm. Study samples were initially taken from the DYNAMICS study. Demographic, biochemical, and hemodynamic data was collected for the patients selected.

4.2.1 Categorization of Patients

In the DYNAMICS study, patients had been classified as septic or nonseptic according to the criteria outlined in Appendix A. Of the entire pool of patients in the DYNAMICS study (404 septic patients and 380 nonseptic ICU patients), we selected 100 patients (50 patients from the septic arm and 50 patients from the nonseptic arm) with APACHE scores between 22 and 29 for our study. These 100 patients were further stratified
according to the presence or absence of infection using more standardized criteria, as outlined in the International Sepsis Forum (ISF) Definition of Infection in the ICU Consensus Conference (Calandra, Cohen, & International Sepsis Forum Definition of Infection in the Intensive Care Unit, 2005). Data for blood cultures, urine culture, peritoneal fluid, or chest X-rays taken within the first 24 h were obtained, and using the following criteria, and it was noted whether patients tested positive or negative for infection. All patients with positive findings were classified as infection-positive. Nine healthy controls were also included in our study.

The most common infections were identified, including the following:

(1) Pneumonia

The criteria used to diagnose pneumonia was derived from the ISF Definition of Infection in the ICU based on one of the three categories: (a) microbiologically confirmed or definite, (b) probable, and (c) possible (Calandra et al., 2005).

According to the ISF Definition of Infection, (a) microbiologically confirmed diagnosis is defined as a new or progressive infiltrate seen on radiography associated with a high clinical suspicion of pneumonia or a positive sputum sample/tracheal aspirate/bronchoalveolar lavage, (b) microbiologically probable diagnosis is one in which a new or progressive radiographic infiltrate with a high clinical suspicion of pneumonia in respiratory secretions, and (c) microbiologically possible diagnosis is evidence of an abnormal chest radiograph of unknown etiology in a patient with a low or moderate clinical suspicion of pneumonia.

(2) Bloodstream Infections (BSIs)

According to the ISF, BSIs were divided into two categories: (a) primary BSIs, which were defined as unknown in origin in patients without an identifiable focus of infection, as well as intravascular catheter-related bloodstream infection; and (b) secondary BSIs, which were defined as being caused by a microorganism related to an infection at another site (Calandra et al., 2005).
(3) Peritonitis

Patients with peritonitis were classified as follows: evidence of a microbial infection of the peritoneal fluid in the absence or presence of a gastrointestinal perforation, abscess, or other localized infection within gastrointestinal tract (Calandra et al., 2005).

(4) Urinary Tract Infections

1. For this study, urinary tract infections were defined by two of the following features (Calandra et al., 2005):
   a. Clinically: presence of fever (temperature greater than 38°C) urgency, frequency, dysuria, pyuria, hematuria
   b. Imaging: suggestive imaging
   c. Microbiology: positive dipstick for leukocyte esterase and/or nitrate or pyuria (≥10 WBCs/µL or ≥3 WBCs/high-power field of unspun urine), organisms seen on Gram stain of unspun urine, frank pus expressed around the urinary catheter, organism counts >1 × 10^3 cfu/mL, or if the patient reported symptoms

For the subanalysis, our 100 patients were categorized according to admitting diagnosis. These subdivisions consisted primarily of 17 cardiothoracic patients, 4 patients with a respiratory-related admitting diagnosis, and 6 patients admitted with a neurological- or trauma-related diagnosis. Additionally, one patient was admitted with abdominal obstruction, one was patient admitted with acute kidney injury, and three patients were admitted with systemic lupus erythematosus flares or active vasculitis. Six patients were admitted with culture-negative sepsis, and 62 patients were admitted with infection.

4.3 Measurements

For the 100 patients (50 in the septic arm and 50 in the nonseptic arm), plasma samples from the first 24 h of recruitment into the DYNAMICS were used for further analysis. Plasma samples from the nine healthy controls underwent the same analysis. All plasma sample preparations and analysis were blinded to infection status.
4.3.1. MPO Fluorometric Activity Assay

MPO was measured using a MPO Fluorometric Activity Assay Kit (Sigma-Aldrich). This assay measured both MPO protein levels and MPO activity. An enzyme-linked immunosorbent assay (ELISA) for MPO was developed using high-binding flat-bottom 96-well immunoplates (Costar, USA). This kit contained five vials: MPO assay buffer, MPO substrate stock, MPO positive control, MPO probe, and fluorescein standard. All vials were protected from light and allowed to reach room temperature (after storage at −20°C) before usage. Vials were briefly centrifuged before opening. A 5-μM standard solution was initially prepared by combining 5 μL of 1 mM fluorescein standard solution with 995 μL of the MPO assay buffer. Standards were created by adding 0, 2, 4, 6, 8, and 10 μL of the 5-μM standard solution to a 96-well plate, generating 0 (blank), 10, 20, 30, 40, and 50 pmol/well standards. MPO assay buffer was added to each well to bring the volume to 100 μL.

MPO was bound via a monoclonal antibody to the plate, and its activity was measured using a substrate that produces a fluorescent product. The plate was then washed, and a polyclonal anti-MPO antibody used to determine the amount of MPO protein bound to the plate. The specific activity of MPO was calculated from the ratio of these two measurements.

4.3.1.1 Sample Preparation

In the uninfected group, there were a total of 17 patients admitted with a cardiothoracic-related diagnosis. Plasma from these patients was compared to that of 17 randomly chosen patients in the infected group. These samples were analyzed for MPO activity using a MPO ELISA kit. In this assay, each well contained 25 μL of plasma from the patient combined with 25 μL of MPO assay buffer and 50 μL of master reactive mixture (master reactive mixture was composed of 46 μL of fluorescein standard, 2 μL of MPO substrate, and 2 μL of the MPO probe) for a final volume of 100 μL per well.
4.3.2 Measuring MPO Activity

The entire plate was covered, protected from light, and placed on a horizontal shaker to completely mix the wells. The plate was incubated at room temperature (37°C) for 2 min. Afterward, an initial measurement (T\textsubscript{initial}) and the fluorescence intensity (FLU\textsubscript{initial}) were taken. Production of the fluorescence product was measured using FLU \( \lambda_{\text{ex}} = 485 \text{ nm} \), \( \lambda_{\text{em}} = 525 \text{ nm} \) over 5 min. A standard curve was then plotted for the fluorescence changes at 5 min versus the concentration of MPO. The MPO activity in the samples was determined using the standard curve.

4.4 HNP ELISA

4.4.1 HNP Preparation and Purification

A mixture of HNP-1, HNP-2, and HNP-3 isolated from the sputum of patients with cystic fibrosis was used. HNP was purified as described previously (Vaschetto et al., 2007; Voglis et al., 2009). The purification of HNP was confirmed by acid-urea-polyacrylamide gel electrophoresis and mass spectroscopy at the Mass Spectrometry Laboratory, Molecular Medicine Research Centre, University of Toronto according to previously described procedures (Elston & Geddes, 2007; Sagel, Chmiel, & Konstan, 2007; Terheggen-Lagro, Rijkers, & van der Ent, 2005; Tirouvanziam et al., 2008). Briefly, sputum was pooled from at least 20 patients with cystic fibrosis before purification and prepared into different batches of purified HNP. The average composition of the 20 batches of purified HNP mixture was 74.1% for HNP-1, 15.2% for HNP-2, and 10.7% for HNP-3, as measured by mass spectrometry. Purified HNP was reconstituted in 0.01% acetic acid and tested by bacterial killing and endotoxin assays before use (Voglis, 2009). Working HNP solutions were prepared from a 4 mg/mL stock solution, and a HNP-free vehicle solution was prepared with 0.01% acetic acid.
4.4.2 HNP ELISA Reading

The 96-well plates were coated overnight with mouse anti-human HNP-1–3 monoclonal antibodies (0.1 mg/mL) (HyCult Biotechnology, Uden, The Netherlands). The next morning, after thorough washing and blocking with a non-specific protein binding, 50 μL of HNP standards and 50 μL of samples were added to each well. For the pilot study, 29 plasma samples (10 septic patients, 10 nonseptic patients, and 9 healthy controls) were added to each well; thereafter, 109 plasma samples were used (62 uninfected critically ill patients, 38 patients who were infected, and 9 healthy controls). Of note, ELISA preparation and analysis were performed blinded to the infection status of the plasma samples. The wells were then incubated for 1 h with 1:1000 rabbit anti-human HNP-1–3 polyclonal antibody (Host Defence Research Centre, Toronto, ON), followed by incubation for 1 h with 1:4000 peroxidase-conjugated goat-anti-rabbit IgG (Jackson ImmunoResearch). 3,3′,5,5′-Tetramethylbenzidine (Sigma) was added, and the reaction was stopped by using 1 M sulfuric acid. The absorbance was read at 450 nm.

4.5 Statistical Calculations

Statistical calculations were performed using the software SAS 9.4 and Prism.

4.5.1 Serum Lactate Calculations

Plasma lactate levels were examined as a surrogate marker of organ dysfunction. Lactate is the end-product of anaerobic glycolysis. In situations of hypoperfusion or hypoxia, pyruvate will no longer enter the mitochondria to undergo aerobic metabolism, but instead, it is preferentially reduced to lactate, resulting in lactate accumulation in the blood (Zhang Z., Xu, & Chen, 2014).

