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Protein analysis of polymorphonuclear leukocytes from domestic cats

Análise de proteínas de leucócitos polimorfonucleares de gatos domésticos

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Highlights

A total of 102 proteins were identified in cat neutrophils.
Some proteins were less abundant and others were more abundant.
Proteoglycan-3 was the most abundant protein.

Abstract

Neutrophils are the first line of defense against microorganisms, making them a crucial component of innate immunity. This study aimed to characterize the neutrophil proteome of *Felis catus.* Granulocytes were isolated from cat blood and purified, and 1 × 10^7 cells were used to identify proteins. In total, 102 neutrophil proteins from cats were identified and categorized on the basis of their abundance. Proteoglycan-3, which is involved in various biological processes, including neutrophil activation and the immune response, was the most abundant protein. Among the metabolic pathways, glycolysis involved the largest group of identified proteins (nine proteins). Proteins involved in molecular functions, cellular components, and biological processes were also identified. The identification of proteins in this study is expected to aid future research by elucidating certain characteristics of the species, identifying potential markers or drugs, and facilitating rapid, non-invasive diagnosis of diseases that particularly affect felines.

Key words: *Felis catus.* Granules. Neutrophils. Proteomics.

Resumo

Os neutrófilos são a primeira linha de defesa contra os microrganismos, sendo assim, uma célula muito importante na imunidade inata. Objetivou-se com este estudo caracterizar o proteoma de neutrófilo

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de *Felis catus.* Para isso, os granulócitos foram obtidos de sangue de gatos, purificados e utilizados $1x10^7$ de células para identificação das proteínas. Foram identificadas 102 proteínas de neutrófilos de gatos, sendo classificadas conforme a abundância. A proteoglicano-3 associada a alguns processos biológicos como ativação dos neutrófilos e resposta imune, foi a proteína mais abundante. Dentre os processos, nas rotas metabólicas a maior quantidade de proteínas identificadas está envolvida na glicólise, que foram nove proteínas. Verificou-se também as proteínas envolvidas em função molecular, no componente celular e nos processos biológicos. Espera-se que a identificação das proteínas nesse estudo auxilie as futuras pesquisas elucidando algumas particularidades da espécie contribuindo com possíveis marcadores ou fármacos auxiliando no diagnóstico rápido e não invasivo de doenças que acometem em particular os felinos.
Palavras-chave: *Felis catus.* Grânulos. Neutrófilos. Proteômica.

Introduction

Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are the first cells in the innate immune system to be recruited to sites of inflammation or infection (Quinn & Gauss, 2004). Neutrophils employ chemical and enzymatic mechanisms to eliminate harmful agents and necrotic tissues (Gordon, 2016).

For an efficient response, neutrophils depend on preformed molecules stored in intracellular granules. The proteins found in these granules regulate cell recruitment to the necessary site, phagocytosis, and the formation of neutrophil extracellular traps (NETs) (Ley et al., 2018). The granules can either fuse with the phagosome containing the microorganism to kill the pathogen, or attach to the plasma membrane in response to microorganisms that have not been phagocytosed, releasing their contents into the extracellular medium (Brinkmann et al., 2004).

The cytoplasmic granules that constitute neutrophil defense are classified as primary, secondary, or tertiary (Cieutat et al., 1998). This classification is related to the time of granule development in the cytoplasm during the course of neutrophil maturation in the bone marrow: primary granules form during the promyelocyte phase, secondary granules are formed during the myelocyte stage, and tertiary granules appear in the rods (Bainton, 1999; Borregaard & Cowland, 1997). The primary granules, also known as azurophilic granules, contain lysozymes, myeloperoxidase (MPO), neutrophil elastase (NE), lactoferrin, defensins, and other proteins. Secondary or specific granules are mainly composed of lactoferrin and lysozymes, whereas tertiary granules contain gelatinase, collagenase, and metalloproteinases (Borregaard, 2010; Witko-Sarsat et al., 2000). In addition to these structures, secretory vesicles, which can be classified as a part of the granule family, are present in the final stages of neutrophil maturation (Amulic et al., 2012; Borregaard et al., 2007).

With advancements in technology and various approaches, studies such as those by Jethwaney et al. (2007), Uriarte et al. (2008), and Tomazella et al. (2009) have contributed significantly to a greater understanding of this field. These authors

successfully identified and quantified over 500 proteins in human neutrophils and their organelles, including less abundant proteins found in granules, secretory vesicles, and other compartments of neutrophils (McLeish et al., 2013).

Although research on neutrophil proteomics has advanced significantly, most studies have focused on human neutrophils, with some other studies examining rat neutrophils (Piubelli et al., 2002, 2005), and only one study involving cattle neutrophils (Lippolis & Reinhardt, 2005). However, studies on other species, including felines, are lacking. This lack of information, combined with the potential variations in neutrophils across different species, underscores the need for exploratory studies to enhance our understanding of the proteomics of neutrophils in various species. Therefore, this study aimed to identify and characterize the protein profile of neutrophils in cats. The findings of this study may help future studies identify disease markers or in understanding how drugs act based on the abundance or absence of the proteins of interest.

