University of Veterinary and Pharmaceutical Sciences Brno

Faculty of Veterinary Medicine

Small Animal Clinical Laboratory

Evaluation of Nucleated Red Blood Cells in Canine and Feline Peripheral Blood

DIPLOMA THESIS

Mari Dahl Bugge

Supervisor: MVDr. Kristína Řeháková, Ph.D.

Brno 2018

TABLE OF CONTENT

Declaration 4

Abbreviations 5

Introduction 6

Literature review 7

1. Nucleated red blood cells within the bone marrow 7
    1.1. Role in physiological erythropoiesis 7
    1.2. Morphological classification 7
    1.3. Role in erythropoiesis evaluation 8
2. Nucleated red blood cells within the peripheral blood 9
    2.1. Manual quantification and morphological evaluation 9
    2.2. Automatic quantification 10
        2.2.1. Automated quantification by human hematology analyzers 10
        2.2.2. Automated quantification by veterinary hematology analyzers Sysmex XT-2000iV 13
    2.3. Assesment of nucleated red blood cells in peripheral blood 15
        2.3.1. Physiological conditions 15
        2.3.2. Anemic syndrome 15
        2.3.3. Non-anemic conditions 16
3. Nucleated red blood cells within other hemopoietic organs 17
4. The clinical use of nucleated red blood cells 18
    4.1. Nucleated red blood cells in critical illness 18
    4.2. Nucleated red blood cells in neonatal diseases 19
    4.3. Nucleated red blood cells in disorders with systemic inflammatory response syndrome 20
    4.4. Nucleated red blood cells in hematological diseases and chemotherapy 20

Own study 22

1. Aims 22
2. Material and Methods 23
    2.1. Material 23
        2.1.1. Animals 23
        2.1.2. Inclusion criteria 23
        2.1.3. Sample collection, preparation and storage 23

2.2. Methods 23
2.2.1. Automatic peripheral blood analysis 23
2.2.2. Blood smear evaluation 24
2.2.3. Correction of WBC count based on NRBC 25
2.2.4. Sysmex XT-2000iV software settings 26
2.2.5. Statistical analysis 27
3. Results 33
3.1. Animals 32
3.2. Quantitative and morphological evaluation of NRBC population 33
3.3. Evaluation of bias in total WBC count and different WBC population changes caused by NRBC in dogs 38
3.4. Assessment of automatic analysis for detection of circulating NRBC in dogs 39
4. Discussion 41
4.1. Quantification and morphological evaluation of NRBC 41
4.2. WBC count and different WBC population changes caused by NRBC in dogs 44
4.3. Assessment of automatic analysis for detection of circulating NRBC in dogs 45
5. Conclusion 47
6. Summary 48
References 50
Acknowledgements 58

DECLARATION

I hereby declare that this diploma thesis is my original work and it has been written by me in its entirety. I have faithfully and properly cited all sources used in the thesis.

Brno, 09.03.2018 Mari Dahl Bugge

ABBREVIATIONS

AVG	Average
ACAS	Adaptive cluster analysis system
BASO	Basophils
CHOP	Cyclophosphamide, Doxorubicin, Vincristine, Prednisone
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EOS	Eosinophils
EPO	Erythropoietin
FSC	Forward Scatter
HCT	Hematocrit
HGB	Hemoglobin
HGFs	Hematopoietic Growth Factors
IL-6	Interleukin-6
IG	Immature granulocytes
IMHA	Immune Mediated Hemolytic Anemia
EMH	Extramedullary Hematopoiesis
LYMPH	Lymphocytes
MONO	Monocytes
M:E	Myeloid:Erythroid ratio
N:C	Nucleus:Cytoplasmic ratio
NEUT	Neutrophils
NRBC	Nucleated Red Blood Cells
RBC	Red Blood Cell
RET	Reticulocyte
RNA	Ribonucleic acid
SIRS	Systemic Inflammatory Response Syndrome
SSC	Side Scatter
SFL	Side Fluorescence Light
WBC	White Blood Cell
DIFF	Differential White Blood Cell

INTRODUCTION

Nucleated red blood cells can be seen in the peripheral blood of patient in pathologic state. Their presence in circulation indicates the reaction hematopoietic organs has to stress and disease. Nucleated red blood cells are physiologically located in bone marrow and should be cleared by the reticuloendothelial system of the spleen if shifted to the peripheral blood. Release of NRBC occurs in anemic or hypoxic conditions as a respond to excessive demand of erythropoiesis, in splenic disorders or in bone marrow disorders. Both in human and veterinary medicine, detection of nucleated red blood cells has been established to be a negative prognostic marker. In human medicine these cells occur approximately equally in hematological and non-hematological diseases, and the fatality is around 50%. There are several studies on the use and detection of NRBC in human medicine, however there is still very limited information on their general use/aspect in veterinary medicine.

There are several automatic hematology analyzers able to quantify NRBC in human medicine, whereas in veterinary medicine routine quantification of these cells is performed microscopically. This method is demanding and time consuming and requires a certain experience. An automated method in veterinary medicine would be valued.

This study focuses on the evaluation of NRBC occurrence in canine and feline patients, both anemic and non-anemic, and on the inaccuracy in measured total WBC count and different WBC populations caused by interference with NRBC. Additionally, we attempt to use the Sysmex XT-2000iV flow cytometry analyzer for automated detection of NRBC by defining a population area in the WBC differential scattergram.

LITERATURE REVIEW

1. Nucleated red blood cells within the bone marrow

1.1. Physiological erythropoiesis

Nucleated red blood cells (NRBC), also called erythroblasts or normoblasts, are immature erythrocytes from the progenitors for the red blood cell line (Schwarzt et al 1954). Red blood cells (RBC) are continuously produced from primitive stem cells within the extravascular space of bone marrow (Quesenberry 1995). RBC are highly differentiated cells that have no nuclei or cytoplasmic organelles making them incapable of cell division. The erythropoietic progenitors are made from hematopoietic stem cell to the common myeloid progenitors and megakaryocyte-erythrocyte progenitors before they become NRBC. Presence of specific hematopoietic growth factors (HGFs) is needed for the proliferation of the hematopoietic stem cell and its progenitors. This may be produced locally in the bone marrow, or by peripheral tissues and transported to the bone marrow by circulating blood. The HGFs include erythropoietin (EPO), growth factors classified as colony stimulating factors, and different interleukins (Metcalf 1993). Erythroid burst-forming units are the most immature erythroid progenitors and represent the precursor of a rubriblast. This is the most immature morphologically identifiable cell of the erythroid cell line. Proliferation and maturation of these cells lead to four or five mitoses producing metarubricytes, which are no longer capable of division. The cells progressing from rubriblast are prorubricyte, rubicyte (basophilic and polychromatophilic) to metarubricyte before the reticulocyte (RET) stage (Erslev et al 1995).

1.2. Morphological classification

The NRBC identified within the bone marrow are rubriblasts, prorubricytes, rubricytes (basophilic and polychromatophilic) and metarubricytes. The early precursors contain many ribosomes actively synthesizing proteins, and therefore stain with intensely blue cytoplasma. During cell division, the cytoplasmic basophilia decreases together with the overall cell size and with the nuclear:cytoplasmic (N:C) ratio. Condensation of nuclear chromatin increases, and hemoglobin progressively accumulates in the cytoplasm causing the cells to stain more polychromatophilic. A reticulocyte is formed following the extrusion of the metarubricyte nucleus (Zlabolotsky and Walker 2014).

Rubriblast: Is the first and most immature erythroid cell we can identify. It is a large round cell with the highest N:C ratio. The nucleus is large and round, the chromatin has a fine granular pattern. There may be seen one or two pale to medium blue nucleoli. The cells have intensely stained basophilic cytoplasm, from high number of polyribosomes, often seen as a thin rim around the nucleus.

Prorubricyte: This second stage is described when nucleoli are no longer visible. The nucleus is large and round, with a tendency of coarser clumping of chromatin. The cytoplasm is slightly less intense blue. The N:C ratio is slightly reduced compared to that of the rubriblast.

Rubricyte: Is smaller in cell and nucleus size than their precursors. This stage can be divided into basophilic and polychromatophilic rubricytes. In both, the nucleus has especially course chromatin pattern which may resemble cartwheel spokes. The basophilic rubricyte is the less mature form, medium sized and with blue cytoplasm. The polychromatophilic rubricyte is smaller and the cytoplasm is polychromic to polychromatophilic due to higher amount of hemoglobin. The N:C ratio is less than in the previous stage.

Metarubricyte: Is the most advanced stage of nucleated erythroid lineage. The nucleus is very dark to pyknotic. There is very little or no distinguishable chromatin pattern. It is a small cell and the cytoplasm is polychromatophilic or even normochromic (Harvey, J. W. 2001).

1.3. Role in erythropoiesis evaluation

The evaluation of the NRBC within the bone marrow should be done to determine if the erythropoiesis is complete and in order. Each stage of development should generally be progressively increasing in numbers. Rubriblasts should account for about 1% and together with prorubricytes should not exceed 5% of the nucleated cell numbers (Stacy et al 2017). Rubricytes are normally up to three quarters of the nucleated cells while metarubricytes account for one quarter. Changes in these proportions should be expected in response to anemia where one or more populations are increased. Proliferative abnormalities may cause increase in immature erythroid cells and not in later stages. Other abnormal changes include megaloblastic cells from impaired DNA synthesis, frequent binucleated cells and pleomorphic nuclei that can be seen in infectious diseases, toxicity or irradiation. Bone marrow examination is commonly indicated in nonregenerative anemia, where hypocellular or normocellular sample with increased myeloid to erythroid (M:E) ratio can be seen. The ratio between the myeloid to

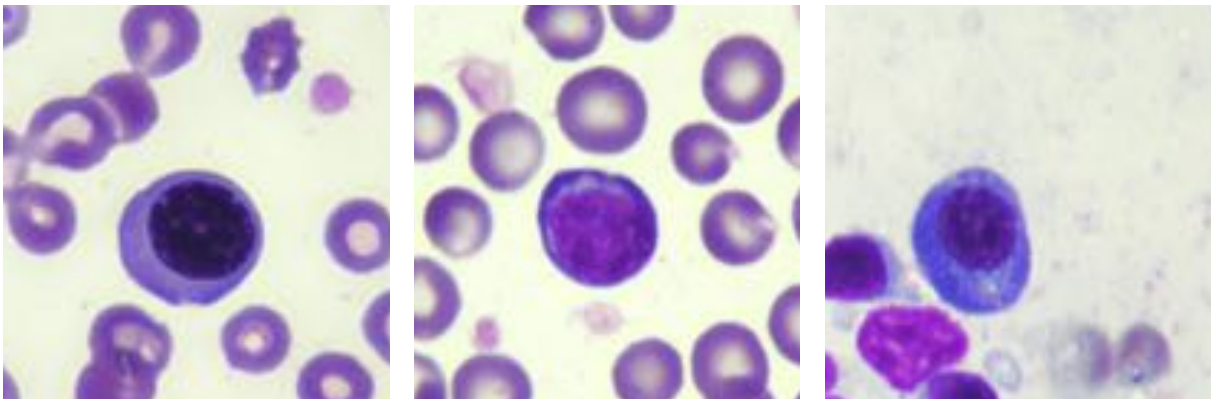
erythroid cells is generally found to be between 0.75 - 2:1 in dogs, and 0,6-4,4:1 in cats. It is generally counted from examining 500 cells and is a difficult task, subjective evaluation can be sufficient for diagnostic purposes. It is interpreted in relation to marrow cellularity and values of the peripheral blood (Grindem et al 2014).