The DYNAMICS database contained plasma lactate levels for only 86 of the 100 patients, including 57 patients with infection, and 29 patients had no infection.
4.5.2 MODS Calculation

MODS, first reported by Marshall et al. in 1995, is a prognostic marker used to quantify organ dysfunction at a point in time or over time. The MODS system uses physiological parameters for six organ systems: cardiovascular, respiratory, hematologic, renal, central nervous, and hepatic systems. Figure 16 describes the various parameters included in the MODS calculation.

Multiple Organ Dysfunction Score

![MODS Criteria](image)

Figure 16. MODS Criteria. MODS is a quantification scoring system of organ dysfunction. It is used to help describe the severity of illness in different patients in the ICUs and enable comparison over time or among groups of patients (Marshall et al., 1995) (Adapted from Crit Care Med, 1995, with permission).

It should be noted that the DYNAMICS database was incomplete, and not all patients had the necessary physiological parameters needed to calculate MODS. From the study population cohort, only 42 patients with infection and 22 uninfected patients had all of the physiological parameters needed to calculate MODS.
4.5.3 Serum Creatinine Calculations

Plasma creatinine is used as an index of renal function. Creatinine is a cyclic anhydride of creatine, which is an end-product of muscle metabolism. It is a low-molecular-weight substance with a molecular weight of 113 Da, it is not protein-bound, it is freely filtered at the glomerulus, and it is not metabolized in the kidneys (Perrone, Madias, & Levey, 1992; Ralston, 1955). It is secreted by the proximal tubules via both the anionic and cationic secretory pathways. In patients with renal dysfunction, this tubular secretion may increase relative to glomerular filtration (Bowers & Wong, 1980; Im et al., 2012; Ralston, 1955).

4.5.4 PaO2/FiO2 Ratio Calculations

The PaO2/FiO2 ratio is the ratio of arterial oxygen partial pressure to fractional inspired oxygen. It is a commonly used indicator of lung function in critically ill patients.

In the absence of a direct reliable marker of lung injury, the PaO2/FiO2 ratio has been used to describe hypoxemia. In addition, clinicians use the ratio to track changes in lung conditions. The PaO2/FiO2 ratio is a central element of the new acute respiratory distress syndrome definition (Berlin definition) (Thompson & Matthay, 2013).
CHAPTER 5—RESULTS

5.1 Study Results

The study included a total of 100 patients selected from the DYNAMICS database (50 patients from the septic arm and 50 patients from the nonseptic arm) who were restratified according to the presence or absence of infection (Figure below). Nine healthy controls, four of whom were age-matched, were included.

5.1.1. Subject Classification and General Demographics

5.1.1.1 Subject Characterization

Patients Subdivided into Presence and Absence of Infection

Figure 17. Classification of the Study Population. The patient cohort derived from the DYNAMICS study database was reclassified according to the presence or absence of infection using the classification outlined in the ISF Consensus Conference on Definitions of Infection in the Intensive Care Unit (Calandra et al., 2005). Of the 100 total patients, the distribution of the study population included 62 patients with evidence of infection and 38 who did not have an infection.
5.1.1.2 Demographics

The study included nine healthy control subjects. Table 4 describes the baseline demographics of each patient population. Patients in the septic group were slightly older than those in the nonseptic group, with mean ages of 67 and 65 years, respectively. Similarly, patients who had an infection were slightly older than those with no evidence of infection (67 and 64 years, respectively). Healthy controls were younger than to the other subgroups, with a mean age of 46 years.

### Baseline Characteristics of Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy Ctrl</th>
<th>Septic</th>
<th>Nonseptic</th>
<th>Infection (+)</th>
<th>Infection (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>50</td>
<td>50</td>
<td>82</td>
<td>38</td>
</tr>
<tr>
<td>Age, Years</td>
<td>46</td>
<td>67</td>
<td>65</td>
<td>67</td>
<td>64</td>
</tr>
<tr>
<td>Gender</td>
<td>4 Males 5 Females</td>
<td>25 Males 25 Females</td>
<td>31 Males 19 Females</td>
<td>34 Males 28 Females</td>
<td>22 Males 16 Females</td>
</tr>
<tr>
<td>APACHE II Score [mean +/- SD]</td>
<td>26.6 +/- 0.5</td>
<td>26.5 +/- 0.7</td>
<td>26.0 +/- 0.4</td>
<td>26.3 +/- 0.4</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Baseline Characteristics of Patients. Patient demographics and APACHE II scores. Study patients’ age and APACHE II scores were similar among the groups. Healthy controls were slightly younger, but this group had an equal gender distribution.
### Comorbidities of Patients

<table>
<thead>
<tr>
<th>Comorbidity</th>
<th>Septic Group n=50</th>
<th>Nonseptic Group n=50</th>
<th>Infection (+) n=62</th>
<th>Infection (-) n=38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes, n(%)</td>
<td>14 (28)</td>
<td>19 (38)</td>
<td>18 (29)</td>
<td>12 (31)</td>
</tr>
<tr>
<td>Chronic Lung Disease</td>
<td>8 (16)</td>
<td>4 (8)</td>
<td>11 (17)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Ischemic Heart Disease/Congestive Heart Failure</td>
<td>9 (18)</td>
<td>18 (36)</td>
<td>17 (27)</td>
<td>9 (24)</td>
</tr>
<tr>
<td>Liver Disease</td>
<td>4 (8)</td>
<td>2 (4)</td>
<td>2 (3)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Chronic Renal Failure</td>
<td>3 (6)</td>
<td>3 (6)</td>
<td>2 (3)</td>
<td>4 (10.5)</td>
</tr>
<tr>
<td>Cancer/Immunocompromised</td>
<td>4 (8)</td>
<td>4 (8)</td>
<td>3 (3.2)</td>
<td>5 (13.16)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Charlson Comorbidities</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21</td>
<td>17</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>12</td>
<td>9</td>
<td>7</td>
</tr>
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<td>3</td>
<td>12</td>
<td>8</td>
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<td>12</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5. Comorbidities of Patients in Each Subgroup. At the bottom, calculated scores are shown (the Charlson Comorbidity Score, Appendix C) for each subgroup. Infection-negative and nonseptic patients tended to have more comorbidities compared to infection-positive patients.

In summary, our two patient subpopulations (infected vs. uninfected) were similar with respect to demographic variables, namely age, gender distribution, number of comorbidities, and APACHE II scores. However, one subpopulation included infected patients, whereas the other group consisted of uninfected patients. This begged the question whether there was a surrogate marker that could enable us to distinguish these two subpopulations. We therefore examined HNPs as a possible surrogate marker.
5.2 Objective 1: To Determine whether HNP Plasma Concentrations are Associated with the Presence of Infection:

![HNP Plasma Concentration](image)

Figure 18. Plasma HNP Concentrations across Subgroups. The graph on the left indicates that healthy controls had the lowest plasma HNP concentration. Nonseptic patients had higher plasma HNP concentrations than septic patients. The plasma HNP concentrations were statistically different between septic/nonseptic patients and healthy controls. The graph on the right indicates that uninfected patients had higher plasma HNP concentrations than both healthy controls and patients with an infection. This difference was statistically significant among the different groups.

As HNPs play a critical role in the host response to infection, we hypothesized that HNP levels are increased in patients with microbiologically confirmed infection. However, whether we stratified patients using the sepsis criteria or by the presence of infection, we found higher plasma levels of HNP in nonseptic and uninfected patients. In fact, a mean HNP level of $8044.5 \pm 3755.1$ pg/mL was recorded in patients with no infection, compared to $4304.8 \pm 3562.9$ pg/mL in those who were infected ($P < 0.001$).
Univariate linear regression illustrated that HNP levels predictive of infection status \([P < 0.001, \text{ odds ratio} = 0.83 \text{ (i.e., the odds of infection would decrease by 17\% with a } 1000-\text{fold increase in HNP levels)}]\). 

Receiver operative characteristic (ROC) analysis was performed for HNP concentrations (Figure 18). The area under the ROC curve (AUC) was 0.69. An optimal cut-off was identified at an HNP value = 7348, where the Youden index (Youden index = Sensitivity + Specificity − 1) was maximized at 0.356.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>AUC (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNP values (unit=1000)</td>
<td>0.828 (0.774, 0.887)</td>
<td>0.691 (0.623, 0.758)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: The odds ratio (OR) was estimated using a 1000-fold increase in HNP levels. CI, confidence interval.

Table 6. Univariate Linear Regression Analysis of HNP Levels Predictive of Infection.
Figure 19. ROC Curve for HNP Levels Predictive of Infection (AUC = 0.691). This suggests that HNP levels predict infection. An optimal cut-off for HNP levels was found at 7348 pg/mL.

From the literature, we know that HNPs are stored in neutrophil granules. This raised the question of whether the number of neutrophils is higher in uninfected and nonseptic patients and therefore whether HNP levels are correlated with the number and activity of neutrophils.