Materials and Methods

Type of study, animal selection, and ethical guidelines

The inclusion criteria were as follows: (1) asymptomatic cats that did not show any abnormalities suggestive of systemic diseases, such as vomiting, diarrhea, weight loss, nasal secretion, or neoplasia, on clinical examination (Collado et al., 2012); (2) cats aged ≥1 year; (3) cats weighing ≥2 kg; (4) cats of both sexes; (5) cats of any breed; and (6) cats with owners. Each animal underwent a complete blood count using the veterinary hematological counter (ABX VET-Horiba®), and serum biochemical assessments using a semi-automatic device (BIO 2000). Additionally, a rapid commercial test based on a cassette immunochromatographic assay was used for qualitative detection of antibodies against Feline Immunodeficiency Virus (FIV) and antigens for Feline Leukemia Virus (FeLV) (Dechra®). The exclusion criteria were as follows: (1) cats showing changes in blood count or clinical biochemistry, and (2) cats showing positive results for FIV and/or FeLV.

The project was approved by the State University of Santa Cruz Animal Use Ethics Committee (CEUA-UESC) under protocol number 024/15.

Blood collection and sample processing

Five animals were selected for the study. Five milliliters of blood were collected from each animal by puncturing the external jugular vein using disposable needles (25 × 8 mm) and a syringe containing the anticoagulant sodium heparin (Cristália Hemofol®) at a concentration of 5,000 IU/mL, using 1 µL of anticoagulant for each mL of blood. Samples were collected on the same day. Two samples were collected from each animal at minimum intervals of 15 days. The samples from these animals were then pooled. Assays were conducted in vitro, starting 2–3 h after blood collection. Fifteen days before blood collection, these animals were dewormed with 5 mg of praziquantel and 14.4 mg of pyrantel per kg of weight (Ciurex Fagra®).

Granulocyte purification

Granulocytes were purified from peripheral blood using a Ficoll-Histopaque® gradient (Sigma-Aldrich). Whole blood was dispensed in a Ficoll gradient with densities of 1.119 and 1.077, respectively, at a ratio of 1.25:1 (Blood:Ficoll). The cells were centrifuged at 400 × g for 30 min at 23°C. After centrifugation, the granulocyte layer formed between Ficoll 1.119 and 1.077 was collected, and the cells were washed in saline solution (0.9% sodium chloride) and centrifuged at 400 × g for 10 min. In cases involving red blood cell contamination, the cells were incubated in lysis solution containing 8.29 g of NH_4Cl 0.15 mol/L, 2 g of $KHCO_3$ 1 mol/L, and 0.074 g of Na_2 EDTA (0.1 mol/L, pH 7.2-7.4) for 3 min, and then washed twice with saline. After washing, the granulocytes were resuspended in 500 µL of RPMI 1640 medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and L-glutamine (Gibco®) for counting using a hemocytometer. Cell viability (≥90%) was assessed using Trypan blue vital dye (Gibco®). On each collection day, processed samples from the cats were grouped into a pool, and the samples obtained on each collection day included a replicate (Figure 1). Pooling was performed to obtain 1 × 10^7 neutrophils for the subsequent analyses.

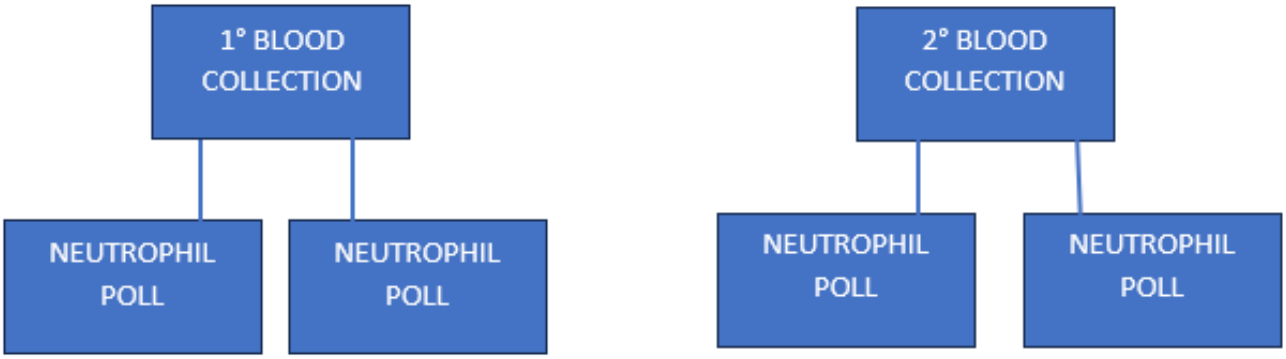

Figure 1. Schematic representation of the pooled collection method for analysis of domestic cats neutrophils.

