Evaluation of the Bactericidal Function of Neutrophils in Cats with Chronic Diseases

by
Parker Hoppe

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biology
(Honors Scholar)

Presented March 6th, 2025
Commencement June 2025

AN ABSTRACT OF THE THESIS OF

Parker Hoppe for the degree of Honors Baccalaureate of Science in Biology presented on March 6, 2025.
Title: Evaluation of Bactericidal Function of Neutrophils in Cats with Chronic Diseases.

Abstract approved:_____

Luiz Bermudez

Neutrophils are phagocytic cells involved in innate immunity, the first line of defense against foreign pathogens. These cells traffic to the bloodstream reaching tissues in which pathogens are present through activation of the complement system, a cascade of enzymatic reactions that opsonizes, or marks, a pathogen for neutrophilic attack. Upon recognition of the pathogen, neutrophils can employ several methods of eradication, such as engulfment, deployment of neutrophil extracellular traps (NETs) and exposure to toxic antimicrobial proteins. Chronic diseases, such as kidney disease, diabetes mellitus, and leukemia can result in premature release of neutrophils from their maturation sites and subsequently impair neutrophil function compared to that of healthy individuals. This research sought to evaluate the neutrophil function of felines with chronic diseases compared to cats without a diagnosis of chronic disease. Inoculated *Escherichia coli* strain OP50 (*E. coli*) was added to samples of fresh whole blood. Following incubation, blood samples were plated to determine the number of bacteria present at successive time points. This process was then repeated with isolated blood plasma. Feline patients with chronic diseases had a higher mean neutrophil count compared to feline patients without a chronic disease diagnosis. Additionally, feline chronic disease patients experienced an increase of E. coli clearance from 2 hours to 24 hours post-infection on average compared to control patients without chronic disease.

Keywords: neutrophils, neutrophil extracellular traps (NETs), kidney disease, diabetes mellitus, leukemia, *Escherichia coli*

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Introduction

The immune system is comprised of various cells that perform the function of protecting the body from infection (bacteria, fungi, or parasites) and tumors.[1] Innate and adaptive immunity are two types of immunity that act together to establish a barrier against pathogens. Adaptive immunity, the immunological memory, develops over time via repetitive exposure to antigens, such as when the body encounters an infectious microbe. This immunity is highly specific and generally takes 1-2 weeks post-exposure for the adaptive immune response to be detected.[2]

In contrast, innate immunity includes the mechanisms in place at birth to combat microbes, such as surface barriers (epithelial, mucosal) and circulating phagocytic cells in the bloodstream as well as those residing in tissues. Responding much faster than the adaptive immune response, it is considered the first line of defense against pathogens. Its effects can be immediate or even minutes to hours following exposure to a pathogen.[3] Cells associated with the innate immune system include phagocytes and natural killer cells.[3] Phagocytic cells (such as neutrophils, macrophages, and monocytes) surround pathogens, release enzymes that damage them, and ingest and kill the foreign aggressor.

The complement system is an integral component of innate immunity leading to the opsonization of pathogens, which facilitates phagocytosis by nearby neutrophils and macrophages. In addition, activation of the complement system stimulates the release of cytokines and chemokines, which promote chemotaxis of neutrophils to the injury site.[4] The complement system is activated through three different pathways: classical, lectin, and alternative. In the lectin pathway, when a pathogen first enters the body, its pathogen-associated molecular pattern (PAMP) is recognized by pathogen recognition receptor proteins (PRRs) circulating through the blood and lymph.[5] This recognition prompts a cascade of enzymatic reactions that cleaves complement proteins in the

blood, making them enzymatically active. Some complement proteins are cleaved into two segments: a and b. Cleavage of complement protein C3 exposes a thioester bond that becomes reactive with the hydroxyl and amino groups on the pathogen's surface, allowing segment C3b to form a covalent bond with the surface of the pathogen.[5] Opsonization of the pathogen by complement protein C3b is recognized by neutrophils in combination with the release of cytokines by macrophages, which aid in the attraction of neutrophils to the correct location within the tissue.[6]

Neutrophils mature in the bone marrow. Following maturation and upon entering the bloodstream, neutrophils can be attracted to specific tissues by chemotaxis, which involves detection of a gradient that redirects the neutrophils to the site of the pathogen or injury.[7] Capillary endothelial cells contain Weibel-Palade bodies, which release P-selectin when cytokines are detected.[8] P-selectin is a cell adhesion molecule (CAM) that allows circulating neutrophils flowing near the blood vessel wall to adhere to the endothelial cells and leave the bloodstream by a processes called margination and diapedesis to move towards the site of inflammation.[8]

Upon arrival at the infected area, neutrophils utilize many mechanisms to eliminate pathogens. Among them, phagocytosis and deployment of neutrophil extracellular traps (NETs). In phagocytosis, the neutrophil engulfs the pathogen, which triggers the production of superoxide anion via an electron transfer from NADPH to oxygen (O_2^-) on the membrane of the neutrophil.[9] Superoxide anion is especially toxic to bacteria that lack superoxide dismutase (SOD), an enzyme that catalyzes the transformation of superoxide to hydrogen peroxide. Alternatively, a NET is used for pathogens that are too large to engulf, including fungi and parasites. NETs are also used for pathogens that produce enzymes to neutralize reactive oxygen species (SOD and

catalase) and thus are not harmed by superoxide anion upon being engulfed. NETs are made up of DNA and histone fibers and can entrap pathogens that are then exposed to toxic antimicrobial proteins (such as, myeloperoxidase, elastase, and Cathepsin G).[10]