2. Nucleated red blood cells within the peripheral blood

Nucleated red blood cells should be quantified correctly as they may reveal significant disease. Several studies have shown that NRBC occurring in the peripheral blood is an important marker of many different diseases. They occur in both hematological and non-hematological diseases, occurrence in non-hematological diseases ranges from 26-54%. These diseases often have unfavorable outcomes and with fatality up to 50% in patients with these finding. Nucleated red blood cells are not associated with specific disease but is a symptom of stressed erythropoiesis (Tsuji et al 1999).

2.1. Manual quantification and morphological evaluation

Morphological differentiation of nucleated red blood cells from white blood cell lines on the blood smear is important (Das et al 2017). The most commonly seen NRBC in the peripheral blood smears are metarubricytes, less often also its more immature precursors, rubricytes. Lymphocyte is the closest cell the NRBC can mimic in the peripheral blood (Pictures 1-3). Typical features of NRBC compared to lymphocytes are a more condense or pyknotic nucleus whereas lymphocytes have a lighter and less condense chromatin pattern from a mixture of euchromatin and heterochromatin. The cytoplasm of lymphocytes is pale blue with a high N:C ratio and the border is more rounded and less ruffled then that of a NRBC. A reactive lymphocyte, with a deeper blue color of its cytoplasm, may resemble the color of a basophilic rubricyte. Basophilic rubricytes have deeper blue cytoplasm and more clumped chromatin. Later stages, metarubricytes, have higher amount of hemoglobin in cytoplasm giving it a more purple to red color (Harvey 2011, Zabolotzky et al 2014).



Pictures 1-3: Rubricyte (left), Reactive Lymphocyte (middle), Plasma cell (right)

A white blood cell (WBC) count determined by hematological analysis corresponds truly to a nucleated blood cell count, which includes NRBC population. Automated cell counts cannot differentiate nucleated red blood cells from white blood cells and therefore give falsely increased WBC count if not corrected. Manual evaluation is used to quantify the number of nucleated red blood cells per 100 white blood cells to determine a corrected white blood cell count. The number of NRBC per 100 WBC is recorded during the differential leukocyte count when performing a blood smear examination and used together with original WBC count for correction (Akhtar and Mahure 2015, Angel et al 2016).

The formula used to correct the WBC count:

$$Corrected\ WBC\ Count = Original\ WBC\ Count\ x\ \frac{100}{no.of\ NRBC + 100}$$

Nucleated red blood cells can be discovered only by chance if a microscopical blood smear evaluation is performed. In general, the manual quantification of NRBC is labor-demanding and requires an experienced and skilled examiner to avoid misclassification of NRBC as lymphocytes. In samples with low levels of NRBC, there is a high degree of intra- and interobserver imprecision due to low number of cells counted and heterogeneity of cell distribution in blood smears with an average coefficient of variation of 40%. (20-110%, Tsjui et al 1999, Rin et al 2017).

2.2. Automatic quantification

2.2.1 Automatic quantification by human hematology analyzers

Detection of nucleated red blood cells by hematology analyzers is an important indication for the morphological smear examination (Tantanate et al 2015, Hwang et al 2016). Automatic quantification of NRBC has been challenging due to the difficulty in separating these cells from WBC. Their morphological properties are similar to small lymphocytes, and often quantified together with this population. A standardized automated NRBC count would be valuable as the peripheral blood smear examination is one of the most labor demanding practices in clinical laboratories.

The automatic quantification of the NRBC was problematic for first generation of automated hematology analyzers. Generally, the limitations have been low sensitivity and specificity of the available hematological softwares (Bruegel et al 2015, Rin et al 2017). Determining NRBC became possible with the help of antibody-mediated flow cytometry. Different types have in common that an antibody sensitive against surface CD antigens, CD71 which is only expressed on the red blood cell line, or CD45 which is only expressed on the white blood cell line. These are used as positive or negative selection respectfully. This method has good accuracy, however, it is expensive, time consuming and requires specialized personnel (Tsuji et al 1999). Comparing an automated count to a manual count, the volume of cells counted in the automated one is much larger than in the manual. The presence of low number of NRBC in peripheral blood of a patient with otherwise normal CBC parameters is detected by improved sensitivity of an automated hematology analyzer. Recent studies show that if at a small amount of NRBC is detected, it may represent a physiological finding compared to zero tolerance for occurrence of NRBC on the blood smear reported per 100 WBC (Hwang et al 2016).

Currently used hematology analyzers, such as *Sysmex XN* (Sysmex, Japan), *Sapphire* (Abbott, USA) *LH 750* (Beckman Coulter, USA), *ADVIA* 2120 (Siemens Diagnostics, USA) are able to separate NRBC from the WBC count and report them quantitatively on default. *Penta 120DX* (ABX-Horiba, France) and *Sysmex XE-2100* (Sysmex, Japan) must have specific programming for determination of this parameter (Buttarello et al 2008, Tantanate et al 2014, Rin et al 2017). An extra programing and additional specific reagents are required, which may not be time or cost-efficient.

Sysmex XE-2100 and XE-5000 have a NRBC channel, where a special reagent lyses the red blood cell membranes, leaving only the nuclei of the NRBC intact. The reagent also perforates

the WBC membrane, staining both organelles and nuclei. The NRBC nuclei stain more intensely than the WBC, which results in two separate populations and allows the automatic hematology analyzer to correct the white blood cell count if NRBC are detected (Wang et al 2003, Hwang et al 2016, Rin et al 2017).

The detection can also be performed using signature position on the impedance size WBC histogram, just below the WBC size threshold. It uses volume, conductivity and scatter which is detected in a specified region in the differential scattergram. Presence of these populations in both areas allows the analyzer to obtain the number of NRBC. This is used in *Coulter UniCel DxH 800* (Rin et al 2017). Another method used is identifying NRBC in the peroxidase negative area of the peroxidase channel, while in the basophilic channel, the NRBC is identified by nuclear density and is located in the neutrophile/eosinophile area. The difference between the number of nuclei in this area in the basophil channel and the sum of the neutrophils and eosinophils in the peroxidase channel represent the NRBC count. This principle is used in *Siemens ADVIA 2120i* (Rin et al 2017). A fully automated hematology analyzer counts NRBC in a special channel by means of scatter of laser light at two angles and fluorescence signals. Three-dimensional analysis of the targeted cell identifies NRBC. This method is used in *Mindray BC-6800* (Rin et al 2017).

In a large comparison of five current main routine hematology analyzers, a good correlation for CBC parameters was found in all the instruments. The comparison used the median of all five analyzers as a reference because without the use of a true reference method for most of the investigated parameters, interpretation of results of different analyzers is difficult. A rather low concordance for NRBC counts was observed between the different instruments (Bruegel et al 2015). A later comparison, specifically comparing the automated nucleated red blood cell count of five commercial hematology analyzers, was found to be excellent in terms of precision. There were a few analyzers who displayed constant errors, which is connected to their approach of detecting the NRBC. It is therefore important to understand the ability and disability of the analyzer used for the correct interpretation of the result (Rin et al 2017).

Sysmex has recently upgraded their veterinary or multispecies systems in July 2017, called XN-V model, with a new improved channel for WBC and NRBC count (WNR). This channel uses SFL and SSC to identify the nucleic acid content of NRBC together with the WBCs. FSC is used for cell size measurements. The WNR scattergram reveals NRBC as a separate population from WBC (Sysmex America 2017).

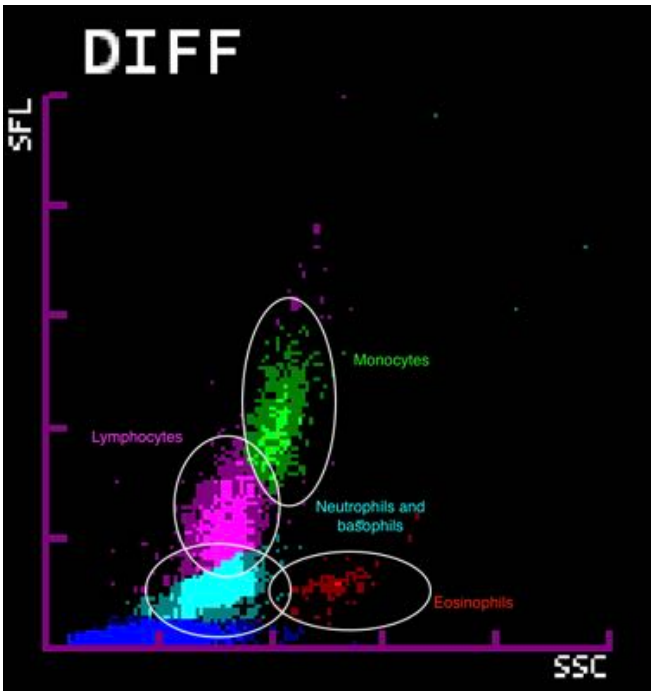
2.2.2. Automatic quantification by veterinary hematology analyzer Sysmex XT-2000iV

The number of studies focused on application of automatic hematology systems in veterinary medicine is increasing. These automatic analyzers use the same technology of laminar flow impedance, flow cytometry, and fluorescent optical cellular analysis as those in human medicine (DeNicola 2011). However nucleated red blood cell counts are rarely mentioned in veterinary studies and reported results are inconclusive (Lilliehöök et al 2009a).

The Sysmex XT-2000iV hematology veterinary analyzer is able to analyze blood samples from many different species. There are 10 predefined animal profiles, including dog, cat, horse, rabbit, guinea pig, rat, mouse, monkey, cattle and pig. The software structure of this instrument is open, allowing the user to define cell populations if needed. The graphics can be outlined and marked with manual gating functions, allowing the possibility of analyzing unusual profiles and saving them for similar tests at a later time. By this way, it is possible for new species and also different body fluids to be analyzed. The system ensures that no original results are overwritten or lost, allowing for full traceability of all samples.

Fluorescence flow cytometry technology profits from a photodiode laser used in combination with a patented polymethine dye. Cells are exposed to this fluorescence dye which binds to intracellular RNA and DNA. The populations are separated based on cells size in the forward scatter (FSC) light, cell internal structure or complexity are detected in signals of side scatter (SSC) from the dichroic mirror. Side fluorescence light (SFL) gives information about the DNA/RNA content (Picture 4). Thus, cytochemical properties decide where each cell population will be located in the scattergram. Cells of similar properties fall within the same area but may be separated using an advanced software algorithm. There are three channels using different combinations of these lights, the WBC DIFF channel, WBC BASO channel and the RET channel.