Proteomics analysis

Protein extraction and quantification

The cells were centrifuged for 15 min at 12,900 × g/4°C; the supernatant was removed; and the pellet was collected with 750 µL of the extraction buffer (100 mmol/L Tris-HCl [pH 8.3]; 5 mmol/L ethylenediamine tetraacetic acid [EDTA]; 100 mmol/L KCl; 1% dithiothreitol [DTT]; 30% sucrose; and one mini protease inhibitor tablet (Sigma)). Next, 750 µL of buffered phenol was added, vortexed for 10 min at 4ºC, and then centrifuged at 12,900 × g for 10 min at 4ºC. The supernatant was collected, and five volumes of ammonium acetate in methanol (100 mmol/L) were added. The samples were incubated at -20ºC overnight and then centrifuged at 15,100 × g at 4ºC for 60 min. The pellet was washed twice with 2 mL of 0.2% acetone DTT solution, each wash separated by an interval of 1 h, at -20ºC, and then centrifuged for 30 min at 15,100 × g at 4ºC. After drying at room temperature, the pellet was resuspended in 8M urea and then stored at -20°C. Subsequently, 1D gel electrophoresis was performed to assess the integrity of the proteins, which were quantified using a 2D-Quant Kit (GE Healthcare Life Sciences) according to the manufacturer's recommendations.

Peptide digestion

One hundred micrograms of protein from the total extract of each sample was reduced with DTT, alkylated with iodoacetamide (IAA), and digested with trypsin according to the methodology described by Villén and Gygi (2008), with modifications.

The solution containing the tryptic peptides was desalted using C18 resin tips (100 µL; Thermo Fisher®). The peptides were eluted in 30 µL of a solution containing 50% acetonitrile, 50% ultrapure water, and 0.1% formic acid. The peptides were stored at 4°C until analysis by mass spectrometry.

Mass spectrometry

The peptides were analyzed using a liquid chromatography system (Agilent 1290 Infinity II HPLC) coupled with quadrupole/time-of-flight mass spectrometry (LC/QTOF; Agilent 6545). Three technical replicates of 10 µL each were injected for every sample. The samples were separated using a reversed-phase C18 column (AdvanceBio Peptide Mapping, 2.1 × 250 mm; Agilent) at a temperature of 55°C. A 20-min gradient was applied using mobile phases A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The percentages of phase B during the gradient were as follows: 5% to 35% (1-10 min), 35% to 70% (11-14 min), 70% to 100% (16-18 min), and 100% (16-20 min), followed by a final period of 5 min programmed for spinal stabilization.

The samples were injected into the QTOF unit via an electrospray source in the AutoMSMS acquisition mode, with a maximum of 10 precursors selected per cycle. The parameters for selecting the precursors were as follows: threshold, 1000; counts per spectrum, 10,000; purity

restriction, 100%; purity cutoff, 30%; isotopic model for peptides; and load preference, 2, 3, >3, and unknown. The collision energy (V) was set according to the formula V = *(slope)* (m/z)*/100+*Offset,* where m/z represents the mass-to-charge ratio of the precursor. The slope and offset values ranged from 3.1 to 5 and from -4.8 to 10, respectively, and varied based on whether the precursor charge was 2, 3, >3, or unknown. The settings for the instrument were as follows: gas temperature, 325°C; gas flow, 13 L/min; capillary voltage, 4000 V; skimmer voltage, 56 V. Nitrogen gas was used for collision-induced dissociation. Instrument control (HPLC and QTOF) and parameter configurations were determined using the Agilent MassHunter Acquisition software.

Identification of peptides in protein banks

The resulting spectra were processed in triplicate for peptide identification using Spectrum Mill software (Rev B.06.00.203 SP1; Agilent). The parameters for spectral extraction were as follows: MSNoise threshold, 10 counts; fixed modifications, carbamidomethylation; MH+ precursor range, 200 to 6000 Da; retention time tolerance, +/- 60 s; m/z tolerance, +/- 1.4; and precursor charge, find. After extraction of the MS/MS spectra, a database search was conducted. The benches used were the *Felis catus* sequences downloaded from Universal Protein Knowledgebase [UniProt] (2023) (https://www.uniprot.org). The parameters used for comparing MS/MS spectra in the protein database were as follows: maximum number of missed cleavages, 24; fixed modifications, carbamidomethylation (C); variable modifications, oxidized methionine (M), pyroglutamic acid (N-termQ), deamidated (N), phosphorylated S (S), phosphorylated T (T), and phosphorylated Y (Y); minimum combined peak intensity, 10%; and precursor mass tolerance, +/- 20 ppm. The search results were validated and filtered to include only those with a false-positive rate (FDR) of less than 1%, score > 5, and Scored Peak Intensity (SPI) > 60%. The results were exported in protein-protein comparison mode as Mass Profiler Professional (MPP) APR files.