5.3 Objective 2: To determine whether HNP plasma concentrations are associated with blood neutrophil activity:

5.3.1 WBC and Neutrophil Counts

All patients in the study group had higher WBC counts than healthy controls, who had a mean plasma value of $5.5 \times 10^9 \pm 0.9 \times 10^9$ cells/L. Both infected and uninfected patients
had similar WBC plasma concentrations, with means of $16.7 \times 10^9 \pm 8.2 \times 10^9$ and $15.6 \times 10^9 \pm 6.8 \times 10^9$ cells/L, respectively. Despite slightly higher WBC values in infected patients relative to uninfected patients, this difference was not statistically significant.

Figure 20. WBC Counts in Patient Cohorts. Both patients with sepsis and those with infections had higher WBC counts than nonseptic and uninfected patients. The difference in the number of blood WBCs between septic and nonseptic patients was statistically significant, whereas that between infected and uninfected patients was not significant. Healthy controls had the lowest number of WBCs in the study population, and this difference was statistically significant.
5.3.2 Association of HNP Levels with WBC Counts

We examined whether there was a relationship between plasma HNP concentrations and the number of WBCs in blood and found a weak correlation between the variables (Rho = 0.15, P = 0.02).

Mean Square Calculation for Group

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>group</td>
<td>3</td>
<td>843.0952003</td>
<td>281.0317334</td>
<td>5.44</td>
<td>0.0015</td>
</tr>
<tr>
<td>HNP_value</td>
<td>1</td>
<td>129.2675310</td>
<td>129.2675310</td>
<td>2.50</td>
<td>0.1165</td>
</tr>
</tbody>
</table>

Estimated Least Square Means for Each Group

<table>
<thead>
<tr>
<th>group</th>
<th>WBCs Least Square MEAN</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection</td>
<td>17.086993</td>
<td>15.197310</td>
</tr>
<tr>
<td>control</td>
<td>6.800914</td>
<td>1.800423</td>
</tr>
<tr>
<td>non-infection</td>
<td>15.032997</td>
<td>12.541416</td>
</tr>
</tbody>
</table>

Results of Contrasts in Differences of WBCs

| Parameter                                           | Estimate | Standard Error | t Value | Pr > |t| |
|-----------------------------------------------------|----------|----------------|---------|------|---|
| Difference between infection and non-infection      | 2.0539964| 1.63890536     | 1.25    | 0.2126|
| Difference between infection and control            | 10.2860787| 2.62452590     | 3.92    | 0.0001|
| Difference between non-infection and control         | 8.2320823| 2.92719461     | 2.81    | 0.0058|

Table 7. ANCOVA of HNP Levels and WBC Counts. ANCOVA results indicate significant differences in WBC counts between patient groups (P < 0.0001). HNP concentrations were not significantly different (P = 0.12).
Figure 21. Blood Neutrophil Counts across Patient Cohorts. The study group population had higher neutrophil counts than healthy controls, and this difference was statistically significant ($P < 0.05$). Using the DYNAMICS classification, the septic population had higher circulating neutrophil counts than nonseptic patients and healthy controls. The difference in blood neutrophil counts was not statistically different between septic and nonseptic patients. When patients were divided according the presence or absence of infection, infected patients had the highest blood neutrophil count. The difference in blood neutrophil counts between infection-positive and infection-negative patients was not statistically significant.

The association was further explored using ANCOVA. ANCOVA (Table 7) results revealed significant differences in neutrophil counts between patient groups ($P < 0.0001$), whereas HNP concentrations were not significantly different ($P = 0.4$).
Table 8. ANCOVA of HNP Levels and Neutrophil Counts for our Patient Cohort. The analysis revealed a significant difference between the groups regarding neutrophil counts but no relationship with plasma HNP levels.

When we examined the WBC differential, we found that neutrophils were predominately represented in all study group patients. All study group patients had statistically higher plasma levels of neutrophils than the healthy controls, who had a mean value of $1.5 \times 10^9 \pm 0.19 \times 10^9/L$. Septic patients had more plasma neutrophils than nonseptic patients, with means of $15.5 \times 10^9 \pm 1.1 \times 10^9$ and $11.7 \times 10^9 \pm 1.1 \times 10^9/L$, respectively. Similarly,
patients with infection had a mean blood neutrophil count of $14.1 \times 10^9 \pm 7.1 \times 10^9 /L$, versus $12.5 \times 10^9 \pm 6.7 \times 10^9 /L$ for uninfected patients.

We examined the plasma HNP concentration and the number of circulating neutrophils. To assess the activity of neutrophils, we examined the concentration of HNPs per neutrophil in plasma. For this calculation, we divided the plasma concentration of HNPs by the number of plasma neutrophils for each patient. Although this measurement does not reflect intracellular HNPs, this was an indirect measurement to determine whether plasma HNP levels are correlated with plasma neutrophil counts. Figure 22 demonstrates that uninfected critically ill patients had a higher plasma HNP concentration per number of neutrophils (1189 pg/mL/neutrophil) than infected critically ill patients (358 pg/mL/neutrophil) and healthy controls (288 pg/mL/neutrophil). These differences were statistically significant ($P < 0.05$). This suggests that neutrophils are more active in uninfected patients than in infected patients and therefore release more of their stored HNP into plasma.
Figure 22. Concentration of HNPs per Neutrophil across Patient Cohorts. The figure illustrates that uninfected patients had a higher plasma HNP concentration per neutrophil than infected patients.

We found that in the uninfected subgroup, patients admitted with a cardiothoracic diagnosis had the highest plasma HNP concentration. The majority of these patients had underwent coronary bypass or valve replacement surgery, suggesting that they may have
Table 9. Subclassification of Patient Cohorts According to the Admitting Diagnosis. If patients were infected, then they were classified as infection-positive irrespective of their admitting diagnosis. The cardiothoracic group had the highest plasma HNP concentration, and patients with infection had the lowest plasma HNP concentration.

mounted a greater systemic inflammatory response, thereby activating more neutrophils, and as a result, more HNPs were released into their plasma compared to the other subgroups.
Table 10. Patient Subgroups with Corresponding WBC and Neutrophil Counts. Patients were categorized according to their admitting diagnosis. Patients with infection had the highest WBC and neutrophil counts among the subgroups. However, when we examined the number of neutrophils, we found that all patients in the uninfected subgroup had similar numbers of neutrophils. This suggested that despite similar amounts of plasma neutrophils, neutrophils are more active in uninfected critically ill patients.
Table 11. Distribution of Groups with Respective Plasma HNP Levels and Neutrophil and WBC Counts.

To determine the activity of neutrophils, we examined MPO activity. MPO is a peroxidase enzyme involved in modulating the phagocytic activity of neutrophils. Two subgroups were chosen according to plasma HNP concentrations. The lower plasma HNP concentration group consisted of infected patients, whereas patients with higher plasma HNP concentration were uninfected patients. Among the uninfected patients, patients admitted with a cardiothoracic-related diagnosis had the highest plasma HNP concentration. Plasma samples from all 17 cardiothoracic patients (these patients represented the uninfected group) and 17 randomly selected patients with an infection were compared using an MPO assay. The MPO activity was higher in patients who had been admitted with a cardiothoracic-related diagnosis (fluorescence intensity of 71 ± 8.7) than in infected patients (fluorescence intensity of 31.1 ± 1.9). This difference was statistically significant (P < 0.0001). These results suggest that neutrophils in uninfected
patients are more active than those in infected patients, indicating that uninfected patients
mount a larger systemic inflammatory response than infected patients.

![MPO Activity Curves](image)

*Figure 23. MPO Activity Curves. The figure illustrates the difference in fluorescence
intensity between the uninfected cardiothoracic group (left) and the infected group
(right).*

5.4 Objective 3: To determine whether HNP plasma concentrations reflect organ
perfusion and dysfunction:

5.4.1 Plasma HNP Concentrations and Organ Dysfunction

5.4.1.1 MODS

Patients with infection had a mean MODS of 7.9 ± 2.8, and patients without an infection
had a MODS of 8.3 ± 3.9. When the patients without an infection were subdivided, the 17
cardiothoracic patients had MODS of 9.8 ± 4.0. These differences were not statistically
significant.
Table 12. Summary of HNP and Lactate Levels and MODS by Patient Group. The uninfected group had higher HNP and lactate concentrations and MODS values than the infected group. In the uninfected group, cardiothoracic patients had the highest mean HNP, lactate, and MODS values.

5.4.1.2 Association of HNP Levels with MODS

There was a moderate correlation between HNP levels and MODS (rho = 0.39, P < 0.001, Table 12).