The leukocyte count and differentiation of WBC take place in 2 channels, the WBC-BASO and WBC DIFF channel. The WBC DIFF channel uses a surfactant (Stromatolyser-4DL) causing lysis of red blood cells, and at the same time slight perforation of white blood cell the membranes. A marker (Stromatolyser-4DS) migrates into the WBC and binds to nucleic acid and cell organelles. Flagging systems are made for identification of abnormal cells (e.g. abnormal WBC scattergram). The WBC differential count is performed as a 5-part count, a semiconductor laser is settled at wavelength of 633nm. An adaptive cluster analyzer system (ACAS) identifies different nucleated populations.



Picture 4: WBC Differential scattergram

In WBC DIFF scattergram, on the Y-axis, SFL measures the amount of DNA/RNA, and the X-axis measures inner cell complexity in SCC. Monocytes are large cells with a large nucleus, causing a greater SFL signal and thus to be located raised on the scattergram. Lymphocytes are represented by round cells with lack of granules and a dens nucleus, locating this cell population below monocytes and above the granulocytes. The granulocytes have similar nucleus size, locating these populations on the same level of SFL, below monocytes and lymphocytes. Eosinophils have prominent cytoplasmic granules which causes a greater SSC, moving the population to the right on the X-axis compared to other WBC populations. Neutrophils and basophils are detected together in the WBC DIFF channel, located on the same SFL level as eosinophils but with less SSC.

In the WBC BASO channel, an acidic reagent (stromatolyser-FB) lyses the red blood cells and shrinks white blood cells to bare nuclei, with the exception of basophils, which are not affected. The resulting differences between basophils and other cells are analyzed using FSC and SCC information. It also accounts for the default method for measurement of the total WBC count, this also takes place in the WBC DIFF channel and works as an internal control. The RET channel has a surfactant which labels nucleic acid of cells like WBC, NRBC, reticulocytes and platelets. Forward scatter light and fluorescent signal is used to differentiate reticulocytes from the other cells. According the fluorescent density, reticulocytes are divided into three different stages of maturity.

The Sysmex XT-2000iV has been evaluated for peripheral blood in several veterinary studies (Lilliehöök et al 2009a, b, Bauer et al 2011, Bauer et al 2012). It has also been validated for effusions, providing a precise and accurate total nucleated cell count and having moderate concordance with cytological findings for classifying canine and feline effusions (Pinto da Cunha et al 2009). In comparison to a different hematology analyzer, it was found to be excellent and favorable (Bauer et al 2011, Bauer et al 2012). The cell differentiation also allows the recognition of abnormal circulating cells, e.g. atypical lymphocytes in patients with chronic lymphocytic leukemia (Novacco et al 2015).

2.3. Assessment of nucleated red blood cells in peripheral blood

Hemolysis and blood loss, bone marrow disorders, neoplasia, extramedullary hematopoiesis, and systemic diseases with complex conditions can all lead to NRBC release into the peripheral blood. It is important to consider the whole clinical picture to differentiate the causes of premature release of NRBC (Constantino et al 2000).

2.3.1. Physiological conditions

In human medicine, nucleated red blood cells are physiologically found in peripheral blood of neonates up to the fifth day of life. This is due to the developmental immaturity of reticuloendothelial system of the spleen, also called hyposplenic state. Per 100 WBC, 0-10 NRBC are within normal limits. The NRBC numbers should fall 50% by first 12 hours after birth (Angel and Geothe, 2016). Nucleated red blood cells are also normal finding in puppies (Goggs 2014). Breeds like miniature schnauzers and dachshunds have physiologically higher NRBC numbers than other breeds, < 7 NRBC per 100 WBC (Valenciano et al 2014).

2.3.2. Anemic syndrome

Anemic syndrome is a major cause of erythropoiesis stimulation. Nucleated red blood cells may occur in the peripheral blood in both, regenerative and non-regenerative anemia. Regenerative anemia is one of the most common causes. The occurrence of NRBC does not necessarily indicate a regenerative response, since they also occur in non-regenerative anemia. In severe anemia, tissue hypoxia causes a raise in erythropoietin synthesis, leading to activation of compensatory erythropoiesis and NRBC release from bone marrow into the peripheral blood (Akhtar and Mahure 2015). If the compensatory erythropoietic response is effective, it will lead to intense marrow reaction and only a few NRBC together with reticulocytosis and polychromasia will be seen. Ineffective response will result in more NRBC released into the

peripheral blood, and dysplastic changes of erythrocytes may be seen. Dacryocytes reflect dyserythropoiesis and may be seen together with NRBC (Constantino et al 2000).

Examples of increased NRBC in peripheral blood were reported in dog with hemolytic anemia caused by babesia (Irizarry-Rovira et al 2001) and in marked regenerative anemia in cats infected by mycoplasma hemofelis (Valenciano et al 2014). Non-regenerative anemia due to cobalamin deficiency was reported with circulating erythroblasts in a dog as well (Morgan and McConnell 1999).

2.3.3. Non-anemic conditions

In adults, low numbers of NRBC may be present secondary to conditions such as splenic contraction, splenic disease or after splenectomy. Erythroblasts which escape the bone marrow should normally be cleared by the spleen. Therefore, disorders with impaired clearance capacity of the spleen cause increased numbers of NRBC in peripheral blood.

Other conditions where NRBC are seen in the circulation are connected with myeloproliferative disorders, thermal injury, traumatic bone fractures or sepsis/endotoxemia, hyperadrenocorticism, uremia, liver diseases, cardiovascular diseases and steroid administration. Changes in the microvasculature (leukemia, multiple myeloma, metastatic tumors, lead poisoning, heat stroke, infections) of the bone marrow cause a disruption of the marrow-blood barrier, resulting in release of immature erythroblasts and other progenitors (Constantino et al 2000).

In cardiopulmonary hypoxia, the pathogenesis for the limited oxygen available for tissues differs from that in anemic syndrome (Akhtar and Mahure, 2015). Red blood cells are produced in accordance to oxygen demands from tissues, and not from the level of hemoglobin. The production continues until the supply of oxygen meets the tissues demands. Therefore, a hypoxic state can be seen with NRBC and polychromasia together with normal to high hemoglobin concentration. Increased NRBC in a dog with pulmonary masses and enlarged right atrium and ventricle was reported (Faunt et al 1998).

3. Nucleated red blood cells within other hematopoietic organs

Hematopoietic progenitors including nucleated red blood cells are found within organs of extramedullary hematopoiesis (EMH). This occurs when pathophysiological alterations affect the bone marrow microenvironment.

Anemic syndrome or any disorder leading to hypoxic state are common stimulus for increased erythropoiesis. If the demand is higher than the bone marrow reserve, EMH occurs by reactivation of hematopoietic fetal tissue of the liver or spleen, or from activation of circulating hematopoietic precursors. Spleen, lymphnodes, liver, myocardium, lung, kidneys, skeletal muscle and fat are tissues where these cells have been found, along with adrenal glands and central nervous system (Carre et al 1997, Wright et al 2001, Kim 2010).

Extramedullary hematopoiesis has rarely been associated with clinical signs in veterinary medicine, and only one report exists about neurological clinical signs attributed to EHM within the choroid plexus (Bienzle et al 1995). Human reports of clinical signs from nonhepatosplenic EMH are wide, the most clinical manifestations are mainly neurological signs or hemorrhagic effusions (O'Malley 2007).

Sinusoidal endothelium of the spleen is a common site where EMH is seen. Nucleated red blood cells found in spleen cytology samples are not indicative of splenic erythropoiesis alone, as the splenic reticuloendothelial system is responsible for their clearance. Presence of high numbers of early or all stages of NRBC together with mitotic figures would support the erythroid EMH (Christopher 2003).

Degenerative and inflammatory conditions in the spleen, like lymphoid hyperplasia, hematomas and thrombosis, are the most common causes of splenic EMH in dogs and cats. This is often seen without obvious hematological abnormalities. Extramedullary hematopoiesis is seen better in cytological samples than in histological biopsies from dogs and cats with disorders like fungal splenitis, reactive lymphoid hyperplasia, histiocytic hyperplasia or neoplasia (Ballegeer et al 2007).

Other causes for EMH in animals are immune mediated hemolytic anemia (IMHA), pyruvate kinase deficiency, babesiosis, zinc toxicosis, and other disorders causing shortened erythrocyte lifespan (Wozniak et al 1997).

Myelofibrosis with EMH in liver and spleen has been found in a study of five female beagles. It could be caused by pyruvate kinase deficiency, response to iron overload or treatment with high dose of human erythropoietin seen in these dogs (Tolle et al 1977).

Extramedullary hematopoiesis is identified in different neoplasms of both, humans and animals. In dogs, it was found in patients with hemangiosarcoma, mixed mammary tumors, histiocytic sarcoma, and in a wide range of neoplasia in humans (Bertazzolo et al 2005, O'Malley 2007, Grandi et al 2010). Distinguishing leukemia or polycythemia vera from EMH can be challenging if all developmental stages of erythroid cells are present. These conditions are quite rare in animas, and immunohistochemistry can be used for diagnostics (O'Malley et al 2005). Detection of nucleated red blood cells in peripheral blood together with finding of EMH is reported, however it is uncertain whether these NRBC are from the extramedullary site or not. Clear evidence that EMH alone results in leukoerythroblastosis is missing (Johns et al 2012). The main underlying disorders were connected to the bone marrow, resulting in the uncertain extramedullary aspect. Similarly, it is seen in a report of leukoerythroblastosis in cats (Hammer and Wellman 1999). A review by Clifford in 1966 hypothesizes that it seems probable that circulating immature cells are derived from sites of EMH where tissue organization and cell release is less controlled than in bone marrow.

4. The clinical use of nucleated red blood cells

4.1. Nucleated red blood cells in critical illness

Multiple extensive studies on NRBC evaluation and their prognostic value have been made. Their presence in peripheral blood is associated with high mortality rates in critically ill patients (Stachon et al 2002). The pathophysiology behind is not completely understood, although hypoxemia, acute or chronic anemia and severe infections are connected with their appearance. Increased concentration of bone marrow stimulators, erythropoietin, interleukins 3 and 6 are associated with increased numbers of NRBC (Stachon et al 2005). Concentration of hemoglobin, platelet numbers and C-reactive protein values do not significantly correlate with NRBC counts (Stachon et al 2007). Positive finding of NRBC was associated with poor outcome in medical and surgical intensive care patients (Stachon et al 2007, Shah et al 2012). Moreover, increasing numbers correlated with increase in mortality, while decreasing numbers to zero was found to be protective (Shah et al 2012). Critical trauma patients were less likely to have abnormal NRBC compared to critical non-trauma patients, however, their presence indicated similar outcome. According to dynamic changes of NRBC counts, intensive care

patients with increased numbers of NRBC should not be relocated to normal ward, ongoing intensive care treatment should be continued (Stachon et al 2007).