Identification of protein abundance

The relative abundance of proteins was analyzed using the MPP software version 15.1 (Agilent). The abundance of each protein was calculated using the median peptide abundance. The results of the technical replicates were then compared. The proteins were filtered on the basis of peptide frequency using the following criterion: proteins that appeared in at least 16.6% of the replicates, or in at least two of the 12 replicates, were retained. Heatmaps and clustering analyses were performed using the same software. Protein abundance values, represented as the mean of the medians of spectral intensities, were normalized to log2 values. The Euclidean distance metric and Ward's linkage method were used.

Functional annotation

ShinyGO 0.77 (2023) (bioinformatics.sdstate.edu/go/) and UniProt (2023) (http://www.uniprot.org) were used to perform functional annotation of the proteins. ShinyGO 0.77 (2023) was used to group the identified proteins according to their functions/attributes, such as metabolic pathways, molecular function, cellular components, and biological processes, whereas UniProt (2023) was used to individually search for each protein found in this study.

Results and Discussion

The protein composition of neutrophils in domestic cats (*Felis catus*) was analyzed, and 102 proteins (Supplementary Material 1) specific to this species were selected. Figure 2 shows a heatmap illustrating the relationships among proteins, highlighting those that were more and less abundant in cat neutrophils. Proteoglycan-3 was the most abundant protein identified in this study.

The identified proteins are involved in several cellular processes. Figure 3 highlights the 10 metabolic pathways involving the identified proteins, with some proteins participating in more than one pathway. Some metabolic pathways were directly related to cellular activation, such as the formation of extracellular neutrophil traps, phagosomes, and phagocytosis. Glycolysis showed the greatest proportional participation of the identified proteins, which accounted for nine of the 61 proteins constituting the glycolytic pathway.

In the ten molecular functions highlighted in Figure 4, many proteins are involved in the binding and structuring of the neutrophil cytoskeleton and actin. We could confirm the significant involvement of the identified proteins in the activity of superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (the identified proteins constituted three of the 11 proteins that make up this function).

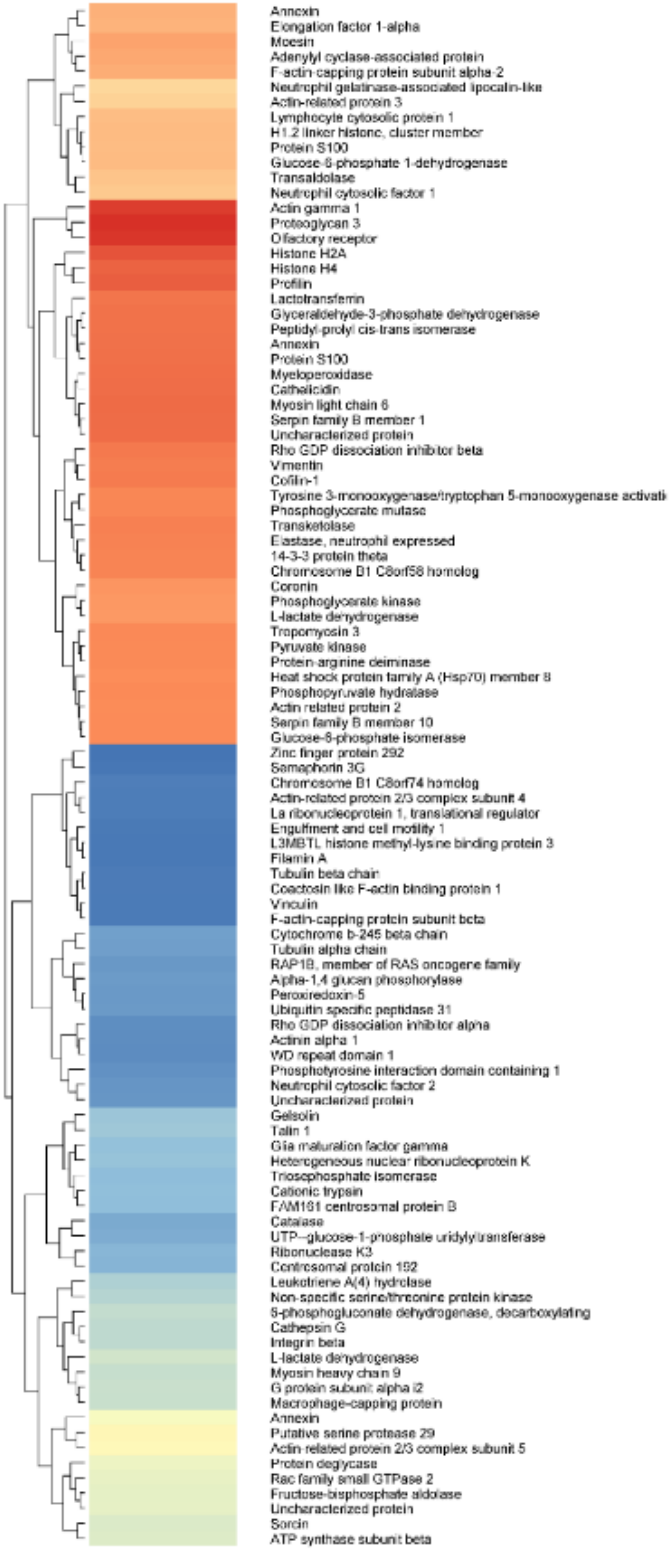

Figure 2. Heatmap of the proteins identified in cat neutrophils, highlighting the most and least abundant proteins. The most intense red color indicates the most abundant proteins, while the most intense blue color corresponds to the least abundant proteins.