Effectiveness of immune responses can be affected by underlying factors such as chronic disease as seen in human patients. These diseases can impede the ability of neutrophils to function effectively in response to pathogens and our understanding of chronic diseases and their role in neutrophil function in felines, nor any species of animal with chronic disease is lacking. In human patients with diabetes, high fasting plasma glucose is correlated with lowered phagocytic activity.[11] However, administration of insulin to lower glucose levels in diabetic rodent patients has been demonstrated to reduce the impairment of the disease on neutrophil function.[12] This is because unregulated, high glucose levels cause saturation of the hexokinase pathway of glucose metabolism and glucose instead goes through the polyol pathway to become sorbitol, which diminishes available NADPH, thus reducing activity of NADPH oxidase.[13] NADPH oxidase is necessary for neutrophil production of superoxide anions, which is necessary to kill bacteria.

Another example is high potassium levels being common amongst patients with chronic kidney disease as the condition impairs their ability to excrete excess potassium from the bloodstream as a result of decreased efficiency of glomerular filtration.[14] Previous research has indicated that neutrophil function may be linked to ATP-sensitive potassium channels.[15] Additionally, a study involving 32 patients with a diagnosis of stage 5 chronic kidney disease being treated with haemodialysis found that the presence of endotoxins in blood resulted in an impairment of neutrophil function compared to patients undergoing alternative forms of treatment, such as peritoneal dialysis and kidney transplantation.[16] This finding illustrates the potential impacts of medication used for management of a chronic condition on neutrophil function. Additionally, the

neutrophil locomotion of patients with chronic liver disease (alcoholic liver disease or chronic hepatitis) was reduced compared to patients with other forms of liver disease (primary biliary cirrhosis or cryptogenic cirrhosis).[17]

In the case of a rapidly progressing disease such as cancer, high demand for neutrophils can result in premature release prior to completion of development, resulting in impaired functionality when compared to neutrophils that complete the developmental cycle.[18]

For more localized diseases such as chronic rhinitis and oral and nasal masses, less is known in terms of the impacts on immune function. However, previous literature cites the role of neutrophils in creating inflammation through attraction of eosinophils and priming of T cells in human patients with allergic rhinitis.[19] This project seeks to address the lack of knowledge of neutrophil function in felines with chronic disease. The hypothesized result is that feline patients with systemic chronic disease, such as diabetes, leukemia, and kidney disease, will demonstrate impaired neutrophil function compared to control patients.

Materials and Methods

Blood collection

Feline blood samples were collected from the Lois Bates Acheson Veterinary Teaching Hospital and Corvallis Veterinary Hospital and stored in 1 mL EDTA tubes. Animals were bled to fulfill diagnostic needs with a small aliquot set aside for our studies. Blood was utilized less than 2 hours after each blood sample was obtained. There were 23 blood samples in total: 11 collected from cats without a specific diagnosis of disease and 12 from felines with diagnoses of various chronic diseases. Neutrophil counts were analyzed at the Oregon Veterinary Diagnostic Laboratory for blood samples obtained from the Lois Bates Acheson Veterinary Teaching hospital and Antech Diagnostics for blood samples obtained from Corvallis Veterinary Hospital.

Ex vivo feline whole blood killing assays

E. coli culture was maintained on Luria-Bertani (LB) agar plates and incubated at 37°C for 24-48 hours before use in the following assays. A concentration of 1 x 10^8 OP50 *E. coli* was created in PBS based on 1 McFarland standard and diluted to a final concentration of 1 x 10^6 CFU/mL. A volume of 0.3 mL of whole blood was infected with 33 µL of a suspension of *E. coli* in PBS (at a bacterial concentration of $1x10^6$). The blood with the bacterial suspension was then placed into an incubator at 37°C and 5% CO_2, to maintain cellular function, for thirty minutes. Then, the blood was removed from the incubator and a volume of 100 µL was plated on LB agar plates before being returned to the incubator and counted after 24 hours of growth.

At 2 hours post-infection, the blood was then removed from the incubator and a volume of 100 µL was serially diluted in water to concentrations of 1:10, 1:100, and 1:1,000 using a sterile 48-well plate. Serial dilutions were then plated onto LB agar plates and incubated at 37°C and 5%

CO_2 for 24 hours, after which this process was repeated to establish a 24-hour bacterial count post-infection.