The association was further explored using ANCOVA (Table 13), which revealed a significant correlation (P = 0.007) between HNP levels and MODS. The differences in MODSs between patient groups were also significant (type III test, P < 0.001).
### ANCOVA of HNP and Predicting MODS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>t Value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>6.303474124</td>
<td>0.97559009</td>
<td>6.46</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Group</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>group Infection</td>
<td>0.442814478</td>
<td>0.85849076</td>
<td>0.52</td>
<td>0.6074</td>
</tr>
<tr>
<td>group cardiothoracic</td>
<td>1.435122392</td>
<td>1.08690796</td>
<td>1.32</td>
<td>0.1905</td>
</tr>
<tr>
<td>group control</td>
<td>-6.669806516</td>
<td>1.33916355</td>
<td>-4.98</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>group non-infection</td>
<td>0.000000000</td>
<td>.</td>
<td>.</td>
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</tr>
<tr>
<td>HNP</td>
<td>0.000260315</td>
<td>0.00009452</td>
<td>2.75</td>
<td>0.0073</td>
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</table>

The Estimated Least Square Means for Each Patient Group

<table>
<thead>
<tr>
<th>Group</th>
<th>MODS</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection</td>
<td>8.162915</td>
<td>7.211154 - 9.114677</td>
</tr>
<tr>
<td>cardiothoracic</td>
<td>9.155223</td>
<td>7.348217 - 10.962230</td>
</tr>
<tr>
<td>control</td>
<td>1.050294</td>
<td>-1.096688 - 3.197277</td>
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<tr>
<td>non-infection</td>
<td>7.720101</td>
<td>6.364792 - 9.075410</td>
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</table>

Results of Contrasts in Differences of MODS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>t Value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difference between infection and non-infection</td>
<td>0.44281448</td>
<td>0.85849076</td>
<td>0.52</td>
<td>0.6074</td>
</tr>
<tr>
<td>Difference between infection and control</td>
<td>7.11262099</td>
<td>1.14654523</td>
<td>6.20</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Difference between non-infection and control</td>
<td>6.66980652</td>
<td>1.33916355</td>
<td>4.98</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Difference between infection and cardiothoracic patients</td>
<td>-0.99230791</td>
<td>1.05053974</td>
<td>-0.94</td>
<td>0.3477</td>
</tr>
</tbody>
</table>

Table 13. ANCOVA of HNP Levels and MODS. The analysis was conducted infection-positive and infection-negative patients as well as cardiothoracic patients.
Figure 24. Linear Regression: MODS vs. HNP. HNP levels are significantly (P = 0.007) correlated with MODSs.

5.4.1.3 Plasma Lactate Concentrations

The mean lactate concentration for patients with infection was $3.7 \pm 3.4$ mmol/L compared to $6.1 \pm 4.3$ mmol/L for patients without infection. For patients admitted with a cardiothoracic diagnosis, the mean lactate was $6.5 \pm 3.9$ mmol/L. The least square mean values indicated that cardiothoracic patients had higher lactate levels than both uninfected and infected patients. Although there was a difference in the mean plasma lactate concentration between infected and uninfected patients, this difference was not statistically significant.
### Estimated Least Square Means for Each Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Lactate Least Square MEAN</th>
<th>95% Confidence Limits</th>
</tr>
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<tbody>
<tr>
<td>Infection</td>
<td>4.118339</td>
<td>3.098023</td>
</tr>
<tr>
<td>Cardiothoracic</td>
<td>5.813764</td>
<td>3.657165</td>
</tr>
<tr>
<td>non-infection</td>
<td>5.626059</td>
<td>4.273712</td>
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</table>

### Contrasts in Differences of Lactate

<table>
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<th>Standard Error</th>
<th>t Value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difference between infection and non-infection</td>
<td>−1.50772034</td>
<td>0.89083796</td>
<td>−1.69</td>
<td>0.0938</td>
</tr>
<tr>
<td>Difference between infection and cardiothoracic patients</td>
<td>−1.69542461</td>
<td>1.24048364</td>
<td>−1.37</td>
<td>0.1749</td>
</tr>
</tbody>
</table>

Table 14. Estimated Least Square Mean and Differences in Lactate Levels. Data were compared among infected patients, uninfected patients, and patients with a cardiothoracic diagnosis.

5.4.1.3.1 Association of HNP Levels with Lactate Levels

There was a moderate correlation between HNP and lactate concentrations (rho = 0.38, P < 0.001 (Table 14).

The association was further explored using ANCOVA (Table 15), with the results indicating that HNP levels are significantly (P = 0.015) correlated with lactate levels. However, the differences in lactate levels between the patient groups were not significant (type III test, P = 0.178).
### Table 15. ANCOVA of HNP and Lactate Levels

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>t Value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
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<td>1.04946606</td>
<td>3.89</td>
<td>0.0002</td>
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<td>Group</td>
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<td>0.89083796</td>
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<td>0.0938</td>
</tr>
<tr>
<td>group cardiothoracic</td>
<td>0.187704264</td>
<td>1.23876898</td>
<td>0.15</td>
<td>0.8799</td>
</tr>
<tr>
<td>group non-infection</td>
<td>0.000000000</td>
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<td>.</td>
<td>.</td>
</tr>
<tr>
<td>HNP</td>
<td>0.000251923</td>
<td>0.00010207</td>
<td>2.47</td>
<td>0.0154</td>
</tr>
</tbody>
</table>

Figure 25. ANCOVA of Serum Lactate Levels in the Patient Cohort. Plasma HNP concentrations increase with increasing plasma lactate levels.
These results illustrated that plasma lactate levels increased with increasing plasma HNP concentrations. Figure 25 demonstrates the correlation of plasma HNP concentrations with plasma lactate levels with a correlation coefficient of 0.73. This increase was most pronounced in patients with plasma HNP levels exceeding 7000 pg/mL. This association suggests that in the setting of a systemic inflammatory response, patients with a greater degree of hypoperfusion/hypoxia had higher plasma HNP concentrations.

**Plasma Lactate**

![Plasma HNP vs. Plasma Lactate](image)

Figure 26. Plasma HNP vs. Plasma Lactate. There is a positive trend between increasing plasma lactate concentrations and increasing HNP concentrations.

In summary, general markers of organ dysfunction revealed a correlation between increasing plasma HNP concentrations and severity of organ dysfunction. Therefore, we sought to investigate the existence of a specific marker of organ dysfunction that best correlates with plasma HNP concentrations.
5.4.2 Specific Organ Dysfunction

Plasma creatinine values, PaO2/FiO2 ratios, plasma bilirubin levels, and the use of inotropes were examined.

5.4.2.1 Plasma Creatinine Concentrations

Plasma creatinine levels in patients without infection were elevated, suggesting greater renal impairment (mean creatinine level of 191.5 ± 170.2), compared to those in infected patients (mean serum creatinine was 145.7 ± 135.5). This difference was not statistically significant.

<table>
<thead>
<tr>
<th>group</th>
<th>N Obs</th>
<th>Variable</th>
<th>N</th>
<th>N Miss</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Minimum</th>
<th>Maximum</th>
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<tbody>
<tr>
<td>Infection</td>
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<td>HNP</td>
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<td>4304.8</td>
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<td></td>
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<td>187.1</td>
<td>104.1</td>
<td>44.0</td>
<td>433.0</td>
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<td></td>
<td></td>
<td>Creatinine</td>
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<td>145.7</td>
<td>135.5</td>
<td>22.0</td>
<td>738.0</td>
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<tr>
<td>cardiothoracic</td>
<td>17</td>
<td>HNP</td>
<td>17</td>
<td>0</td>
<td>8359.3</td>
<td>3537.0</td>
<td>1545.2</td>
<td>11949.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P_F_ratio</td>
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<td>3</td>
<td>254.1</td>
<td>125.1</td>
<td>112.0</td>
<td>514.0</td>
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<tr>
<td></td>
<td></td>
<td>Creatinine</td>
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<td>1</td>
<td>172.3</td>
<td>149.3</td>
<td>44.0</td>
<td>597.0</td>
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<tr>
<td>control</td>
<td>9</td>
<td>HNP</td>
<td>9</td>
<td>0</td>
<td>1407.3</td>
<td>799.8</td>
<td>538.2</td>
<td>2735.1</td>
</tr>
<tr>
<td></td>
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<td>Creatinine</td>
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<td>64.0</td>
<td>11.1</td>
<td>47.0</td>
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<tr>
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<td>HNP</td>
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<td>483.0</td>
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</tr>
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<td></td>
<td></td>
<td>P_F_ratio</td>
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<td>154.5</td>
<td>39.0</td>
<td>573.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Creatinine</td>
<td>36</td>
<td>4</td>
<td>191.5</td>
<td>170.2</td>
<td>19.0</td>
<td>679.0</td>
</tr>
</tbody>
</table>

Table 16. Summary of HNP Levels, PaO2/FiO2 Ratios, and Serum Creatinine Levels by Patient Group P_F_ratio, PaO2/FiO2 ratio.
5.4.2.1.2 Association of HNP Levels with Creatinine Levels

There was a weak correlation between HNP and creatinine levels (rho = 0.21, P = 0.02).

The association was further explored using ANCOVA, which did not reveal a correlation between HNP and creatinine levels after adjusting for the group (P = 0.2). The differences in creatinine levels between patient groups were also not significant (type III test, P = 0.28).