Presence of NRBC in peripheral blood of critical human patients has been shown to be of prognostic value, this was similarly evaluated in critically ill dogs (Müller et al 2014). Mortality was significantly higher in patients with NRBC presence, compared to those without. Higher prevalence of NRBC was seen in anemic dogs compared to non-anemic dogs, and dogs with regenerative anemia had a higher prevalence of NRBC than those with non-regenerative anemia (Müller et al 2014). Presence of NRBC in the absence of anemia is an unusual finding. Mostly, NRBC occur in situations like lead toxicity, splenectomy, pure red cell aplasia, myeloproliferative or dysplastic disorders, hemangiosarcoma, histiocytic sarcoma, feline hepatic lipidosis or upper respiratory infections. Use of NRBC in non-anemic dogs is still unclear due to small sample sizes of research done this far. Association of NRBC count with levels of inflammatory mediators in critically ill small animals remains to be explored (Goggs 2014).

4.2. Nucleated red blood cells in neonatal diseases

Presence of NRBC in neonates is mainly associated with prematurity, hypoxia, maternal diabetes or acute stress. Asphyxia is the major problem in neonates and it is one of the main causes of infant mortality and chronic neurologic disabilities. It can cause severe hypoxic-ischemic damage in organs with long-term or fatal consequences in neonates, making prediction and prevention with targeted treatment a high priority in neonatology and pediatrics (Boskabadi et al 2015). A method of efficiently predicting perinatal asphyxia has been lacking until recently.

Chronic or acute hypoxia is an important cause of elevated NRBC count in neonates (Hermansen 2001). Nucleated red blood cells can be used as an early marker of severity of birth asphyxia during hospital stay and may help in predicting the neurological outcome (Rai et al 2014). Statistically significant relationship between NRBC levels and the outcome of asphyxiated neonates has been found (Boskabadi et al 2017). More than 11 NRBC per 100 WBC and increased hypoxic-ischemic encephalopathy severity with low first hour pH, indicated high mortality and morbidity among asphyxiated neonates.

For critically ill children up to one month of age, NRBC count may be used as a negative prognostic factor. However, this association disappears with age (Schaer et al 2014). In canine neonates, this is confused by the normal findings of NRBC in very young dogs (Goggs et al 2014).

4.3. Nucleated red blood cells in systemic inflammatory response syndrome

Nucleated red blood cells have been seen in systemic inflammatory response syndrome (SIRS). Cardiopulmonary bypass is known to cause SIRS (Espher et al 2014). Patients with long cardiopulmonary bypass time were found to have higher numbers of NRBC, together with increased production of interleukin 6 and erythropoietin, which are considered as markers of inflammation (Frey et al 1999).

Heatstroke in dogs is a clinical syndrome when core body temperature rises above 41 degrees Celsius. It is associated with central nervous system dysfunction and tachypnea, and often leads to systemic inflammatory response syndrome. Studies have shown that heatstroke in dog has an overall mortality of 50-64% (Bruchim et al 2006). Treatment is challenging in these patients because of high rate of systemic complications and fatality, and with an uncertain prognosis. NRBC have been found to be present in patients with heatstroke, where they were not associated with anemia or erythroid hyperplasia (Aroch et al 2009). The number of NRBC should decrease with time after presentation, while consistent high numbers are found in non-survivors. In a case report from Mastrorilli et al 2013, mild thrombocytopenia causing petechias and inappropriate rubricytosis were suggestive of heatstroke and demonstrated ongoing life-threatening vascular derangement.

4.4. Nucleated red blood cells in hematological diseases and chemotherapy

A high number of hematological diseases have NRBC in peripheral blood, related to stress-, ineffective- or altered erythropoiesis. They are detected in most hemato-oncological diseases and also frequently detected during chemotherapy and absent at remission. Chemotherapy causes damage to the bone marrow erythropoiesis releasing NRBC to peripheral blood (Danise et al 2012).

Nucleated red blood cells are frequently seen during monitoring of dogs undergoing chemotherapy. This may mask neutropenia since it causes an increased WBC count. Correction of nucleated cell count is important to obtain the accurate WBC count, because neutropenia is a limiting factor for dogs undergoing chemotherapy (Simon et al 2006). A study on chemotherapy of dogs affected by lymphoma, carcinoma or mast cell tumors found NRBC to be frequent in these patients. Treatment based on CHOP protocol for lymphoma patients was associated with severely increased NRBC count, slighter elevation was observed in mast cell tumor treatment with vinblastine and prednisolone. Detection of NRBC in peripheral blood

seems to be a consequence of chemotherapy and not associated with anemia (Moretti et al 2015).

OWN STUDY

1. Aims

The presence of nucleated red blood cells in the peripheral blood is increasingly used as a negative prognostic marker in human medicine. Similar studies made on dogs have shown their usefulness in prediction of prognosis in certain pathological states. Because of the interference with nucleated leukocytes, only several hematology softwares are developed do detect circulating NRBC in humane medicine. However, no automated analyzer has been validated to identify NRBC in peripheral blood in dogs or cats. In veterinary medicine blood smear examination remains the only way of detection for this specific cell population.

The aims of the study were:

1. To quantify and morphologically evaluate NRBC population in canine and feline patients.
2. To evaluate the significance of bias in total WBC count and different WBC population changes by presence of NRBC.
3. To assess the possibility for identification of circulating NRBC using hematology analyzer based on fluorescence flow cytometry.

2. Material and Methods

2.1. Material

2.1.1. Animals

The collected data for the study derived from dogs and cats treated in the Small Animal Clinic of the University of Veterinary and Pharmaceutical Sciences Brno. All patients were presented to the clinic between July 2016 and February 2018, of all which had a hematology sample taken.

2.1.2. Inclusion criteria

The inclusion criteria in the study were based on finding at least 1 NRBC / 100 WBC during peripheral blood smear evaluation regardless of other pathological findings. With the purpose to differentiate regenerative and non-regenerative anemia, an automatic reticulocyte analysis was performed together with routine hematological examination as a part of diagnostic work-up or as an additional analysis from the same blood sample. For patients with more than one blood collection, the reticulocyte analysis was performed just once.

2.1.3. Sample collection, preparation and storage

For the study, all samples of peripheral blood within EDTA tubes (Dispolab s.r.o., Czech Republic) were handled from samples delivered to the Small Animal Clinical Laboratory of the University of Veterinary and Pharmaceutical Sciences Brno for hematological analysis and processed within 2 hours after collection.

2.2. Methods

2.2.1. Automatic peripheral blood analysis

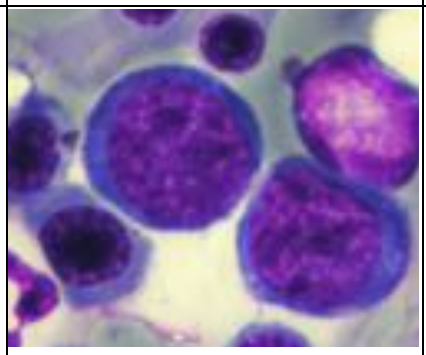
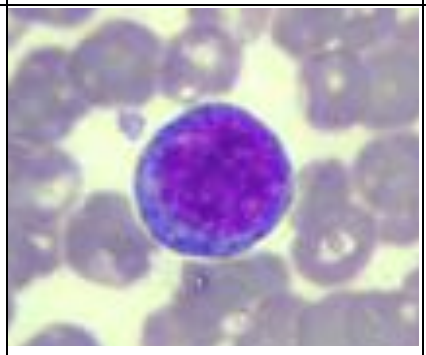
The automated hematological analysis with a 5-part WBC differential count was determined by the Sysmex XT-2000iV analyzer (Sysmex, Japan). For canine samples, the measured hemoglobin concentration was used to differentiate anemic and non-anemic patients with cut-off value 124g/l. In dogs, an addition reticulocyte quantification was made for allowing differentiation of regenerative and non-regenerative anemia based on cut-off value for absolute RET count $60x10^9$/l. Automated results (including numeric results, histograms and

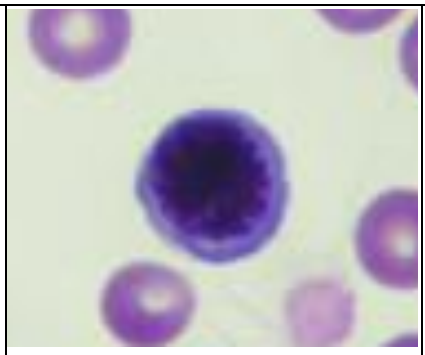
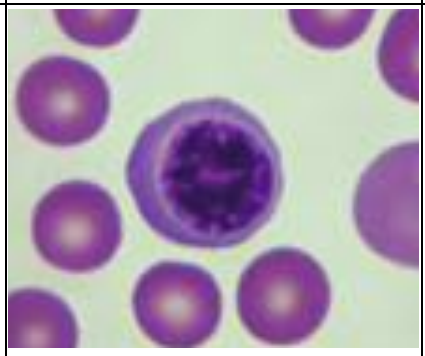
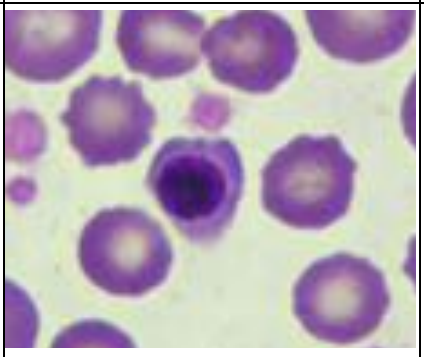
scattergrams as well as primary analytic data) from Sysmex XT-2000iV system were saved for additional analysis using modified software settings (see below).

2.2.2. Blood smear evaluation

All blood smears were prepared from a 5µL sized drop of EDTA blood samples and stained by modified Romanowsky stain (Hemacolor, Germany). Nucleated red blood cell population was evaluated as the total number of NRBC per 100 WBC (Zabolotzky and Walker 2014). According to the number of NRBC found, patients were divided into 5 groups as follows: **Group 1: 1-4 NRBC, Group 2: 5-10 NRBC, Group 3: 11-15 NRBC, Group 4: > 15 NRBC.** Different stages of maturation of NRBC were identified based on morphological classification routinely used for NRBC evaluation in bone marrow samples (Table 1).

Table 1: Morphological classification of nucleated red blood cells

Stage of maturation	Example	Morphology
Rubriblast		- Large cell - High N:C ratio - Fine granular chromatin with nucleoli - Intense basophilic cytoplasm
Probubricyte		- Reduced ratio of N:C - Coarser clumping of chromatin, no nucleoli - Dark blue cytoplasm

Basophilic rubricyte		- Moderate N:C ratio - Course clumped chromatin - Blue cytoplasm
Polychromatophilic rubricyte		- Lower N:C ratio - Course chromatin pattern - Polychromatophilic cytoplasm
Metarubricyte		- Small cell - Deep dark to pyknotic nucleus - Without distinguishable chromatin pattern. Polychromatophilic to normochromic cytoplasm.