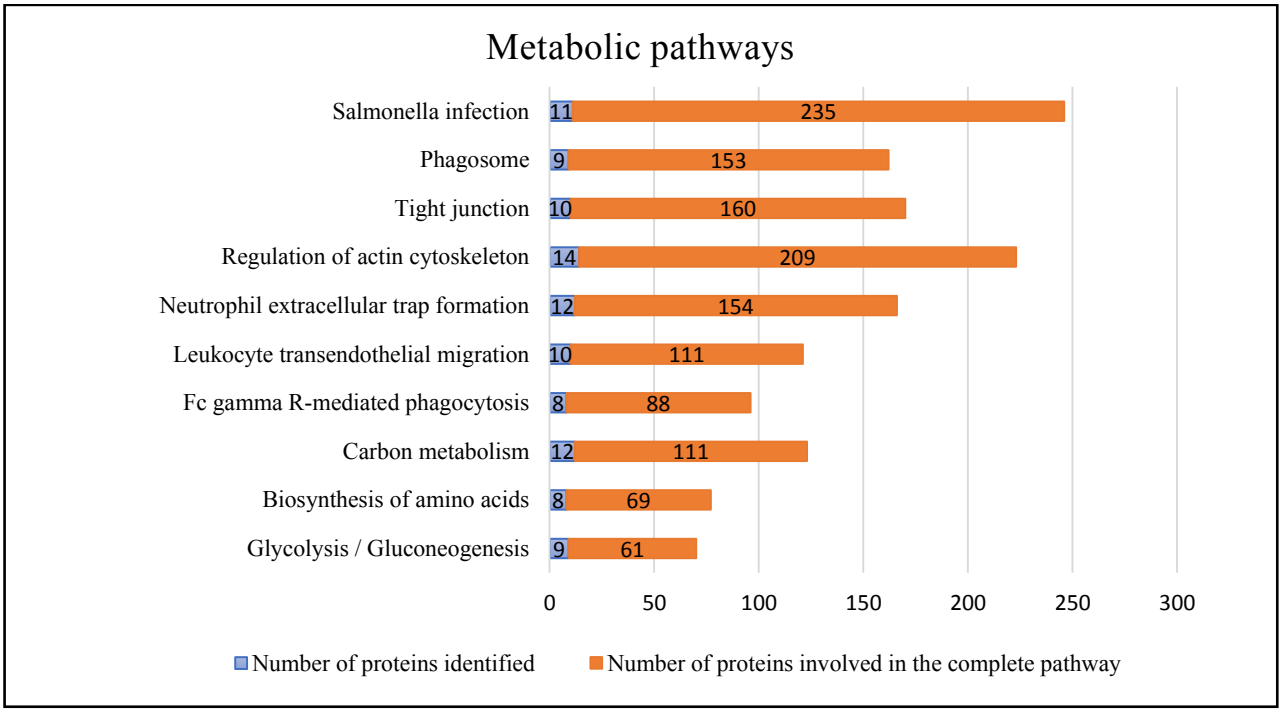

Figure 3. Metabolic pathways involving proteins found in cat neutrophils.