<table>
<thead>
<tr>
<th>ANCOVA of HNP and Group Predicting log(Creatinine)</th>
</tr>
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<tbody>
<tr>
<td>Parameter</td>
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<tr>
<td>Intercept</td>
</tr>
<tr>
<td>Group</td>
</tr>
<tr>
<td>group Infection</td>
</tr>
<tr>
<td>group cardiothorac</td>
</tr>
<tr>
<td>group control</td>
</tr>
<tr>
<td>group non-infection</td>
</tr>
<tr>
<td>HNP</td>
</tr>
</tbody>
</table>

Estimated Least Square Means for Each Group

<table>
<thead>
<tr>
<th>Group</th>
<th>log_creatinine Least Square MEAN</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection</td>
<td>4.746225</td>
<td>4.550624</td>
</tr>
<tr>
<td>Cardiothoracic</td>
<td>4.815705</td>
<td>4.436176</td>
</tr>
<tr>
<td>Control</td>
<td>4.252824</td>
<td>3.735559</td>
</tr>
<tr>
<td>non-infection</td>
<td>4.830787</td>
<td>4.569893</td>
</tr>
</tbody>
</table>

Results of Contrasts in Differences of log(Creatinine)

| Parameter                              | Estimate      | Standard Error | t Value | Pr > |t| |
|----------------------------------------|---------------|----------------|---------|------|---|
| Difference between infection and non-infection | -0.08456271  | 0.17199736     | -0.49   | 0.6239 |
Table 17. ANCOVA of Serum Creatinine and HNP Levels. HNP levels were not correlated with serum creatinine concentrations.

| Difference between infection and control | 0.49340077 | 0.27091567 | 1.82 | 0.0711 |
| Difference between non-infection and control | 0.57796348 | 0.30536816 | 1.89 | 0.0609 |
| Difference between infection and cardiothoracic patients | −0.06948071 | 0.22161081 | −0.31 | 0.7544 |

Figure 27. Residual Plot of Serum Creatinine Concentrations. The residual plot indicated a non-normal distribution. Creatinine was transformed by taking the natural log.
5.4.2.2 Plasma Bilirubin Concentrations

Mean plasma bilirubin concentrations were higher in uninfected patients than in infected patients (20.26 ± 6.4 µmol/L vs. 18.87 ± 3.0 µmol/L), but the difference was not statistically significant. Linear regression analysis also did not illustrate a correlation between plasma HNP and plasma bilirubin levels.

5.4.2.3 PaO2/FiO2 Ratio

Patients with infection had a PaO2/FiO2 ratio of 187.1± 104.1, compared to 257.8 ± 154.5 for uninfected patients. There was no statistical significance difference between the two mean values.

5.4.2.3.1 Association of HNP Levels with PaO2/FiO2 ratios

There was no significant correlation between HNP levels and PaO2/FiO2 ratios (P = 0.91, Table 18).

The association was further explored using ANCOVA (Table 18), with the data indicating that HNP levels were not significantly (P = 0.26) correlated with the PaO2/FiO2 ratio. The differences in PaO2/FiO2 ratios between the patient groups were significant (type III test, P = 0.01).
Table 18. ANCOVA of HNP Levels and PaO2/FiO2 (P/F) Ratios. There was no significant correlation between HNP levels and P/F ratios.

5.4.3 Hemodynamics

5.4.3.1 Vasopressor Requirements

Vasopressor therapy was required more often in patients with higher plasma HNP concentrations than in those with lower plasma HNP concentrations. Among the patients, 58% with plasma HNP concentrations greater than 9999 pg/mL were on vasopressor therapy, whereas only 18% with low plasma HNP concentrations (less than 4000 pg/mL)
required vasopressor therapy. This suggests that critically ill patients with higher plasma HNP concentrations were more hemodynamically unstable than those with lower plasma HNP concentrations, thereby requiring more vaspressors.

<table>
<thead>
<tr>
<th>HNP_Group</th>
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<tbody>
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</tr>
<tr>
<td></td>
<td>95.45</td>
</tr>
<tr>
<td>4000-6999</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>53.33</td>
</tr>
<tr>
<td>7000-9999</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.76</td>
</tr>
<tr>
<td>10000-12999</td>
<td>0</td>
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<tr>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
</tr>
</tbody>
</table>

Cochran-Armitage Trend Test p-value: <0.0001

Table 19. The association between HNP group and Vasopressor Use. There is a significant association between HNP plasma concentration and vasopressor use.

<table>
<thead>
<tr>
<th>group</th>
<th>vasopressor_use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency Row Pct</td>
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<td>Infection</td>
<td>42</td>
</tr>
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<td></td>
<td>68.85</td>
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<tr>
<td>cardiothoracic</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>17.65</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
</tr>
</tbody>
</table>

Fishers’ exact test p-value: 0.0002

Table 20. The association between Infection/Cardiothoracic groups and Vasopressor use. There is a significant association between HNP plasma concentration and vasopressor use.
A significant association between HNP group and Vasopressor use was identified in our study cohort which showed that higher HNP level is associated with more Vasopressor use. Moreover there was also a significant association between Infection/Non-infection groups and Vasopressor use in that there was less Vasopressor use in the infection group compared to the non-infection group. Specifically there was more Vasopressor use in the Cardiothoracic group than the infection group.

5.5 Objective 4: To determine whether HNP plasma concentrations are associated with mortality:

Mortality for our patient population was examined. Overall, there was no difference in survival rates between infected and uninfected patients. Figure 29 demonstrates the total number of patients at risk at a certain time.

Patients with infection had slightly better survival than those without infection, although the difference was not significant.

Figure 28. Kaplan–Meier Overall Survival for the Patient Cohort. Patients with infection had a better survival rate than those without infection, although the difference is not statistically significant.
Figure 29. Kaplan–Meier Survival Curves for our Patient Cohort at 30 Days. The graphs show that patients with infection had a better survival rate than those without infection, although this difference was not statistically significant.

At 15 days, 70% of the patients without infection were alive, compared to 87% of infected patients. At 30 days, the survival rates of these groups were 84.8% and 78.1%, respectively ($P = 0.0857$). The overall survival rate of our patient population was 76.7%, including rates of 82.6% for infected patients and 66.7% for uninfected patients ($P = 0.1182$), suggesting that patients with infection had slightly better outcomes than those without infection.

We examined the survival rate of patients admitted with a cardiothoracic diagnosis compared to that of infected patients. The overall survival rate of uninfected patients who were admitted with a cardiothoracic diagnosis was 50%, compared to 82.6% for infected
patients (P = 0.0010). At 30 days, 50% of patients admitted with a cardiothoracic diagnosis were alive, versus 84.8% of patients with an infection (P = 0.0006).

Figure 30. Kaplan–Meier Survival Curves for Infected vs. Cardiothoracic Patients. The graph illustrates that patients with a cardiothoracic diagnosis did worse than patients admitted with infection.

Potential confounders of plasma HNP concentrations were examined. Given the properties of HNPs as antimicrobial and pro-inflammatory peptides, for our study, we considered (a) APACHE II scores, (b) patient age, (c) patient gender, (d) MODS, (e) the Charlson Comorbidity Index, and (f) survival. In considering these confounders, our data illustrated that plasma HNP concentrations were not correlated with mortality.
HNP Concentration and Mortality

Figure 31. HNP Concentration vs. Mortality. Possible confounders of plasma HNP concentrations were considered, revealing no association of these values with mortality.
 CHAPTER 6—DISCUSSION & LIMITATIONS

The systemic inflammatory response is a host response to various triggers including sepsis. Sepsis is a systemic host response to infection. The septic process triggers a cascade of biochemical and physiological changes that are part of (SIRS. Given the heterogeneity and complexity of critically ill patients, it has been challenging to find a biomarker that can effectively assist in determining their diagnosis and prognosis. To date, there is no biomarker to facilitate the diagnosis and prognosis of critically ill patients with SIRS.

The aim of this research was to assess whether plasma HNP concentrations predict the severity of the inflammatory response in sepsis.

6.1 Is HNP Plasma Concentration Associated with the Presence of Infection?

It is known that HNPs, also known as defensins, are endogenous molecules with a dual physiological role. They have the ability to act as natural antimicrobial molecules as well as pro-inflammatory agents. Studies examining defensins spectrum of activity have found that defensins are active against gram-positive bacteria (GPBs) and gram-negative bacteria (GNBs) in vitro in microbiological medium (Harwig, Ganz, & Lehrer, 1994; Porro et al., 2001). From previous data examining the activity of defensins, it was confirmed that defensins have direct and indirect roles in bacterial killing. Under physiological conditions, cationic defensins kill bacteria both directly via the well-described mechanisms of increasing membrane permeability and cell lysis (Ganz & Lehrer, 1995) and indirectly by producing bactericidal products (Porro et al., 2001).

Both our critically ill subpopulations (infected and noninfected) had higher plasma HNP concentrations than healthy volunteers. Our results indicate that noninfected patients had a mean plasma HNP concentration of 8044.5 pg/mL, compared to 4304.8 pg/mL in infected patients and 1407 pg/mL in healthy volunteers.
6.1.1 Plasma HNP Concentrations and Infection

Our study results demonstrated that plasma HNP concentrations are influenced by the presence or absence of infection. In fact, our univariate linear regression identified HNP levels as predictive of infective status at a statistically significant level (P < 0.001).