2.2.3. Correction of WBC count based on NRBC

For all samples we calculated corrected WBC count based on correction formula (Webb et at 2011):

$$Corrected\ WBC\ Count = Original\ WBC\ Count\ x\ \frac{100}{no.\ of\ NRBC + 100}$$

From the corrected WBC count and the original percentage of each WBC population we calculated the new absolute number for each of them:

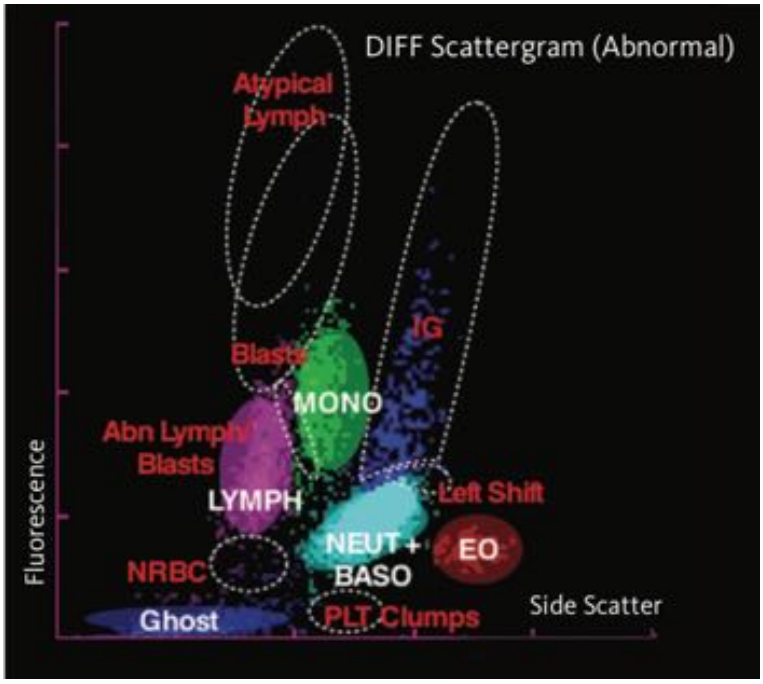
$$Corrected\ DIFF\ Count = Corrected\ WBC\ Count\ x\ \frac{Original\ Population\%}{100}$$

The estimated number of circulating NRBC was calculated as (Aroch et al 2009):

$$Estimated\ NRBC\ Count = Original\ WBC\ Count - Corrected\ WBC\ Count$$

2.2.4. Sysmex XT-2000iV software settings

Based on size, nucleus and cell complexity, nucleated red blood cells resemble lymphocyte population represented by small cells, with high N:C ratio, round nucleus and lack of granules. The hypothetical location of such a population would be located close to the area of small lymphocytes. On the WBC DIFF scattergram, NRBC population should appear on the left side from granulocytes on the X-axis (SSC) and under the area of small to medium-sized lymphocytes and monocytes on the Y-axis (SFL) (Picture 5).

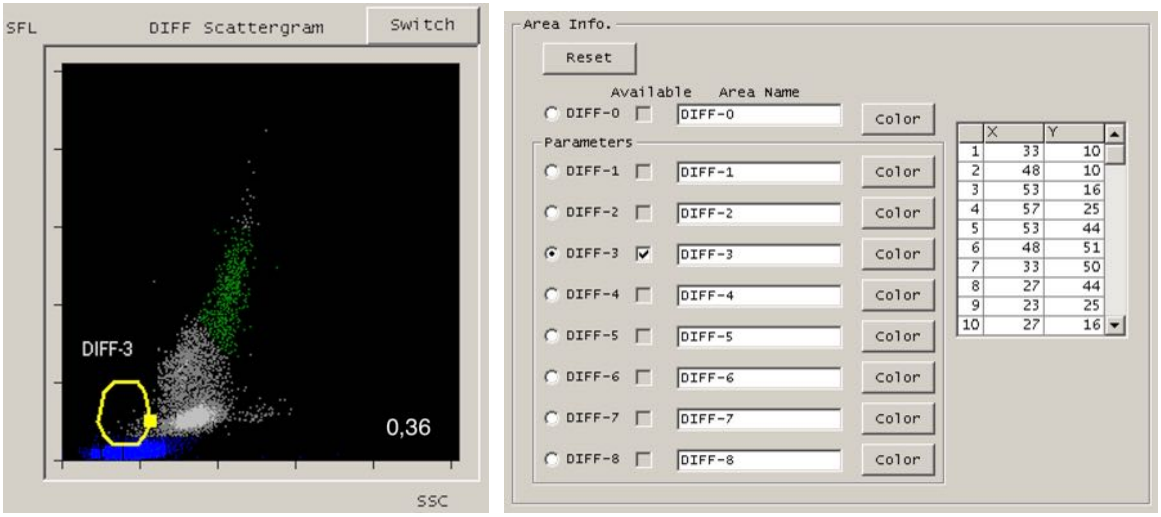


BASO - Basophile
EO - Eosinophile
IG – Immature granulocyte
LYMPH - Lymphocyte
MONO- Monocyte
NEUT - Neutrophile
NRBC – Nucleated red blood cell
PLT - Platelet

Picture 5: Hypothetical location of NRBC area in association with different areas for WBC populations (WBC DIFF scattergram).

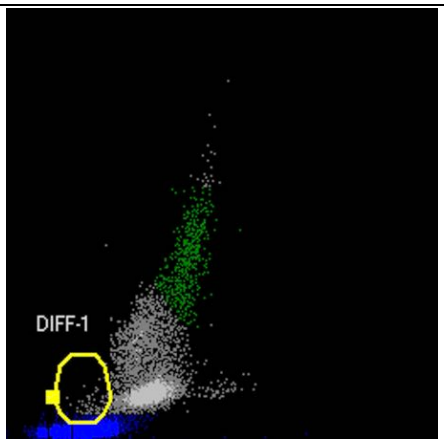
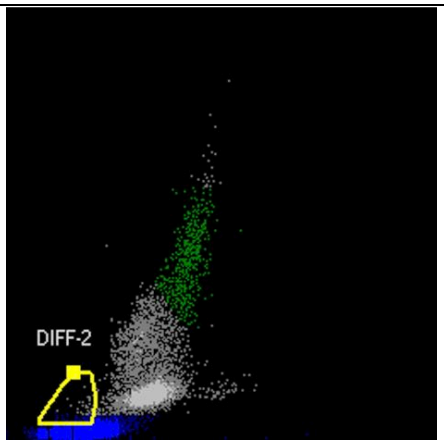
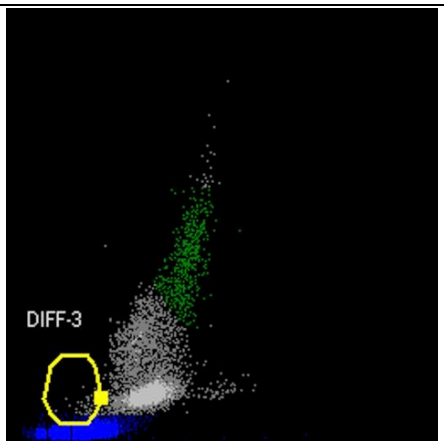
The configuration of Sysmex software setting, with the purpose to identify the specific area of NRBC population, was done in 3 steps. As first we selected samples with highest numbers of NRBC (group 3 and 4) to see a specific area of cells not present in other samples. In the second step we defined a gate of WBC DIFF channel using several crucial points, each defined by X- and Y-axis (Picture 6). In the last step we reanalyzed results of patients from group 3 and 4 according to the new setting. At this time, we also reanalyzed all lymphopenic patients from

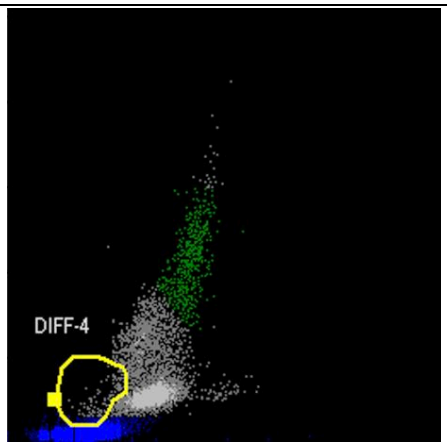
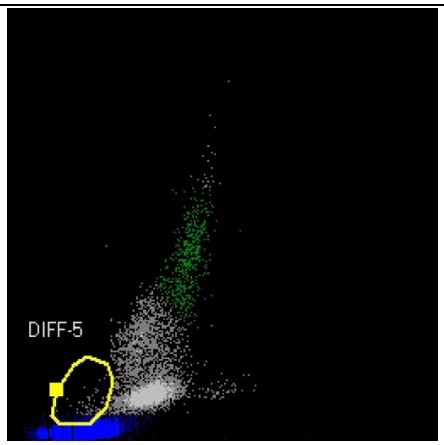
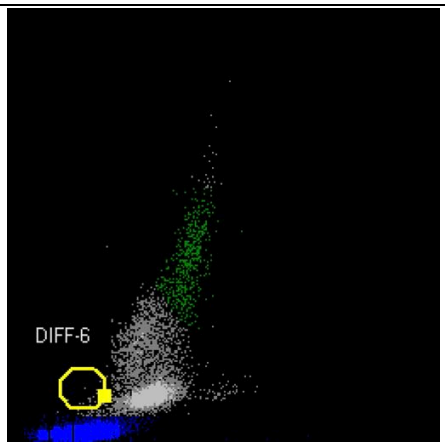
group 1 and 2 (as negative samples for NRBC with no interference with lymphocytes) and all patients with lymphocytosis from group 1 and 2 (as negative samples with possible interference with lymphocytes). For each patient a quantitative result possibly representing NRBC in a predefined area was recorded. Based on these preliminary data, 6 different software settings were tested (Table 2).



Picture 6: Software setting defining hypothetical location of NRBC area with quantitative result possibly representing NRBC in a predefined area

Table 2: Different configurations of Sysmex-XT 2000iV software settings defining hypothetical location of NRBC area

Setting No.	Area on WBC DIFF scattergram	Axis setting for WBC DIFF channel	
		Axis X	Axis Y
DIFF1		38	10
		52	10
		58	16
		62	25
		58	44
		52	51
		38	50
		32	44
		28	25
		32	16
DIFF2		20	10
		40	10
		50	10
		53	24
		50	40
		40	40
		33	33
		20	14
DIFF3		33	10
		48	10
		53	16
		57	25
		53	44
		48	51
		33	50
		27	44
		23	25
		27	16

Setting No.	Area on WBC DIFF scattergram	Axis setting for WBC DIFF channel	
		Axis X	Axis Y
DIFF4		32	10
		52	10
		58	16
		62	25
		70	30
		70	40
		68	44
		52	51
		38	50
		32	44
		28	25
		32	16
DIFF5		32	10
		40	10
		49	10
		59	20
		63	35
		60	45
		48	50
		40	45
		30	30
		27	15
DIFF6		38	20
		52	20
		58	27
		58	37
		52	43
		38	43
		32	37
		32	27

2.2.5. Statistical analysis

Quantitative and morphological evaluation of NRBC population

The incidence of canine and feline patients presented with NRBC in peripheral blood is expressed as percentage from all patients whose blood samples were admitted at the Small Animal Clinical Laboratory during July 2016 to February 2018.

The total numbers of NRBC are expressed as median value (min, max) for all 3 groups of canine patients (regenerative anemia, non-regenerative anemia, non-anemic) and based on assessment of data distribution normality (Kolmogorov-Smirnov test) compared using Mann Whitney test (GraphPad Software, Inc., USA).