Ex vivo complement system assays

Plasma was isolated from the same feline whole blood as above. Blood was placed in a microcentrifuge tube and spun at 1,000 RPM for ten minutes to separate the plasma from the blood cells and platelets. The plasma was then transferred to a separate microcentrifuge tube and frozen at 0°C until future use, with each sample yielding approx. 50 µL of plasma. The same infection protocol described above was repeated with plasma. A volume of 30 µL of plasma was infected with a volume of 3.33 µL of suspension of *E. coli* in PBS solution, yielding a final bacterial concentration of $1x10^5$. The plasma with the bacterial suspension was then placed into an incubator at 37°C and 5% CO_2 for thirty minutes. Thirty minutes post-infection, the plasma was removed from the incubator and a volume of 10 µL was plated onto LB agar plates before being returned to the incubator. The plasma was then incubated at 37°C and 5% CO_2 until 2 hours post-infection, at which time the number of bacterial colonies was determined. This was repeated at the 24-hour time point, plating a volume of 10 µL of each dilution.

Statistical Analysis

Following data collection, a two-sample T-test assuming unequal variances was performed to compare the mean neutrophil count (in CFU/mL) of the eleven samples from control felines to the twelve samples of felines with chronic diseases. Additionally, a second T-test assuming unequal variances was performed to compare the mean CFU/mL at 2 hours post-infection for the three samples from felines with chronic kidney disease to the mean CFU/mL at 2 hours post-infection for the eleven control felines. A third T-test assuming unequal variances was performed

to compare the mean CFU/mL at 2 hours post-infection for the three samples from felines with masses (nasal, oral) to the mean CFU/mL at 2 hours post-infection for the eleven control felines.

Results

Neutrophils in felines with chronic disease alter clearance of *E. coli*

To determine the neutrophil function of felines with various chronic diseases we utilized whole blood challenged with non-virulent *E. coli* and compared them to healthy felines. Neutrophil quantification of blood from 11 felines without chronic disease diagnoses (control) is shown in Table 1. Normal neutrophil counts range from 2.5 x 10^6 to 12.5 x 10^6 cells/mL of blood, and all control patients fell within this range. Neutrophil regulation of *E. coli* infected whole blood was observed at indicated timepoints in control felines (Figure 1). *E. coli* colonies present in 100 µL of whole blood increased in number from 30 minutes to 2 hours post-infection for most patients except P3 and P11. From 2 hours to 24 hours post-infection, all control patients reduced *E. coli* CFUs except P5.

Neutrophil quantification of blood from 12 patients with chronic diseases is shown in Table 2. Notably, the diabetic feline had elevated levels of neutrophils, 17.0 x 10^6 cells/mL. Felines with an oral mass and leukemia, respectively, had neutrophil counts at the top of the normal range (12.0 x 10^6 cells/mL for both). Neutrophil regulation of *E. coli* infected whole blood was observed at indicated timepoints in felines with chronic disease (Figure 2). Patients with chronic disease (hyperthyroidism, liver disease, nasal mass, and chronic rhinitis) experienced an increase in CFUs in whole blood from 30 minutes to 2 hours post infection followed by decreases from 2 hours to 24 hours post-infection. Patients with an oral mass, two with chronic kidney disease, diabetes, and a nasal mass (2) experienced an increase in CFUs from 30 minutes to 2 hours post-infection. Agar plates at 24 hours post-infection did not grow *E. coli* colonies. The remaining patients (lymphoma, hypertrophic cardiomyopathy, and CKD1) experienced a decrease in CFUs from 30 minutes to 2 hours post-infection. Again, agar plates at 24 hours post-infection had no *E.*

coli colony growth. It is unknown whether the patients with masses are cancerous. We then concentrated on comparing the reduction in E. coli from 2 hours to 24 hours post-infection for felines with chronic disease by determining the percent decrease in CFUs. The patient with liver disease experienced a less than 10% reduction in CFUs from 2 hours to 24 hours post-infection, while the patients diagnosed with hyperthyroidism, a nasal mass, and chronic rhinitis experienced 71%, 67%, and 56% reductions respectively (Figure 3).

Bactericidal activity of plasma isolated from felines with chronic disease

To determine the contribution of complement activation against *E. coli*, uninoculated whole blood from the same patients was centrifuged to collect plasma. The activation results in the formation of the membrane attack complex (MAC), which forms pores in the cell membranes of bacterial pathogens. The plasma of felines without diagnoses of systemic disease experienced a decrease in CFUs between 30 minutes and 2 hours post-infection for P1, P3, P6, P7, P10, and P11, while P2, P4, P5, P8, and P9 experienced an increase during this same period (Figure 4). The plasma of the patients with hyperthyroidism, diabetes, hypertrophic cardiomyopathy, and both patients with nasal masses experienced an increase in CFUs from 30 minutes to 2 hours post-infection, then a decrease from 2 hours to 24 hours post-infection. The patients with liver disease, lymphoma, chronic rhinitis, an oral mass, and all three patients with chronic kidney disease (CKD) experienced a decrease in CFUs over time. As seen previously, some patients had no *E. coli* colony growth on agar plates 24 hours post-infection (Figure 5).

Direct comparisons between control felines and chronic disease felines

The mean neutrophil count per mL of blood for chronic disease patients was then compared to that of the control patients. Felines with chronic disease had a mean of 5.1 x 10^6 neutrophils/mL of blood (N = 12) while the control patients had a mean of 8.0 x 10^6 neutrophils/mL. The mean neutrophil count of felines with chronic disease was significantly higher than control felines (Welch's t-test, t = -2.25, df = 15, p = 0.040) (Figure 6).