Similar to previous studies, our healthy volunteers had extremely low plasma HNP concentrations (Panyutich et al., 1993; Panyutich, Hiemstra, van Wetering, Ganz, 1995; Voglis et al., 2009) compared to the study patients. Contrary to previous data demonstrating that bacterial infections increase plasma HNP concentrations to as high as 170 mg/mL (Ihi et al., 1997; Panyutich et al., 1993), our data illustrated that plasma HNP levels were lower in critically ill patients with infection than in uninfected patients. ROC curve analysis revealed an AUC of 0.69 with a cut-off of 7348 pg/mL. This suggests that patients with higher plasma values are likely not to be infected, whereas those with lower values are likely to be infected.

The discrepancy in plasma HNP concentrations could be attributable to HNP’s dual role as an antimicrobial molecule with pro-inflammatory properties. That is, in patients with infection, the antimicrobial properties predominant over the pro-inflammatory properties thereby consuming HNP to fight infection, this leads to lower plasma HNP concentrations. Whereas plasma HNP levels are higher when the pro-inflammatory properties are accentuated, such as the case in patients with SIRS-like symptoms but no evidence of infection.

6.2 Is HNP plasma Concentration Associated with Neutrophil Activity?

Our data indicate that critically ill patients had higher numbers of neutrophils circulating in their plasma compared to healthy controls. This suggests that during an inflammatory response, the activation of cytokines stimulates the production of more neutrophils.

From the literature, physiologically, neutrophil homeostasis is maintained via a careful balance between neutrophil production and release from the bone marrow and neutrophil clearance from the periphery. Under normal conditions, approximately $1 \times 10^{11}$
neutrophils are generated in the human bone marrow daily (Cartwright, Athens, & Wintrobe, 1964; Lerman & Kim, 2015) where they undergo terminal differentiation from myeloid precursors. Bone marrow hematopoietic cells can be subdivided into three groups: the stem cell pool, the mitotic pool, and the post-mitotic pool. The stem cell pool consists of undifferentiated hematopoietic stem cells, the mitotic pool refers to the multipotent progenitor cells that are undergoing differentiation, and the post-mitotic pool consists of fully differentiated cells (Demetri & Griffin, 1991; Summers et al., 2010). As new leukocyte production is required to replenish the dead and dying cells, multipotent progenitor cells differentiate into either a lymphoid or myeloid lineage by producing either common lymphoid progenitor cells or common myeloid progenitor cells respectively (Friedman, 2007). In the absence of infection or inflammation, myeloid differentiation commitment pathway serves as the default (Kondo et al., 2003). Common myeloid progenitor cells in turn can give rise to either megakaryocyte-erythrocyte progenitor cells or granulocyte-monocyte progenitor cells (Iwasaki & Akashi, 2007).

Following the commitment of granulocyte-monocyte progenitor cells to granulocyte lineage development, terminal neutrophil differentiation includes the myeloblast, promyelocyte, myelocyte, meta-myelocyte, and segmented (mature) neutrophil stages. It is known that neutrophils are key players in the acute inflammatory response that act as key effectors in host defenses against microbial infections through oxidative and nonoxidative mechanisms (Ganz & Lehrer, 1994; Ganz et al., 1985; Lehrer, Ganz, Selsted, et al., 1988; Tsutsumi-Ishii et al., 2000). Severe sepsis, as well as other diseases associated with SIRS, is characterized by a marked increase (up to 10-fold) in the bone marrow production of neutrophils and the recruitment of immature neutrophils into the circulation (Smith, 1994). This is commonly referred to as a “left shift” with increased circulating hypossegmented neutrophils (“band forms,” up to 30–50% (Orr, Taylor, Bannon, Geczy, & Kritharides, 2005). Neutrophil migration is critical for pathogen clearance and host survival during severe sepsis (Lerman & Kim, 2015). Neutrophils circulate in the bloodstream and emigrate into tissues in response to signals released in the microenvironment of infection and tissue injury (Marshall et al., 2005). Neutrophil chemotaxis is promoted by signals emanating from the inflammatory focus, and it results
in the accumulation of large numbers of neutrophils at the site of an acute threat (Marshall, 2005).

Importantly, previous studies indicated that circulating neutrophils during sepsis are less mature overall than those obtained from healthy subjects. It has also been revealed that circulating neutrophils from patients with sepsis and SIRS are slightly less potent in supporting innate immune defenses than mature neutrophils (Drifte et al., 2013).

Furthermore, under normal conditions in the circulation, neutrophil turnover is extremely rapid, with estimates of neutrophil half-lives ranging from 6–10 h to 5 days (Kolaczkowska & Kubes, 2013; Summers et al., 2010), after which they undergo apoptosis spontaneously. Neutrophil survival can be prolonged by exposure to a wide variety of inflammatory mediators of both host and microbial origin (Savill, 1997). In fact, in the presence of pro-inflammatory mediators, microbe-derived molecules such as endotoxin (LPS) and in particular environmental conditions such as hypoxia, the neutrophil life span is significantly extended (Kolaczkowska & Kubes, 2013; Lerman & Kim, 2015). In sepsis, a profound inhibition of neutrophil programmed cell death has been reported (Lerman & Kim, 2015; Tamayo et al., 2012). Prolongation of the neutrophil lifespan is a process requiring new gene expression and protein synthesis through a number of poorly understood mechanisms, including the inactivation of pro-apoptotic enzymes and upregulation of anti-apoptotic proteins (Marshall, 2005). Overall, the deregulation of neutrophil programmed cell death is associated with the accumulation of activated neutrophils at the site of inflammation. This, in turn, can contribute to the exacerbation of the systemic inflammatory response and, consequently, the induction of host tissue damage as well as consecutive organ failure in critically ill patients.

In our study, septic or infected patients had higher neutrophil counts than nonseptic patients and uninfected patients. Despite not a significant difference between the groups, these data suggest that in the presence of infection, there are more neutrophils circulating in plasma than in the noninfected state. Despite higher number of neutrophils in the circulation, they may be immature and not as functional as those found in noninfected patients. Moreover as outlined previously, this difference in circulating neutrophil
numbers could be explained by delayed apoptosis or sequestration of neutrophils at sites of infection.

This raised the question whether the activity of neutrophils affects the plasma HNP concentration? Although, we found no significant correlation between plasma HNP concentrations and plasma neutrophil numbers (rho = 0.11, P = 0.1), from our data, critically ill patients who were not infected had higher plasma HNP concentrations than their infected counterparts. This suggests that the difference could be attributable to neutrophil activity.

From the literature, it is known that when a patient becomes infected or septic, there are various stages of the process that in turn can affect neutrophil activity. In early sepsis, there is evidence of excessive migration and activation of phagocytes and DCs (Bosmann & Ward, 2013). Excessive neutrophil migration during the early stages of sepsis may lead to an exaggerated systemic inflammatory response with associated tissue damage and subsequent organ dysfunction. As sepsis progresses, however, there is progressive functional deterioration of these cells (Bosmann & Ward, 2013), which results in degraded innate immune functions (such as phagocytosis, chemotaxis, and the ability to kill phagocytized organisms). Furthermore, dysregulation of migration and the insufficient migratory response that occurs during the later stages of severe sepsis can impact neutrophils’ ability to contain and control infection and result in impaired wound healing (Lerman & Kim, 2015). Perhaps, noninfected critically patients have more active neutrophils releasing more HNP into their plasma. Specifically, in critically ill patients who were not fighting an infection but nevertheless had a systemic inflammatory response secondary to their critical illness, their neutrophils were more active in their ability to phagocytose and release their granules containing HNPs.

Also importantly, neutrophils contain a rich supply of the green heme enzyme MPO, which in combination with H2O2 and chloride constitutes a potent antimicrobial system (Klebanoff, 2005). MPO accounts for approximately 5% of the total neutrophil protein and is a major granule constituent (Klebanoff, 2005). Activated neutrophils and macrophages generate amounts of ROS and RNS (Anderson, Hazen, Hsu, & Heinecke,
1997); additionally, activated neutrophils produce H2O2, and neutrophil MPO generates derivatives of H2O2 (Anderson et al., 1997). These compounds, in turn, are involved in the intracellular killing of bacteria and are also linked to tissue damage, increased vascular permeability, and organ injury (Welbourn et al., 1991; Windsor, Mullen, Fowler, & Sugerman, 1993).

In our study, MPO activity was measured in two subgroups: patients who were infected and those who were not infected but had a systemic inflammatory response. Critically ill patients had higher MPO activity than patients without evidence of infection. These findings suggest that in patients with infection, either (a) MPO may be consumed to help fight infection or (b) their neutrophils are too immature that their ability to be activated is compromised or (c) perhaps in patients with infection, administration of antimicrobial therapy can dampen the systemic inflammatory response and thereby lower MPO activity.

Another possible explanation for this discrepancy is that noninfected patients have a greater systemic inflammatory response. This in turn activates more neutrophils which release MPO resulting in higher plasma HNP concentrations, and increased MPO activity and possibly greater effects on tissue dysfunction.