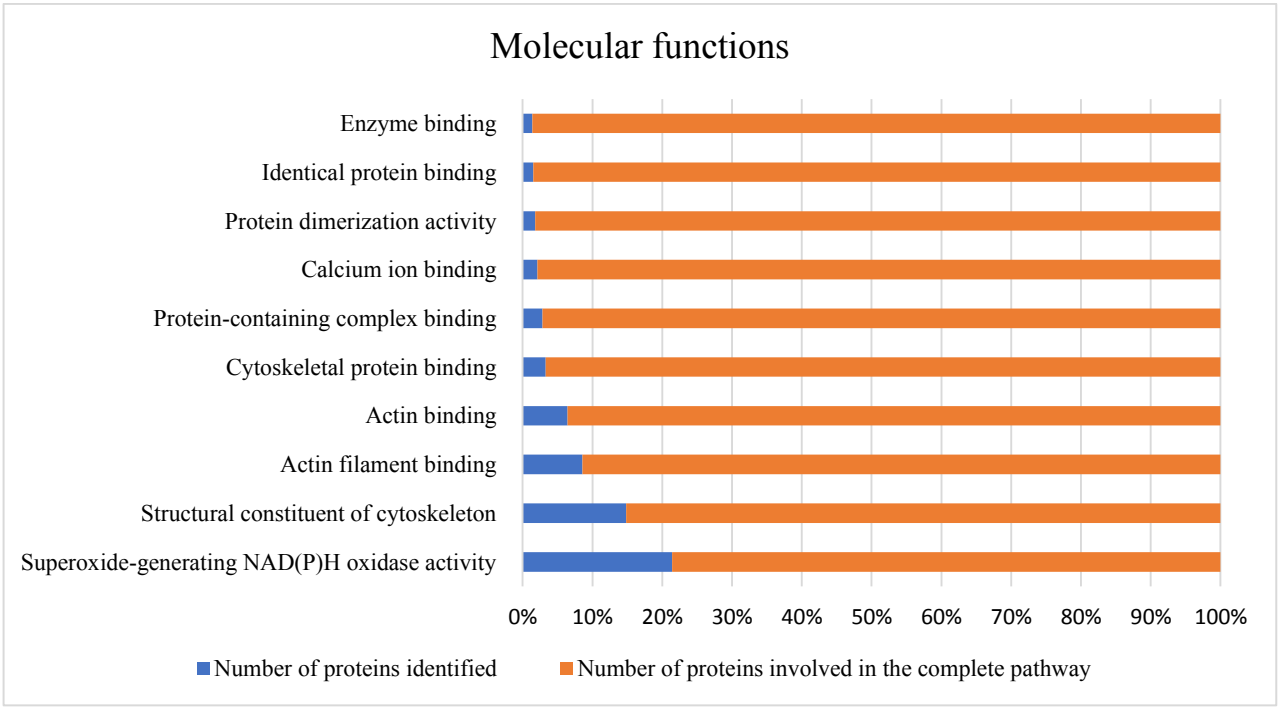

Figure 4. Molecular functions involving proteins found in cat neutrophils.

Figure 5 shows the proteins involved in cellular composition, highlighting the variations in cellular composition and showing that most of the identified proteins involved in cellular composition were components of the cortical region of the cytoskeleton. In terms of biological processes (Figure 6), most of the identified proteins were associated with actin, either by regulating or organizing this protein.

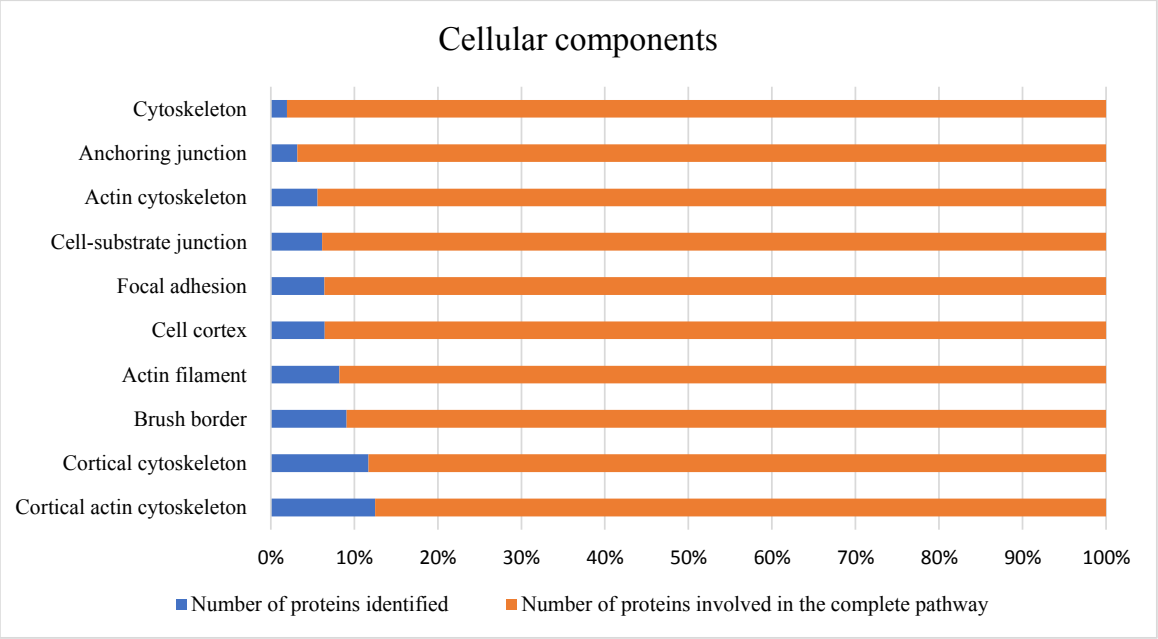

Figure 5. Cellular components involving proteins found in cat neutrophils.