When comparing mean CFUs of *E. coli* in whole blood of felines with chronic kidney disease (N = 3) at 2 hours post-infection to control felines (N = 11), a significant reduction in bacterial load was observed (Welch's t-test, t = 2.69, df = 6, p = 0.036) (Figure 7).

When comparing mean CFUs of E. coli in whole blood of felines with masses (N = 3) at 2 hours post-infection to control felines, no significant effects to bacteria load were observed (Welch's t-test, t = 1.99, df = 12, p = 0.074) (Figure 8).

Table 1. Neutrophil counts (in cells/mL) for control patients obtained from analysis at the Lois Bates Acheson Veterinary Teaching Hospital. All values fall within the range of normal for felines (between 2.5 x 10^6 and 12.5 x 10^6 cells/mL). Control patients are numbered 1-11.

Patient Number	Neutrophil count (cells/mL)
P1	8.0 x 10^6
P2	4.0 x 10^6
P3	5.0 x 10^6
P4	9.0 x 10^6
P5	5.0 x 10^6
P6	7.0 x 10^6
P7	7.0 x 10^6
P8	5.0 x 10^6
P9	5.0 x 10^6
P10	4.0 x 10^6
P11	5.0 x 10^6

Figure 1. *E. coli* CFUs per 100 µL of whole blood at 30 minutes, 2 hours, and 24 hours post-infection for each of the 11 control patients on a logarithmic scale. Control patients are labeled P1 through P11. The blue bar (leftmost) describes the CFUs at 30 minutes post-infection. The orange bar (middle) describes the CFUs at 2 hours post-infection. The green bar (rightmost) describes the CFUs at 24 hours post-infection.

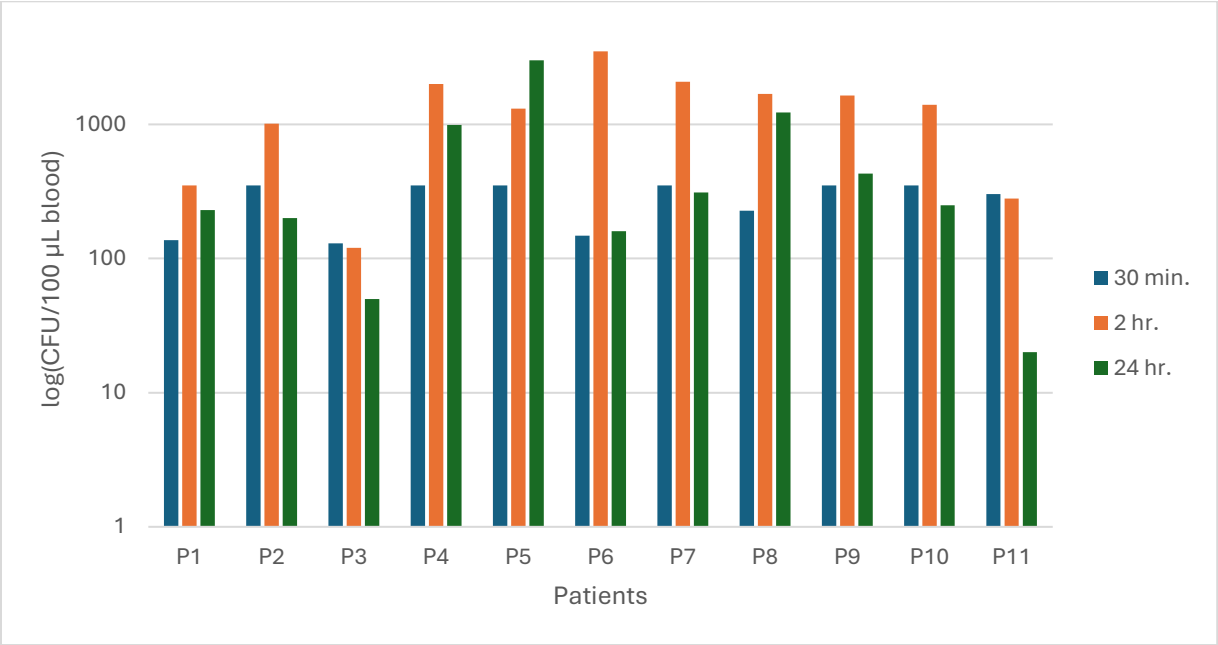


Table 2. Neutrophil counts (in cells/mL) for patients with chronic disease obtained from analysis at the Lois Bates Acheson Veterinary Teaching Hospital and by Antech. The patient with diabetes has a neutrophil count that falls outside of the normal range (>12.5 x 10^6 cells/mL) while the rest of the patients fall within the normal range (2.5 x 10^6 to 12.5 x 10^6 cells/mL). Four patients have neutrophil counts greater than 10.0 x 10^6 cells/mL, unlike the control patients which were all <10.0 x 10^6 cells/mL.