In keeping with this, our study results also showed that noninfected patients required more hemodynamic support with vasopressors. From the literature, we know that catecholamine use affects leukocyte activity, in that catecholamines modulate the phagocytosis process by enhancing peripheral blood neutrophil phagocytic function (Ortega et al., 2005). This suggest that neutrophils in critically ill patients who were not infected were more active. That is their phagocytic abilities were greater thereby by doing so releasing more HNP in their plasma.
6.3 Is HNP Plasma Concentration Associated with Organ Perfusion and Dysfunction?

Our study examined biochemical markers currently used in clinical practice as surrogate markers of overall organ dysfunction, namely MODS and plasma lactate concentrations. Specific organ markers were also examined including plasma creatinine, PaO2/FiO2 ratios, and hemodynamic instability as reflected by vasopressor use.

In our study, MODS scores differed between critically ill patients with infection and those without infection; specifically, critically patients with no infection had worse MODS scores (mean 8.3 ± 3.9) compared to those with infection (mean 7.9 ± 2.8). In the noninfected group, patients admitted with a cardiothoracic diagnosis had the worst MODS scores (mean 9.8 ± 4.0). This difference could be explained by the ability to treat an identifiable cause (infection) in patients with infection and thereby dampen the systemic inflammatory response which in turn prevent organ dysfunction. On the contrary patients who were noninfected, had no identifiable etiology that could be treated and therefore their systemic inflammatory response became unregulated thereby leading to organ dysfunction and higher MODS scores.

We know that patient plasma samples were drawn within the first 24 hours of recruitment into the DYNAMICS study, but we do not know whether samples were obtained before or after the administration of antibiotics. Assuming that patients who were deemed septic were immediately administered antibiotics, the use of antimicrobial therapy may have dampened the systemic inflammatory response in our infected population, whereas in noninfected patients, the systemic inflammatory response remained activated, resulting in an imbalance between pro-inflammatory and anti-inflammatory mediators and as a consequence greater organ dysfunction and worse MODSs (Schulte, Bernhagen, & Bucala, 2013).

We further examined whether there was a relationship between plasma HNP concentrations and MODSs. A moderate correlation between plasma HNP concentration and MODSs was found (rho = 0.39, P < 0.001). The association was further explored
using ANCOVA, and we found that plasma HNP concentrations were significantly (P = 0.007) correlated with MODSs. Moreover, the difference in MODSs between infected and noninfected patients was also significant (P < 0.001). In summary, these findings suggest that higher plasma HNP concentrations in our patient population were associated with worse MODSs and therefore greater organ dysfunction.

Similarly, serum lactate content was more strongly elevated in critically ill patients who were not infected than in those who were. In particular, patients admitted with a cardiothoracic diagnosis had the highest mean lactate plasma concentration. The correlation between plasma lactate and plasma HNP concentrations was moderate (rho = 0.38, P < 0.001).

According to the literature, plasma lactate is affected by the degree of hypoperfusion. Therefore, these results suggest that ischemia-reperfusion leads to clinical sequelae, including activation of pro-inflammatory mediators and eventually organ injury. It was possible that the critically ill patients in our study who were not infected had a greater degree of hypoperfusion relative to infected and critically ill patients.

Moreover, many of the critically ill patients in the noninfected group were postoperative patients with histories of coronary bypass surgery, valve replacement, trauma, or postmyocardial infarction. According to the literature, after prolonged ischemia, the restoration of blood flow induces the production of ROS and cytokines, namely TNF-alpha, ILs, and peptide-activating factor. These in turn increase neutrophil infiltration. Neutrophils secrete pro-inflammatory cytokines and chemokines to create a positive feedback loop of neutrophil recruitment and activation. Although these mediators are important for host defense, they also promote tissue damage (Lerman & Kim, 2015). Interestingly, patients in the uninfected group had higher serum lactate levels and plasma HNP concentrations than infected patients and healthy controls. These results suggest that plasma HNP may have diagnostic value in distinguishing critically ill patients who have a higher degree of hypoperfusion and are thereby at greater risk for organ dysfunction.
6.3.1 Specific Organ Dysfunction:

No specific organ dysfunction index was revealed to have a significant correlation with plasma HNP concentrations.

Noninfected patients exhibited a slighter higher degree of renal impairment, as reflected in their mean serum creatinine concentration, than infected patients. Similarly, cardiothoracic patients displayed a moderate degree of renal impairment. Given the large number of patients in the noninfected group who had histories of surgery or trauma, this difference could most likely be due to pre-renal causes (mainly ischemia or hypovolemia). Meanwhile, serum creatinine levels were not correlated with plasma HNP concentrations in our population cohort.

PaO2/FiO2 ratios were compared for the different subgroups. All groups exhibited some degree of lung injury. Patients with infection had lower PaO2/FiO2 ratios, suggestive of more lung injury in this subpopulation. This could either be explained by pneumonia being the primary cause of septic shock and therefore compromising respiratory status. This difference was statistically significant between groups.

There was no significant correlation between the plasma HNP concentration and PaO2/FiO2 ratio, underlining the fact that lung function contributes to the systemic inflammatory response but does not significantly influence the release of HNPs by neutrophils.

Hemodynamics and vasopressor requirements were examined as surrogate markers of instability and organ dysfunction. Patients with higher plasma HNP concentrations had more cardiovascular instability. In fact, 58% of patients with plasma HNP concentrations exceeding 9999 pg/mL had more hemodynamic instability requiring more inotropic support. Similarly, patients with plasma HNP concentrations exceeding this threshold had a higher temperature (38.3°C) than those with lower plasma HNP concentrations (37.3°C). These differences affected physiological responses, which included neutrophil activation and degranulation, and later measurements of biochemical organ dysfunction.
In summary, ischemia-reperfusion injury in any organ can result in SIRS and multi-organ failure. Neutrophil migration and involvement in the inflammatory cascade are fundamental. The systemic inflammatory cascade affects tissue injury, hemodynamic instability, and organ dysfunction. Moreover, during the early stages of sepsis, pro-inflammatory chemokines and cytokines prime and guide neutrophils out of circulation to the sites of infection, thus allowing efficient bacterial phagocytosis and apoptotic cell clearance to occur. Proteolytic enzymes stored in azurophilic granules within neutrophils are released in response to a variety of stimuli at infected sites, including HNPs. Thus, in uncontrolled inflammatory conditions such as severe sepsis, during which many neutrophils become activated at the endothelial surface and in the underlying extravascular space, excessive inflammation leads to severe microvascular damage and dysfunction (Wright, Moots, Bucknall, & Edwards, 2010).

Our data indicate that plasma HNP concentrations are correlated with neutrophil activity. Furthermore, plasma HNP concentrations are correlated with worsening hemodynamics, MODSs, and plasma lactate levels. Therefore, based on our results the ability of HNPs to identify overall organ dysfunction appears to be correlated with plasma HNP concentrations in critically ill but not uninfected patients.

6.4 Is HNP Plasma Concentration Associated with Mortality?

Our data indicated that infected patients had a better overall survival rate than uninfected patients. At 30 days, 84.8% of patients with infection were alive, compared to only 66.7% of patients who were not infected. Patients admitted with a cardiothoracic diagnosis had the worst mortality rate of 50%. This discrepancy could be explained by the fact that in patients with infection, antibiotics were administered, which could have affected their systemic inflammatory response, thereby suppressing HNP release and in turn preventing organ dysfunction and mortality.

Further, patients with a cardiothoracic diagnosis may have had a greater systemic inflammatory response, thereby resulting in greater numbers of neutrophils being activated and more HNPs being released into their plasma and leading to further organ dysfunction and finally death.
6.5 Plasma HNP Concentrations and Confounders

Previous studies indicated that HNPs have both pro-inflammatory and antimicrobial properties and that their plasma levels are affected by a number of factors including age, gender, and comorbidities (Ganz & Lehrer, 1995; Hazlett & Wu, 2011). For our patient population, when we considered potential confounders of HNPs, we found that there was no correlation between plasma HNP concentrations and mortality.

6.6 Limitations

Our study had several potential limitations. First, a portion of our study was based on retrospective analyses of a large database. Although the information is more readily available, retrospective analyses of databases are often times associated with limitations. These include both patient recruitment and data selection biases. Our study patients were recruited using the selection criteria outlined in the DYNAMICS study. Many patients were misclassified as nonseptic (and vice versa). Moreover, a retrospective study does not account for confounders, which could be of importance when interpreting the potential abilities of a biomarker.

Additionally, an ideal control group for our study would have been patients who were age-matched to our study population with the same gender distribution and similar with respect to the number and type of comorbidities (calculated using the Charlson Comorbidity Index Score) and exhibiting APACHE II scores of less than 22. Our two populations derived from the DYNAMICS study were septic and non-septic. The non-septic population consisted of a very heterogeneous group of patients and perhaps this is not an ideal control group. Despite this, patients in both septic and non-septic groups were divided into presence or absence of infection. HNP was helpful in discriminating patients who were infected and non-infected ones who were initially categorized in the sepsis group. Unfortunately it was not possible to investigate whether HNP can predict the occurrence of a secondary infection in a patient initially admitted for another reason. This would have required monitoring microbiology for a number of days for the diagnosis of infection.
Despite there were some imbalances with respect to comorbidities in the two groups, the noninfected group was used as a control. Chronic renal failure, immunocompromise were not distributed equally between infected and noninfected patients. Although the non-infected group of patients had different admitting diagnosis, all these patients had some form of inflammatory response. This observation is further observed in non-infected patients and their use of vasopressors. Vasopressors affect leukocyte activation and degranulation. They also affect lactate production.