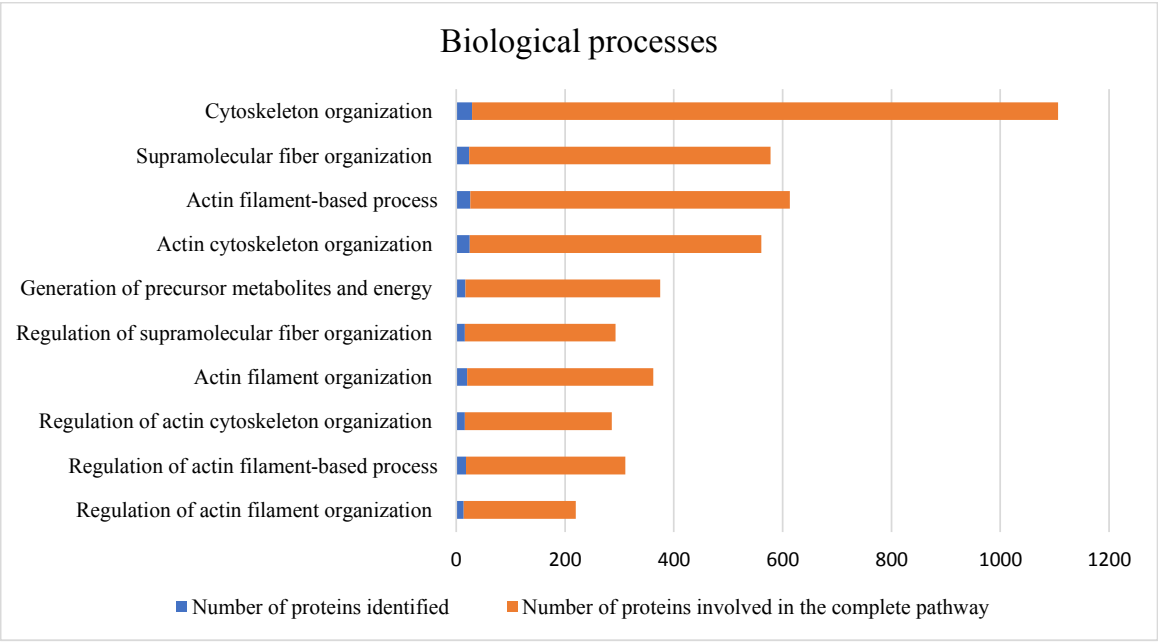

Figure 6. Biological processes involving proteins found in cat neutrophils.

The present study, which used proteomics analyses to evaluate cat neutrophils, identified 102 proteins using this methodology. To date, only studies involving neutrophils from humans (Tomazella et al., 2009), rats (Piubelli et al., 2002), and cattle (Lippolis & Reinhardt, 2005) have been conducted. The proteins identified in this study were involved in the fundamental functions of neutrophils, including those of the domestic cat (*Felis catus*) neutrophil proteome.

The most abundant of the 102 proteins was proteoglycan-3, which plays a role in several biological processes, including neutrophil activation, immune response, superoxide anion generation, upregulation of interleukin-8 production, and leukotriene biosynthesis. These functions have been documented using UniProt (2023) (http://www.uniprot.org/uniprotkb/M3WEX9/entry). Leukotrienes are lipid mediators that act in a paracrine manner at low concentrations and produce different responses depending on the target cell. They are biosynthesized by neutrophils and other leukocytes (Haeggström, 2018; Haeggström & Funk, 2011), and some leukotrienes are involved in cell adhesion, chemotaxis, and leukocyte activation (Peters-Golden & Henderson, 2007).

Proteoglycan-3 upregulates interleukin-8, which is a crucial pro-inflammatory (Moser & Loetscher, 2001) chemokine for polymorphonuclear cells in humans, stimulating these cells to infiltrate tissues (Baggiolini et al., 1995; Stillie et al., 2009). Interleukin-8 is not conserved across species, and its chemokine functions may vary across species (Gonzalez-aparicio & Alfaro, 2019). Consequently, it may show alterations in functions that are specific to *Felis catus.*

Glycolysis is the metabolic pathway that involved the greatest proportion of the identified proteins. Glycolysis is a source of adenosine triphosphate (ATP), which is essential for neutrophil dynamics and functions such as chemotaxis, degranulation, and pathogen control or elimination (Borregaard & Herlin, 1982). Neutrophils use ATP generated by glycolysis to adjust their energy supply according to the energy demand (Sadiku et al., 2021). Neutrophils have been shown to store glycogen in their granules. This reserve can be altered based on the demands of circulating cells, which may be influenced by the oxygen-sensing response and the stimulation of pro-inflammatory mediators (Robinson et al., 1982).

In addition to glycolysis, other pathways are directly involved in the primary functions of neutrophils. We identified several related proteins, such as those associated with the Fcγ receptor (FcγR)-mediated phagocytosis pathway. These receptors are responsible for the phagocytosis of opsonized IgG particles and play a crucial role in the innate immune response. Phagocytosis of opsonized targets through signaling leads to the fusion of protease-rich granules with the phagosome, triggering an oxidative burst (Botelho et al., 2002; Witko-Sarsat et al., 2000).