Patient Diagnosis	Neutrophil count (cells/mL)
Hyperthyroidism	7.0 x 10^6
Liver disease	4.0 x 10^6
Nasal mass	6.0 x 10^6
Rhinitis	7.0 x 10^6
Oral mass	12.0 x 10^6
CKD1	11.0 x 10^6
CKD2	8.0 x 10^6
CKD3	5.0 x 10^6
Lymphoma	12.0 x 10^6
Diabetes	17.0 x 10^6
Cardiomyopathy	6.0 x 10^6
Nasal mass 2	7.0 x 10^6

Figure 2. *E. coli* CFUs per 100 µL of whole blood at 30 minutes, 2 hours, and 24 hours post-infection for each of the 12 patients with chronic disease on a logarithmic scale. CFU counts at 2 hours post-infection are lower on average compared to the control group. Multiples of the same disease are numbered. Patient CKD2 has a secondary diagnosis of anemia and patient CKD3 has a nasal mass. The blue bar (leftmost) describes the CFUs at 30 minutes post-infection. The orange bar (middle) describes the CFUs at 2 hours post-infection. The green bar (rightmost) describes the CFUs at 24 hours post-infection.

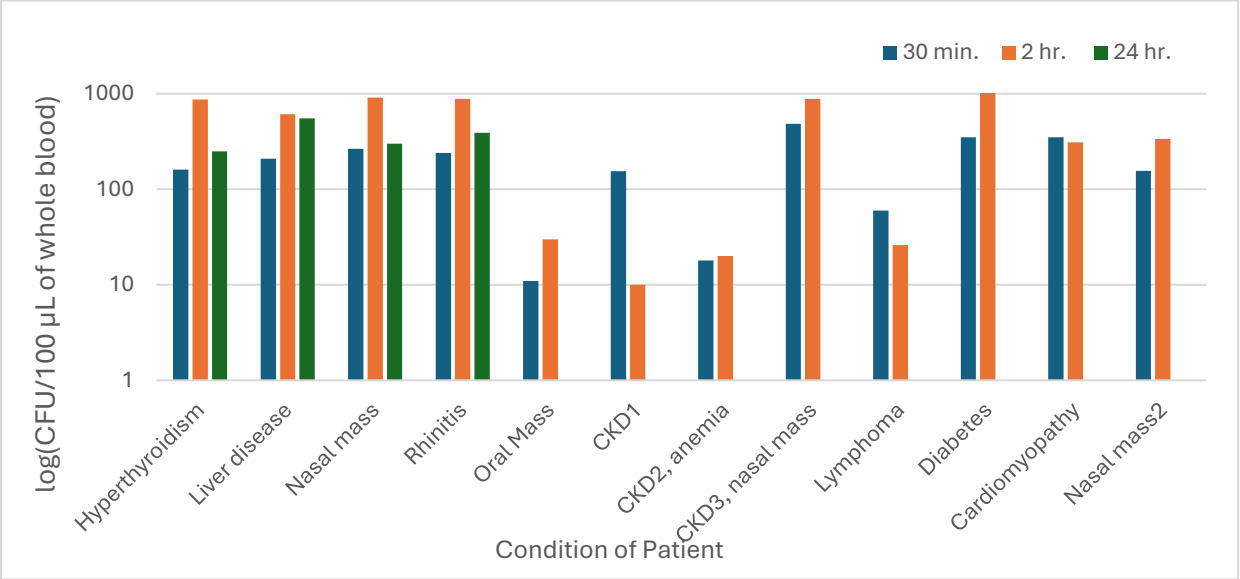


Figure 3. Comparison of the percent decrease in *E. coli* CFUs from 2 hours to 24 hours post-infection for the 4 patients that had countable colonies at 24 hours post-infection. The patient with liver disease experienced a smaller decrease in CFUs (9.8%) compared to the other three patients (71%, 67%, and 56%). Each patient is labeled by their respective condition and percent decrease in CFUs from 2 hours to 24 hours post-infection is represented by a blue bar.