The analysis between neutrophil activity and HNP has some limitations. Any posthoc subdivision of a cohort introduces a significant risk of reporting associations due to chance.

Furthermore, many patients in our study had incomplete patient records. This affected various data calculations, and our initial numbers could not be included in all our calculations. For example, inotropic data were minimal. The database simply indicated whether the patient was or was not using inotropes. The information unfortunately did not indicate the type or duration of inotropic therapy. This in turn may limit the generalization of our results and thereby affect our overall conclusions in that these results do not properly represent the biological features of HNPs as a marker of organ dysfunction.

Importantly, our study was relatively small for the proper clinical assessment of HNP’s properties as a diagnostic and prognostic biomarker. Our small sample size may limit the power of our study to confirm the prognostic abilities of this biomarker. In addition, the smaller sample size may limit our ability to detect significant interactions, which in general requires a larger sample size.

Lastly, our data indicated that higher plasma HNP concentrations are correlated with a greater degree of organ dysfunction in uninfected patients, in particular cardiac patients. Blocking or inhibiting HNP activity in patients who are not infected could be potentially
beneficial in the clinical setting.

CHAPTER 7 – CONCLUSIONS AND FUTURE DIRECTIONS

In summary, our study results demonstrate that despite similar plasma neutrophil counts, plasma HNP concentrations are higher in critically patients without infection than in critically ill patients with infection or healthy controls. This suggests that in uninfected patients, the pro-inflammatory properties of HNPs are predominant over their antimicrobial properties. This in turn results in a greater inflammatory response and higher neutrophil activity but not in a higher plasma neutrophil count.

Our study results indicated that plasma HNP levels are correlated with the severity of MODS and plasma lactate levels. Our results further indicated a positive trend between vasopressor use and plasma HNP concentrations. Higher plasma HNP concentrations were correlated with a greater degree of hemodynamic instability, higher MODSs, and higher plasma lactate levels. The correlations between these markers of organ dysfunction were strongest for patients without infection, in particular cardiothoracic patients. This suggests that HNPs could potentially be used as a marker of organ dysfunction in uninfected critically ill patients.

From our data, HNPs exhibit characteristics that may be valuable in distinguishing and diagnosing critically ill patients who are infected from those who are not infected. This can be useful in overall treatment and can perhaps influence prognosis.

Moreover, our results also demonstrated that plasma HNP concentrations are correlated with neutrophil activity. Higher plasma HNP concentrations were linked to greater neutrophil activity. This increased neutrophil activity affects the overall inflammatory response, which influences organ dysfunction and perhaps overall mortality. Lastly, as plasma HNP concentrations can predict the severity of the systemic inflammatory response and the degree of organ dysfunction in critically ill patients, decreasing plasma HNP concentrations could potentially dampen the inflammatory response and overall organ dysfunction.
7.1.1 Future Directions

With respect to future directions, a larger prospective study would be useful. Our results can help guide the design of a larger prospective study to properly evaluate the potential of HNPs as a prognostic biomarker of infection in critically ill patients. Given that plasma HNP concentrations provide a measure of ischemic reperfusion injury, it would be interesting to follow plasma HNP concentrations over time in a patient population.

Furthermore in devising a larger prospective study, a control population that was closer to the study population could be selected. This would allow for better control of HNP confounders and better collection of data.

Moreover a larger study would substantiate our results and perhaps an inhibitor of HNP could be designed to help change the course of the systemic inflammatory response and even mortality.
References


Appendix A

DYNAMICS Study (Clinical Trials.gov Identifier: NCT01355042).

INCLUSION criteria for Severe Sepsis / Septic Shock:
Patients must have A or B and C
A. Severe Sepsis
   1. ≥ 3 SIRS criteria (systemic inflammatory response syndrome)
   2. Infection (suspected or confirmed)
   3. ≥ 1 acute organ dysfunction
B. Septic Shock
   1. All of the above in “A” and currently on vasopressors
C. Patient expected to remain in ICU for ≥ 72 hrs

INCLUSION criteria for Non-Sepsis:
Patients must have A or B or C
A. 1. Multiple Trauma with episode of Shock on presentation
   2. Patient expected to remain in ICU for ≥ 72 hrs
B. 1. Critically Ill
   i.e.: - ICH, SAH, SDH
   - No shock
   - No organ failure
   2. Patient expected to remain in ICU for ≥ 72 hrs
C. 1. Non-Septic Shock
   i.e.: - Cardiogenic - Hypovolemia
   - Heat Shock - Pulmonary Embolism
   - Burns: - AAA rupture
   2. Patient expected to remain in ICU for ≥ 72 hrs

Demographic Data
1. Baseline
   - Date of Birth; Gender; Height and Weight;
   - Admission diagnosis; Admission Type - Medical vs Surgical
   - Severity of Illness Measurement; APACHE
2. Presence of SIRS Criteria
   - Fever, Tachycardia, Tachypnea, Leukocytes
3. Presence of Organ Dysfunctions at the time of enrolment
   - Cardiac-SBP, Respiratory-P/F Ratio, Kidney-Creatinine, Hematological-Platelets
4. Chronic Health Variables

Daily Data
1. Interventions and therapies—Dialysis, Central Lines, Arterial Lines, Pulmonary Artery Catheters, Intra Aortic Balloon Pumps, Mechanical Ventilation, Insulin, Therapeutic Anticoagulation, Therapeutic Steroids, Inotropes
2. Specimen acquisition for Cell free DNA
3. New Sepsis (suspected or Confirmed)
4. Laboratory Results Relevant to Sepsis (i.e. White Cell Count, Lactate, p/f ratio etc)
5. ICU specific scoring systems for Critical Care: MODS and SOFA
6. Vital Signs—Including Temperature, Blood Pressure, Central Venous Pressure, Respiratory Rate
7. Fluid status—quantity of fluids for intake and output

Sepsis Specific Data
1. All microbiology culture data—including organisms, and site of specimen
2. All antibiotics, antimicrobial and antiviral agents administered
Appendix B

Blood Collection from Healthy Controls

Research ethics board approval obtained. Informed consent obtained.

Performed by: Medical Doctor

Protocol:

1. In a sterile fashion, blood is drawn from the examinee’s arm

2. Two vials of a 4.5-mL Vacutainer will be used to draw blood from each healthy volunteer

3. Twenty microliters of whole blood will be taken from one of the 4.5-mL vials and placed in a COULTER® AcT diff2™ Hematology Analyzer for neutrophil counting

4. The remainder of the blood in the vials will be stored on ice (−4°C) until the neutrophil counting is completed (i.e., 5 min)

5. The two 4.5- mL vials will be centrifuged at 1500 × g for 10 min at 4°C using a Allerga 6 R Centrifuge (Beckman Coulter)

6. Plasma will be collected and aliquoted in Eppendorf tubes at approximately 200 μL per tube

7. The plasma is immediately stored at −80°C

Total Amount of Blood Collected:

1 vial (BD Vacutainer, Buffered Cit. Na (9NC) = 4.5 mL. Total of two vials per healthy volunteer each time
Appendix C

Charlson Comorbidity Index

(http://www.fpnotebook.com/prevent/Exam/ChrlsnCmrbdtyIndx.htm)

AKA: Comorbidity-Adjusted Life Expectancy

1. Indication
   1. Assess whether a patient will live long enough to benefit from a specific screening measure or medical intervention
2. Scoring: Comorbidity component (Apply 1 point to each unless otherwise noted)
   1. Myocardial infarction
   2. Congestive heart failure
   3. Peripheral vascular disease
   4. Cerebrovascular Disease
   5. Dementia
   6. Chronic obstructive pulmonary disease (COPD)
   7. Connective tissue disease
   8. Peptic ulcer disease
   9. Diabetes Mellitus (1 point uncomplicated, 2 points if end-organ damage)
  10. Moderate to severe chronic kidney disease (2 points)
  11. Hemiplegia (2 points)
  12. Leukemia (2 points)
  13. Malignant lymphoma (2 points)
  14. Solid tumor (2 points, 6 points if metastatic)
  15. Liver disease (1 point if mild, 3 points if moderate to severe)
  16. Acquired immune deficiency syndrome (AIDS) (6 points)

3. Scoring: Age
   1. Age <40 years: 0 points
   2. Age 41–50 years: 1 point
   3. Age 51–60 years: 2 points
   4. Age 61–70 years: 3 points
   5. Age 71–80 years: 4 points

4. Interpretation
   1. Calculate Charlson Score or Index (i)
      1. Add comorbidity score to age score
      2. Total denoted as ‘’ below
   2. Calculate Charlson Probability (10-year mortality)
      1. Calculate $Y = e^{(i * 0.9)}$
      2. Calculate $Z = 0.983^Y$
      3. where Z is the 10-year survival

5. References
   1. Charlson, 1987