At least one of the proteins was identified in each of the following metabolic pathways: phagosome and NET formation, transendothelial leukocyte migration, and FcγR-mediated phagocytosis. The proteins $p47^{phox}$/neutrophil cytosolic factor 1 (NCF1),

p67^{phox}/neutrophil cytosolic factor 2 (NCF2), and gp91^{phox}/cytochrome b-245 β chain (CYBB) are essential for the assembly of the NADPH oxidase complex, which plays an important role in host defense against microorganisms (Roos & Boer, 2013). These proteins were also identified in our study as having molecular functions in NADPH oxidase activity. When neutrophils are at rest, these proteins are expressed in different parts of the cell: p47^{phox}, p67^{phox}, and p40^{phox} remain in the cytoplasm, whereas p22^{phox} and p91^{phox} are located in the membranes of secretory vesicles and specific granules. After stimulation, the cytoplasmic proteins migrate to the membrane to assemble active oxidases (Babior, 1999).

Several granule proteins have been identified during NET formation, including cathelicidin (Jann et al., 2009), cathepsin G (Folco et al., 2018), and elastase (Rodrigues et al., 2020). Protein-arginine deiminase type 4 (PADI4) plays a crucial role in the citrullination of histones. The identified histones were H2AC1 (histone H2A) and H4C4 (histone H4). However, in human NETs, additional histones are also involved (Chen et al., 2021; Hong et al., 2022).

Regarding molecular functions (Figure 4), alpha actin 1 was prominent and involved in 70% of the functions, most of which were related to neutrophil binding and structure. However, this protein was absent in the activity of NADPH oxidase, which generates superoxide as a constituent of the cytoskeleton, and in enzymatic binding. The cytoskeleton of eukaryotic cells is composed of actin filaments, microtubules, and intermediate filaments. The structures and proteins that constitute the cytoskeleton are important because they are responsible for organizing and maintaining intracellular compartments. Polymerization and depolymerization of actin drive changes in cell shape (Fletcher & Mullins, 2010). Actin plays a crucial role in motility, cell division, and degranulation, and inhibition of the actin cytoskeleton can compromise NETosis, the process of cell death induced by NETs (Sprenkeler et al., 2022a). In our study, the alpha-1 actin protein was involved in metabolic pathways, molecular function, and biological processes, particularly in cell composition, and participated in all 10 processes related to cellular structure (Figure 5). This underscores its importance at all neutrophil stages. Other actins are also involved in cell composition. Coronine (CORO1A) regulates cytoskeletal-dependent processes involving actin; assists in neutrophil trafficking; enhances adhesion, dissemination, and migration (Pick et al., 2017); and is important in phagocytosis and phagocytic vacuole formation. It also binds to p47^{phox} (Grogan et al., 1997).

Some proteins in cat neutrophils appear to be duplicated or tripled. The corresponding proteins analyzed in this study were annexin, L-lactate dehydrogenase (LDH), and S100. Analysis of the UniProt (2023) database indicated that they are encoded by different genes. In this study, the ANXA1, ANXA5, and ANXA6 isoforms of annexin were identified. Annexins are a family of proteins found in eukaryotes that bind to calcium ions (Salzer et al., 2002). Hundreds of annexins have been identified in various species (Mirsaeidi et al., 2016). Some annexin genes may have been lost or duplicated in certain species such as bony fish (Postlethwait et al., 2000). This duplication of the annexin genes has also been observed in humans

(Smith et al., 1994). LDH and annexins are encoded by different genes, with LDH-A and LDH-B being isoforms. These isoforms can be grouped together to form up to five different combinations, each with specific functions. LDH-A is involved in converting pyruvate to lactate, whereas LDH-B converts lactate back to pyruvate, contributing to oxidative metabolism and resulting in NADH production (Certo et al., 2021). Both contain proteins that are 100% identical to those of other feline species.

The S100 proteins identified were S100A8 and S100A9, none of which had any isoforms. Proteins of the S100 family, which are expressed in vertebrates, interact with annexins. S100 proteins are involved in many functions, including structural regulation of the cell, calcium homeostasis, and mediation of muscle contraction (Mirsaeidi et al., 2016). S100A8 and S100A9 belong to a subgroup of S100 proteins, known as calgranulins, characterized by their ability to bind calcium and high expression in granulocytes. They are also expressed in various cell types during acute and chronic inflammatory processes (Goyette & Geczy, 2011). They are not released during exocytosis of primary and secondary granules, but during NETosis (Sprenkeler et al., 2022b).

Conclusion

The results obtained indicate that many proteins were identified in the proteome of domestic cat neutrophils, and variations in the abundances of these proteins are likely directly related to their cellular functions. In this study, we identified several proteins that influence metabolic pathways and other cellular functions ranging from general to specific roles in neutrophils. The absence of certain proteins previously mentioned in the literature underscores the dynamic nature of proteomics findings, which reflect the state of cells at a given time without any specific stimuli in this study. We emphasize the importance of using tools such as mass spectrometry to gather extensive and relevant information about the proteins present in domestic cat neutrophils using complex samples.

Acknowledgments

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