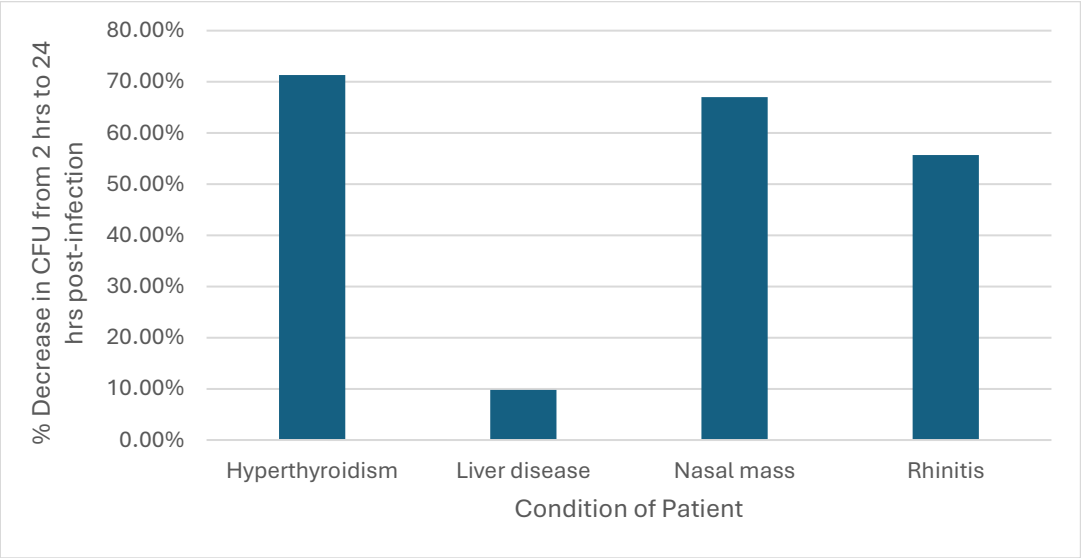


Figure 4. *E. coli* CFUs per 100 µL of plasma at 30 minutes, 2 hours, and 24 hours post-infection for each of the 11 control patients on a logarithmic scale. Most CFU counts are lower in the plasma compared to the whole blood. The blue bar (leftmost) describes the CFUs at 30 minutes post-infection. The orange bar (middle) describes the CFUs at 2 hours post-infection. The green bar (rightmost) describes the CFUs at 24 hours post-infection.

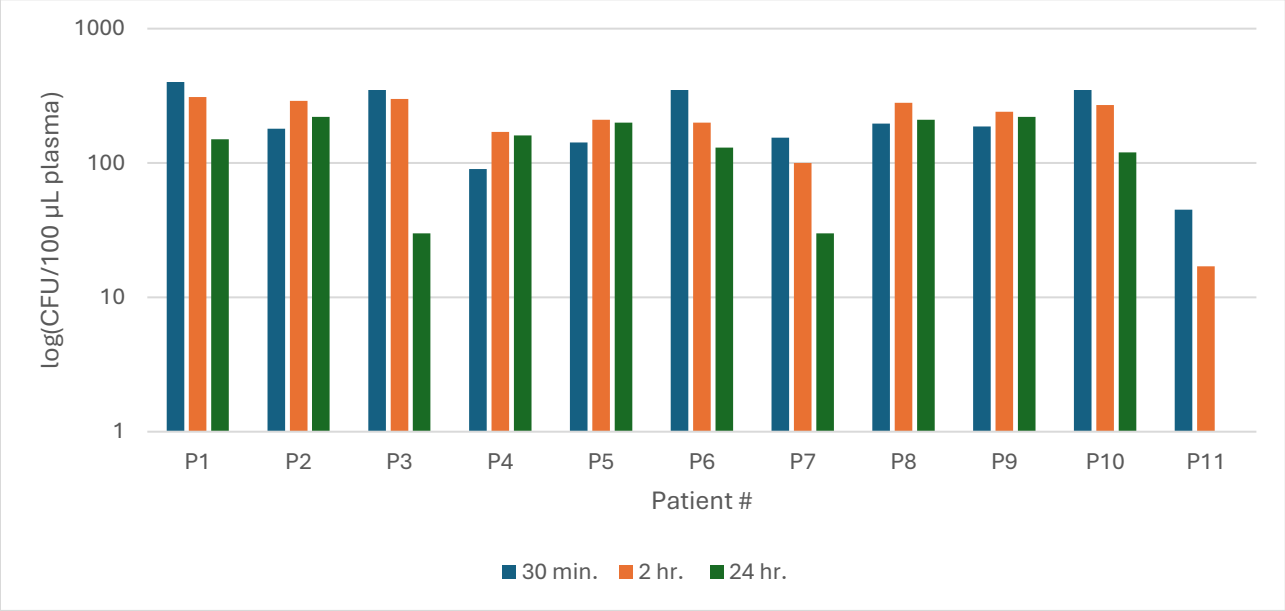


Figure 5. *E. coli* CFU count per 100 µL of plasma at 30 minutes, 2 hours, and 24 hours post-infection for each of the 12 patients with chronic disease on a logarithmic scale. Most CFU counts are lower in the plasma compared to the whole blood. The blue bar (leftmost) describes the CFUs at 30 minutes post-infection. The orange bar (middle) describes the CFUs at 2 hours post-infection. The green bar (rightmost) describes the CFUs at 24 hours post-infection.