Evaluation of bias in total WBC count and different WBC population changes caused by NRBC in dogs

The bias in WBC counts caused by NRBC was assessed by correction of total WBC count and all WBC populations based on the number of NRBC found on the blood smear. Changes are reported as number of cases where corrected result appeared inside or outside the reference range compared to original value (Table3).

Clinical significance of bias between original and corrected WBC count and different WBC populations is expressed as percentage of change (median, min, max) for each group of patients (groups 1-5). Based on assessment of data distribution normality (Kolmogorov-Smirnov test), statistical significance of bias in WBC counts caused by NRBC was assessed by Wilcoxon test for paired data within all 5 groups test (GraphPad Software, Inc., USA).

Table 3: Reference values for WBC count and DIFF count in dogs

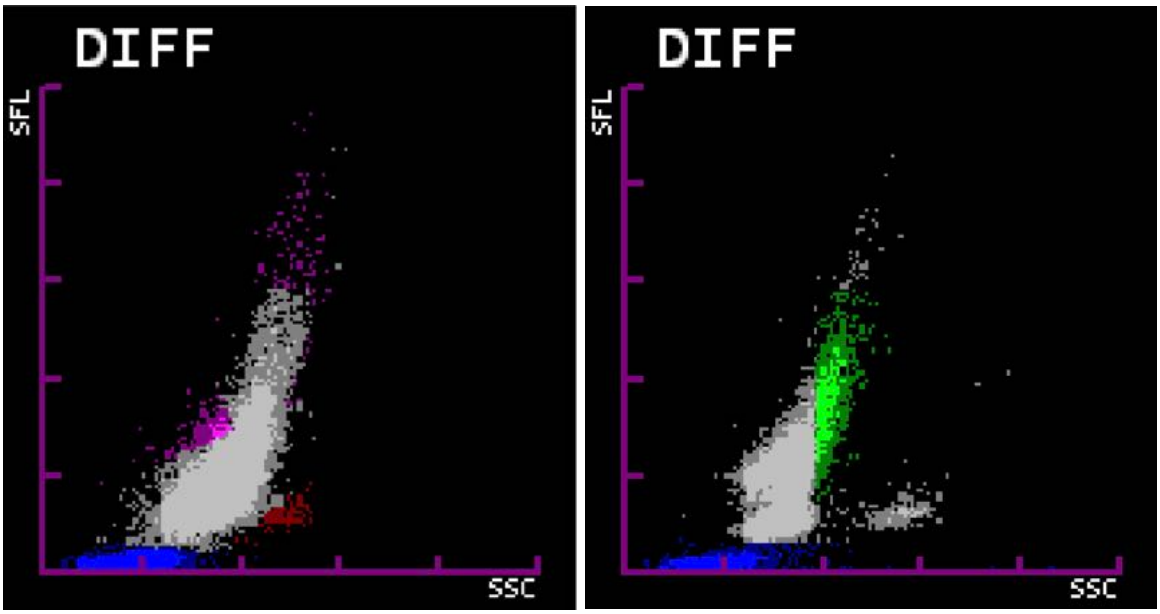
	WBC	**Neutrophils**	**Lymphocytes**	**Monocytes**	**Eosinophils**	**Basophils**
Unit $x10^9/l$	6- 18	3,3 - 11,0	1,0 - 3,6	< 0,7	< 0,8	< 0,1

Assessment of automatic analysis for detection of circulating NRBC in dogs

For each configuration of Sysmex software setting, the primary accuracy based on morphology was evaluated as number of true positive and true negative results divided by all samples. For morphologically identified NRBC, as true positive we defined a patient with ≥ 5 NRBC on the blood smear and a cut-off value for predefined area equal or higher than 0,10 (DIFF1), 0,03 (DIFF2), 0,03 (DIFF3), 0,22 (DIFF4), 0,07 (DIFF5) and 0,01 (DIFF6), respectively. As true

negative we defined patients with 1-4 NRBC on the blood smear and the quantitative result possibly representing NRBC in a predefined area lower than the cut-off value. In order to identify all morphologically positive samples (100% sensitivity), we selected as the detection limit for estimated number of circulating NRBC value ≥ $0{,}3x10^9$/l. Using this limit, accuracy calculation followed (accuracy based on estimated number of NRBC). As true positive we defined patients with estimated number of circulating NRBC ≥ $0{,}3x10^9$/l and a cut-off value for predefined area equal or higher than 0,07 (DIFF1), 0,02 (DIFF2), 0,02 (DIFF3), 0,16 (DIFF4), 0,06 (DIFF5) and 0,01 (DIFF6), respectively. As true negative we defined a patient with estimated number of circulating NRBC less than $0{,}3x10^9$/l and quantitative result possibly representing NRBC in a predefined area lower than the cut-off value.

For all configurations we reviewed data of all available hematological analyses. Based on observed abnormalities we excluded cases with possible interference (patients with leukopenia or flagged with WBC abnormal scattergram (picture 7)) and recalculated the new accuracy for all 6 DIFF with the definition of true positivity as number of circulating NRBC more than $0{,}3x10^9$/l and same cut-off values. The accuracy of this approach was tested as reanalysis of 55 random canine blood samples corresponding to routine caseload at Small Animal Clinical Laboratory at University of Veterinary and Pharmaceutical sciences Brno.



Picture 7: Examples of excluded WBC-DIFF scattergrams

Finally, with the purpose to identify NRBC in canine peripheral blood by automatic analysis an algorithm was formulated as follows:

Patient has no leukopenia or is not flagged with WBC abnormal scattergram and the quantitative result from DIFF area is equal or higher than the cut-off value used for the DIFF setting with the best accuracy for both, collected patient group and random group.

3. Results

Between July 2016 and February 2018 total of 144 samples derived from 112 dogs and 3 cats were microscopically evaluated for NRBC. From all collected data, hematological results (WBC, HGB, RET) were correctly archived in 133/144 samples. From these, an automated total white blood cell count was available for 108 samples. In remaining 25 samples, automated differentiation of leukocyte populations based on fluorescence flow cytometry failed (samples were flagged with WBC Abnormal Scattergram). Because only 3 feline samples were collected, results from cats are presented in a restricted form.

3.1. Animals

From all samples delivered to the Small Animal Clinical Laboratory during July 2016 and February 2018, dogs with NRBC in peripheral blood represented 3,2% (141/4320), cats 0,3% (3/880) of cases. Canine group comprised patients of 42 different breeds, 15 crossbreds and 15 unknown. The different breeds were as follows: Yorkshire terrier (n=10), Labrador retriever (n=7), Dachshund (n=3), Cavalier King Charles Spaniel (n=3), Chihuahua (n=3), German Shepherd (n=3), Jack Russel Terrier (n=3), Maltese (n=3), Pitbull (n=3), American Cocker Spaniel (n=2), Boxer (n=2), English Cocker Spaniel (n=2), Golden retriever (n=2), Irish setter (n=2), Miniature Schnauzer (n=2), Poodle (n=2), Pug (n=2), Shih-Tzu (n=2), Spitz (n=2), Weimaraner (n=2), and one of Beagle, Belgian Shepherd, Bernese Mountain Dog, Bichon Frise, Bohemian Spotted Dog, Bohemian Wire-haired Pointing Griffon, Bolognese Dog, Border Collie, Brussels Griffon, Bull terrier, Caucasian Shepherd Dog, Flat Coated Retriever, French Bulldog, German Pincher, German Spaniel, Papillon, Prague Ratter Dog, Rhodesian Ridgeback, Shiba-Inu, Small Münsterländer, Tibetan terrier and West Highland White terrier. Feline group included 1 Domestic Shorthair cat, 1 Ragdoll and one with no data.

The 112 canine patients included 45 females (14 castrated), 52 males (3 castrated) and 15 patients with unknown data. Median age was 10 years (range: 1,7 to 16 years). Median weight was 11,5 kg (range: 1,5 50 70 kg). In feline patients, there were 2 females (1 castrated) and one with no data. Median age was 3,7 years (range: 2,5 to 4,9 years). Median weight was 2,8 kg (range: 2,1 to 3,5 kg).

From all 112 dogs, 69 dogs (88 samples) suffered from anemia. From these 69 dogs, 59 (77 samples) had regenerative anemia and 10 dogs (11 samples) were presented with non-regenerative anemia. The remaining 43 dogs were non-anemic.

Because only 3 cats were included in the study, feline patients were not divided in anemic or non-anemic group. They were also not evaluated for bias in WBC changes by NRBC neither for automatic detection of NRBC count by fluorescence flow cytometry. In these 3 cats we observed 6, 3 and 4 NRBC on the blood smear.

3.2. Quantitative and morphological evaluation of NRBC population

Based on the number of NRBC, samples were most commonly represented just by one erythroblast. From all 141 samples, 43,3% were included in group 1 (Table 4 and 5). The predominant morphological subtype was metarubricyte representing 88% of all circulating NRBC. Quantitative results together with morphological evaluation for individual cases for all 4 groups are presented in Graphs 1-4.