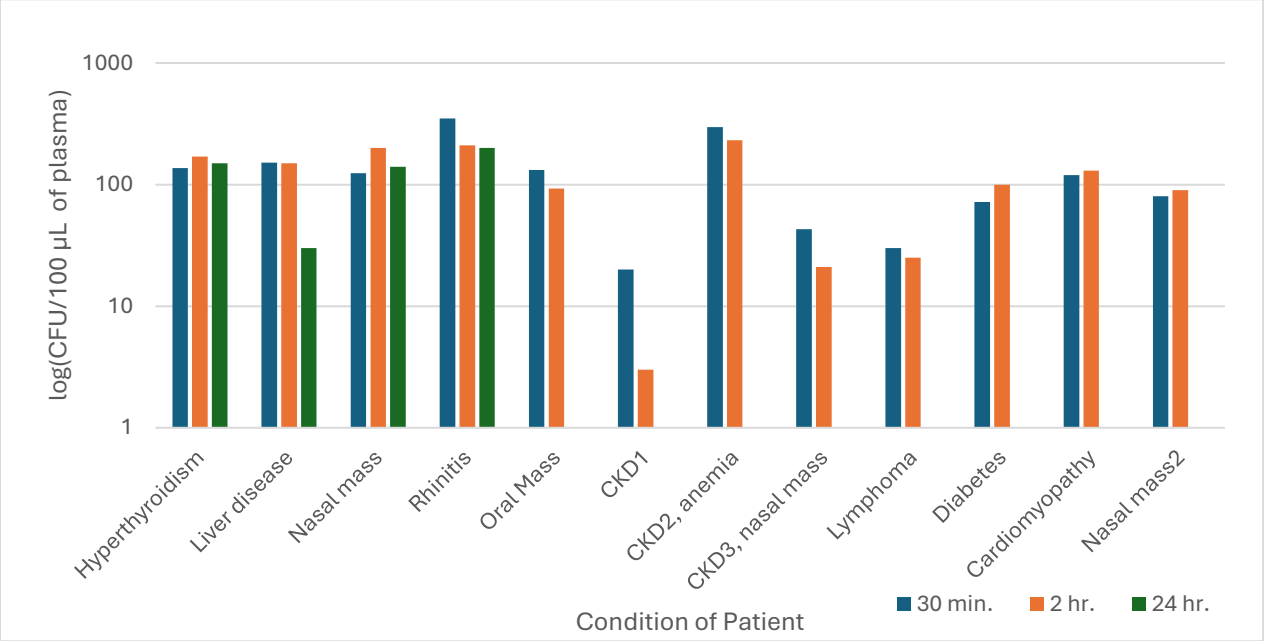


Figure 6. Comparison of the mean neutrophil count per mL of blood for felines with chronic diseases (right) and control patients (left). Chronic disease felines had a mean of 5.1 x 10^6 counts/mL with a sample size of N = 12, while control patients had a mean of 8.0 x 10^6 counts/mL with a sample size of N = 11. Felines with chronic disease have a significantly higher neutrophil count compared to the control patients (Welch's t-test, t = -2.25, df = 15, p = 0.040).

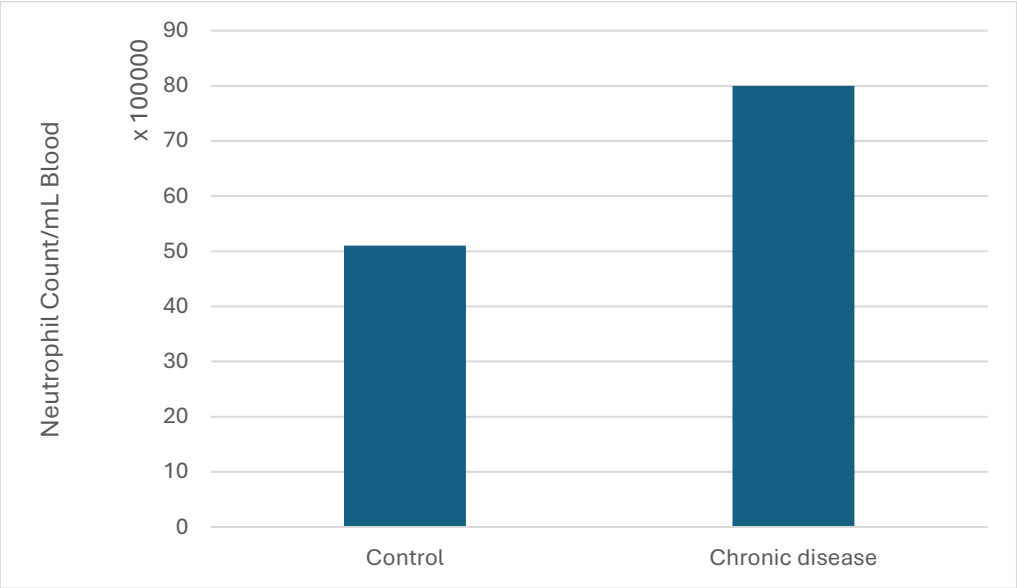


Figure 7. Comparison of the average CFUs of *E. coli* at 2 hours post-infection for the control patients (n = 11) and patients with chronic kidney disease (n = 3). Patients with chronic kidney disease (CKD) had a significantly lower mean CFU at 2 hours post-infection compared to control patients ($p = 0.036$). Blue bars represent the average CFU count at 2 hours post-infection for each of the two groups.

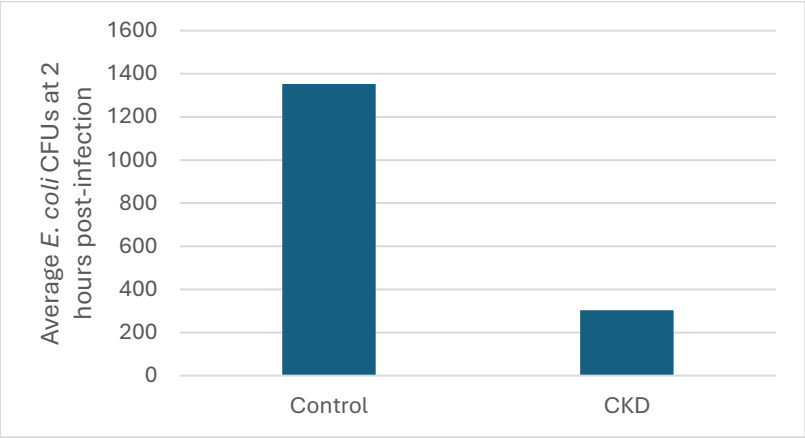
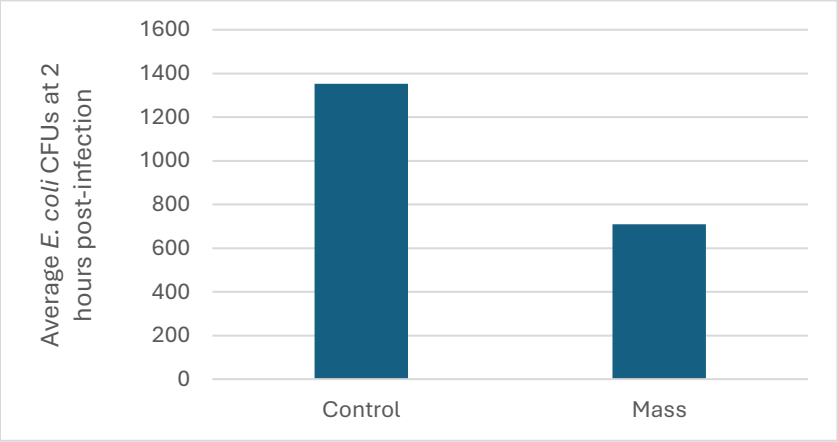


Figure 8. Comparison of the average CFUs of *E. coli* at 2 hours post-infection for the control patients (n = 11) and patients with masses (n = 3). There is insufficient evidence to determine that the means between the two groups are statistically significant ($p = 0.074$). Blue bars represent the average CFU count at 2 hours post-infection for each of the two groups.



Discussion

Initially, this research sought to evaluate the bactericidal function of neutrophils in felines diagnosed with diabetes compared to felines without. This was broadened to include felines with various chronic diseases (many of which are more commonly seen in clinical setting), such as chronic kidney disease and hyperthyroidism.

Complement-mediated opsonization, the method by which pathogens are labeled for phagocytic cells, is a process that begins immediately after the pathogen enters the body.[20] By 30 minutes post-infection, the majority of bacteria will have been opsonized and engulfed. The increase in CFU from the 30 minute to 2-hour time point may be due to engulfed bacteria taking longer than 30 minutes to fully degrade within a phagocytic cell, during which bacteria are able to replicate. Following 2 hours post infection, the effectiveness of the immune system for its ability to eradicate bacteria from the bloodstream appears to be more substantial, as the 24-hour incubation yielded a significant decrease in bacterial CFU compared to the initial count at the 30-minutes.

Analysis of blood plasma in addition to whole blood allows for isolation of the complement system including proteins that form. Results of these experiments demonstrated a reduction in the ability of the whole blood to kill *E. coli* compared to the plasma. This is illustrated by the large increase in CFU in the whole blood from 30 minutes to 2 hours post-infection.

Neutrophils held in the bone marrow may be recruited at higher rates to enter the bloodstream in patients with chronic diseases releasing cytokines to areas of damaged tissue. Certain cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF- α) promote both granulopoiesis in the bone marrow and recruitment of neutrophils to enter the bloodstream.[7]

Felines with chronic disease had lower CFUs at both 2 hours and 24 hours post-infection compared to felines without a diagnosis. This could have been attributed to a higher mean neutrophil count (measured in neutrophil count/mL). The mean neutrophil count of felines with chronic disease diagnoses was 8.0x10^6 compared to 5.1x10^6 for cats without, which is statistically significant at the 5% significance level (*P = 0.023)*. Similarly, it has been found that human neutrophil counts are higher in many patients with advanced forms of tumors.[21] In malignant cancer, heightened demand for neutrophils results in release of neutrophils that are immature and potentially have impaired function compared to a neutrophil with an average maturation time.[22] However, sheer number of neutrophils, even if they are impaired, may increase overall ability to eradicate this specific pathogen.

In the patient with liver disease, whole blood *E. coli* CFUs reduced by less than 10% from 2-hours to 24-hours post infection while felines with various other chronic diseases, such as kidney disease, experienced a greater percent decrease between the two time points. Most complement proteins are produced by the liver and spleen, and injury inflicted by liver disease can reduce the organ's ability to produce these proteins.[23] Reduction in complement protein production also has a profound impact on neutrophil recruitment because complement proteins are necessary for opsonization.

Due to the small number of samples obtained and wide range of diagnoses represented, there are too few patients to determine correlation. Future studies should build upon this concept and acquire a greater sample size, ideally at least 30 feline patients with chronic diseases. Knowledge about treatments and medications given to each patient with chronic disease could also further enhance the meaning of results. Additionally, data about the age of each feline patient is relevant to their immune functionality and should be considered in future research.

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