Table 4: Sample distribution of canine patients according to NRBC numbers

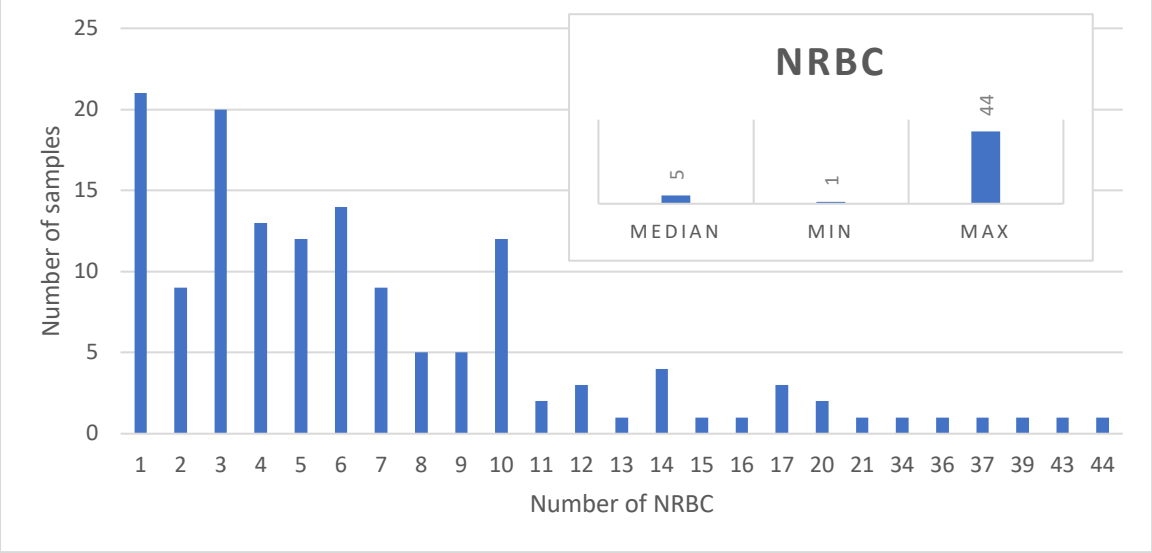
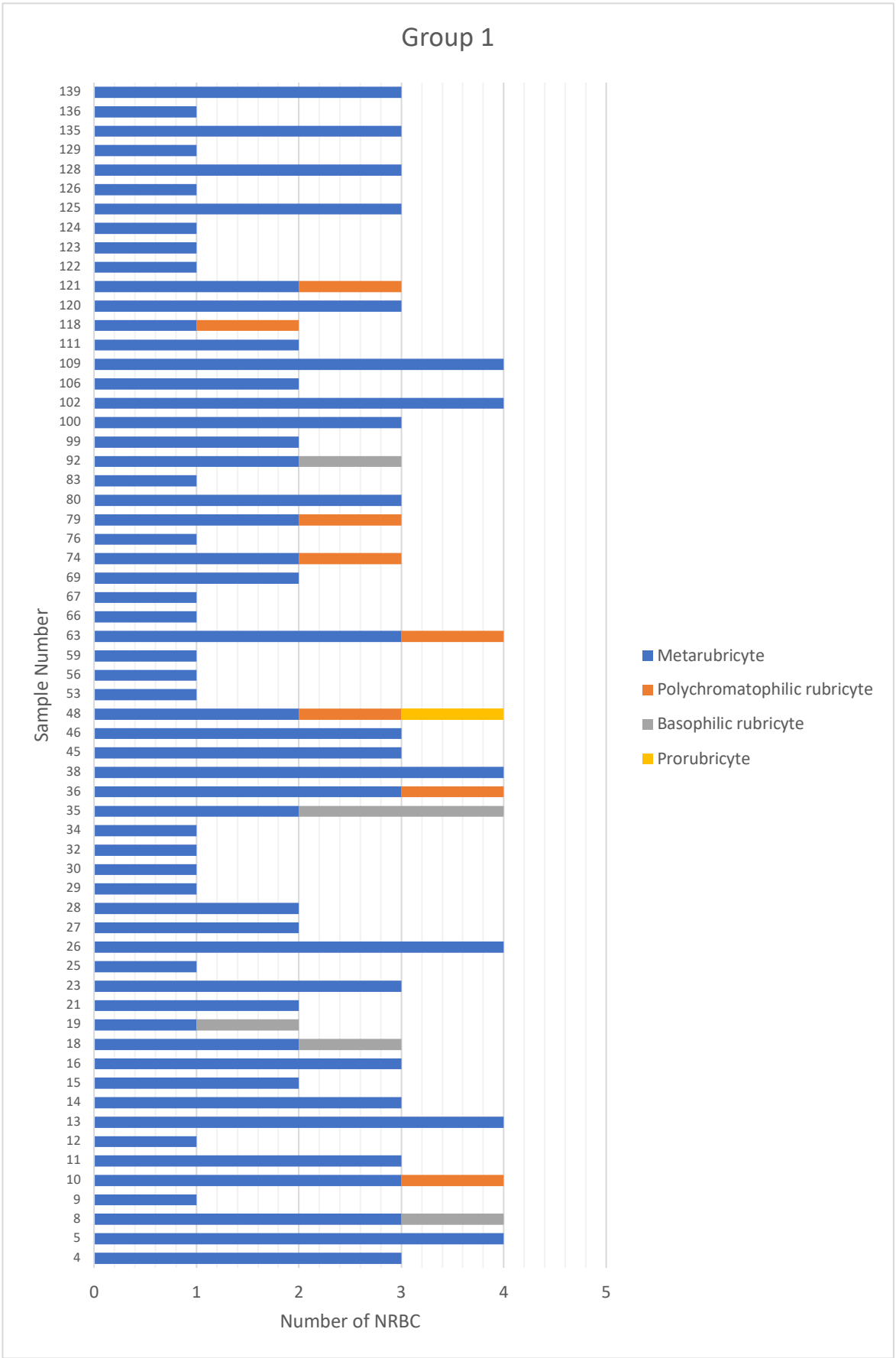


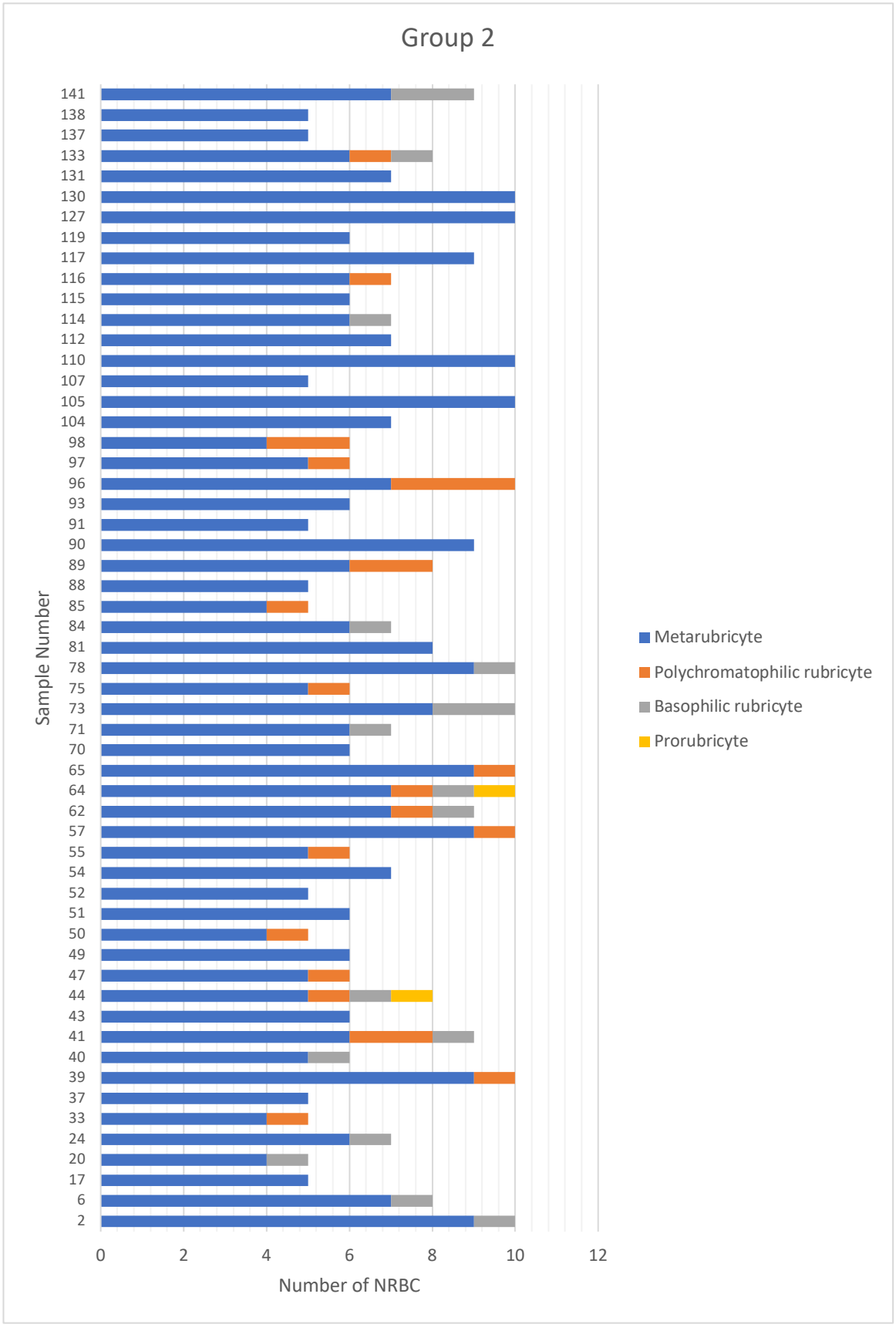
Table 5: Sample distribution among groups of canine patients according to NRBC numbers

	Group 1 1-4 NRBC	**Group 2 5-10 NRBC**	**Group 3 11-15 NRBC**	**Group 4 >15 NRBC**	**Total**
Number of samples	61	56	11	13	141

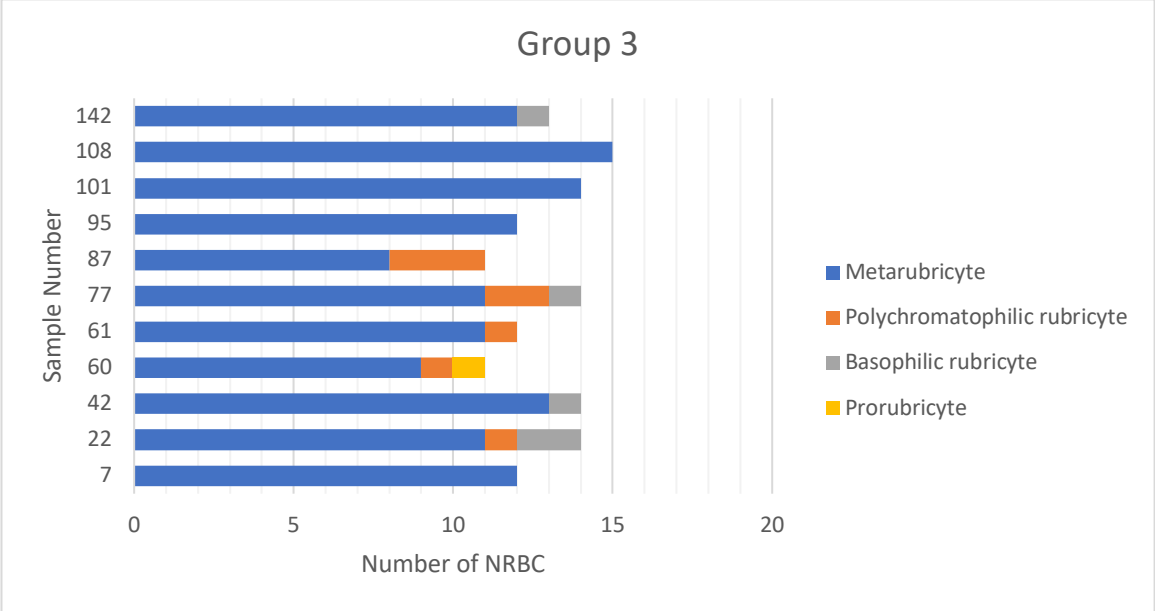
Graph1: Distribution of NRBC morphological subtypes in canine samples (group 1)



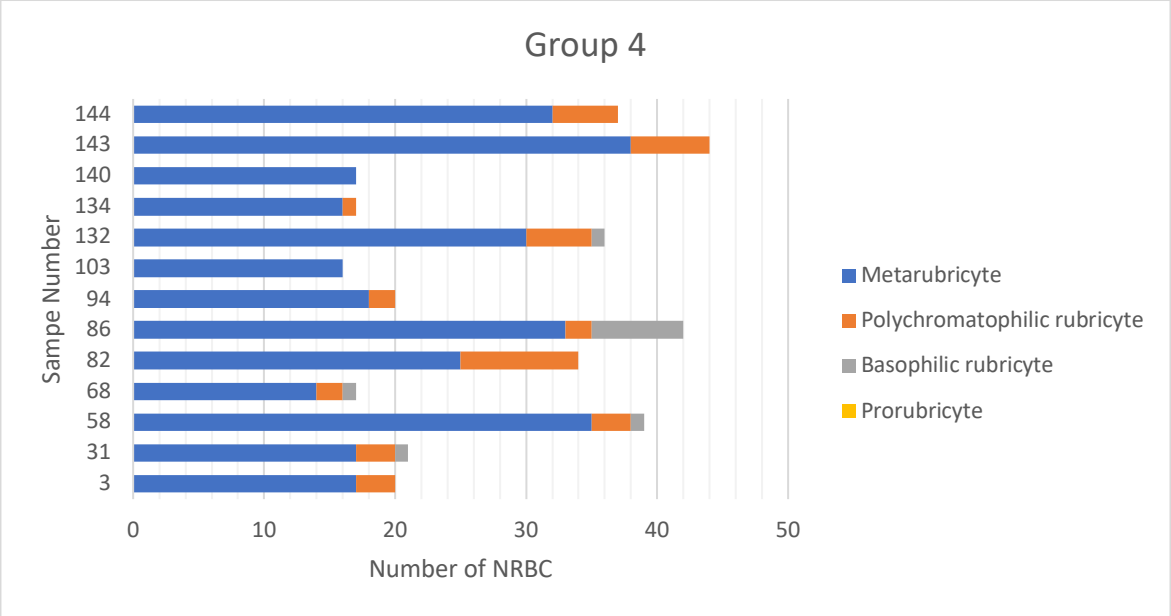
Graph 2: Distribution of NRBC morphological subtypes in canine samples (group 2)



Graph 3: Distribution of NRBC morphological subtypes in canine samples (group 3)



Graph 4: Distribution of NRBC morphological subtypes in canine samples (group 4)



Based on the HGB concentration and RET count, the median value for NRBC count in dogs with regenerative and non-regenerative anemia were 6 (range: 1 to 44) and 3 (range: 1 to 4), respectively. For the non-anemic group, the median value for NRBC count was 5 (range: 1 to 10). Dogs with regenerative anemia had statistically higher NRBC count than patients with non-regenerative anemia ($p = 0{,}00026$) or non-anemic dogs ($p = 0{,}01046$). Patients from non-anemic group had significantly higher NRBC count than dogs with non-regenerative anemia ($p = 0{,}03318$).

3.3. Evaluation of bias in total WBC count and different WBC population changes caused by NRBC in dogs

Based on the correction, 3,67% (4/112) of dogs with leukocytosis turned to normal leukocyte count and only one with normal WBC count turned to leukopenia. From 58 neutrophilic dogs, 3 turned to normal neutrophile count. One dog with normal neutrophile count became neutropenic. From 18 dogs with lymphocytosis, 4 patients turned to normal lymphocyte count. One dog with normal lymphocyte count became lymphopenic. From 92 dogs with monocytosis, 1 turned to normal monocyte count. From 15 dogs with eosinophilia, 3 turned to normal count. From 23 dogs with basophilia, 5 turned to normal count. Percentage of change between original and corrected WBC count and different WBC populations for all canine groups representing clinical significance of bias caused by NRBC is shown in Table 6. The median percentage of change in total WBC count caused by NRBC interference was lower than 5%. The highest interference was confirmed in group 4 with maximal value over 30%.

Table 6: Clinical significance of bias between original and corrected WBC counts based on NRBC count (canine samples)

% of change Orig. / Corr. Count: Median (min, max)	Group 1	Group 2	Group 3	Group 4	Total
WBC count	1,96% (0,99%, 3,85%)	6,54% (4,76%, 9,09%)	11,89% (9,91%, 13,04%)	16,67% (13,80%, 30,55%)	**4,76%** (0,99%, 30,55%)
Neutrophile count	2,02% (0,86%, 3,93%)	6,52% (4,29%, 9,16%)	11,44% (9,92%, 13,08%)	16,59% (13,81%, 27,99%)	**5,59%** (0,86%, 7,99%)
Lymphocyte count	2,46% (0,73%, 4,22%)	6,69% (3,86%, 9,50%)	11,09% (10,10%, 14,23%)	16,72% (10,97%, 28,08%)	**5,43%** (0,73%, 28,08%)
Monocyte count	2,10% (0,27%, 4,51%)	6,39% (1,87%, 9,70%)	11,40% (9,91%, 13,16%)	16,67% (14,06%, 28,02%)	**5,37%** (0,27%, 28,02%)
Eosinophile count	1,96% (0,99%, 3,85%)	6,54% (4,76%, 9,09%)	11,50% (9,91%, 13,04%)	16,67% (13,79%, 28,06%)	**5,66%** (0,99%, 28,06%)
Basophile count	2,91% (0,99%, 3,85%)	6,54% (4,76%, 9,09%)	11,89% (9,91%, 13,04%)	16,67% (13,79%, 28,06%)	**5,66%** (0,99%, 28,06%)

There was significant decrease in corrected total WBC count compared to original total WBC count in all groups of dogs. For basophilic population significant decrease caused by NRBC interference was observed only in group 4 (Table 7).

Table 7: Statistical significance of bias between original and corrected WBC counts based on NRBC count (canine samples)

Wilcoxon test (p-value)	Group 1	Group 2	Group 3	Group 4	Total
WBC	<0,0001	<0,0001	0,0020	0,0010	<0,0001
Neutrophile count	<0,0001	<0,0001	0,0039	0,0078	<0,0001
Lymphocyte count	<0,0001	<0,0001	0,0039	0,0078	<0,0001
Monocyte count	<0,0001	<0,0001	0,0039	0,0078	<0,0001
Eosinophile count	<0,0001	<0,0001	0,0039	0,0078	<0,0001
Basophile count	0,2782	0,2490	0,3828	0,0234	0,0039

The estimated numbers of circulating NRBC calculated as the difference between original WBC Count and corrected WBC count are presented as median value (min, max) for all 5 canine groups (Table 8).

Table 8: Estimated number of NRBC (canine samples)

	Group 1	Group 2	Group 3	Group 4	Total
Estimated Number of NRBC ($x10^9$/l): Median (min, max)	0,30 (0,07, 0,91)	1,17 (1,31, 4,41)	1,97 (0,35, 5,29)	3,48 (1,83, 9,84)	**0,82** (0,07, 9,84)

3.4. Assessment of automatic analysis for detection of circulating NRBC in dogs

Primary accuracy based on both morphologically identified NRBC and estimated number of circulating NRBC, was calculated for 6 selected Sysmex software settings from 115 canine samples (Table 9). In morphologically identified NRBC, the highest accuracy was found for DIFF5 (66%), in estimated number of circulating NRBC, the highest accuracy was observed in DIFF3 (81%).

Table 9: Detection of circulating NRBC based on morphology in Sysmex software settings

Number of cases		True positive	True negative	False positive	False negative	Accuracy (%)
DIFF1	Primary	45	30	21	19	**65**
DIFF2	Primary	49	24	27	15	**63**
DIFF3	Primary	50	24	27	14	**64**
DIFF4	Primary	47	28	23	17	**65**
DIFF5	Primary	48	28	22	17	**66**
DIFF6	Primary	25	43	8	25	**59**

Based on observed abnormalities we excluded cases with possible interference (patients with leukopenia or flagged with WBC abnormal scattergram) and recalculated the new accuracy (Table 10). With the definition of true positivity as number of circulating NRBC more than $0{,}3x10^9$/l and same cut-off values as used for the accuracy based on estimated number of circulating NRBC, the highest accuracy was observed in DIFF3 (82%).

Table 10: Detection of circulating NRBC based on estimated numbers in Sysmex software settings

Number of cases		True positive	True negative	False positive	False negative	Accuracy (%)
DIFF1	Primary	68	15	11	21	**72**
	Recalc.	59	11	7	13	**78**
DIFF2	Primary	77	14	12	12	**79**
	Recalc.	66	10	7	7	**84**
DIFF3	Primary	78	15	11	11	**81**
	Recalc.	64	10	9	7	**82**
DIFF4	Primary	74	15	11	15	**77**
	Recalc.	60	11	8	11	**79**
DIFF5	Primary	69	18	8	20	**76**
	Recalc.	59	13	6	12	**80**
DIFF6	Primary	60	22	4	29	**71**
	Recalc.	49	17	2	22	**73**

To confirm the approach, with the definition of true positivity as number of circulating NRBC more than $0,3x10^9/l$ and cut-off values derived from recalculated accuracy based on estimated number of circulating NRBC, we reanalyzed data of 55 random canine blood samples. The accuracies for all 6 DIFF are presented in Table 11. The highest accuracy for this random group was found for DIFF6 (91%).

Table 11: Detection of circulating NRBC based in a random canine group

Number of cases	True positive	True negative	False positive	False negative	Accuracy (%)
DIFF1	3	43	6	3	**84**
DIFF2	5	33	16	1	**69**
DIFF3	5	34	15	1	**71**
DIFF4	3	43	6	3	**84**
DIFF5	3	43	6	3	**84**
DIFF6	3	47	2	3	**91**

From both, the collected patient group and the random group, we selected 3 DIFF settings with the highest accuracy (green labels, Tables 10 and 11). Settings DIFF1, DIFF4 and DIFF6 were excluded based on results from collected patient group. Setting DIFF2 and DIFF3 were excluded based on results from random group.

Finally, we formulated an algorithm for identification of NRBC in canine blood by an automatic analysis as follows:

Patient has no leukopenia or is not flagged with WBC abnormal scattergram and the quantitative result from DIFF5 area is equal or higher than 0,06.

According to this algorithm, from all false results, 66,6% were false negative in the patient group and 33,3% were false negative in random group.

4. Discussion

Identification of peripheral NRBC should be as important in veterinary medicine as it is in human medicine. Their negative prognostic value is similar in both humans and canine patients (Müller et al 2014, Stachon et al 2004). Presence of NRBC indicates the reaction bone marrow has to stress and disease (Akthar et al 2015). They are not associated with specific disorder but should indicate a pathologic state (Tsjui et al 1999). Correct quantification of NRBC has clinical importance also because they cause a false elevation of WBC count. There are only a limited number of studies focused on NRBC evaluation and their diagnostic value in veterinary medicine.

4.1. Quantitative and morphological evaluation of NRBC

In our study, 144 blood samples with at least one nucleated red blood cell on the blood smear were collected from 112 canine and 3 feline patients. From all samples delivered to the Small Animal Clinical Laboratory during July 2016 and February 2018, dogs with NRBC in peripheral blood represented 3,2% (141/4320), cats 0,3% (3/880) of cases. This shows that NRBC are 10x more commonly present in dogs than in cats. The scarce number of feline samples indicates their rare occurrence, which is probably also why only one study was directed on NRBC population in this species (Hammer and Wellman 1999). In this study, erythroblastosis was presented in 20/313 of feline samples. The possible explanation for this 20x more frequent occurrence may be different method of quantification (S-plus IV Coulter counter, USA).

Nucleated red blood cells in the peripheral blood are physiologically observed in neonates. There were no patients from this age group in our study. All patients were adult and suffered from some clinical disease. From all 112 dogs, 69 dogs suffered from anemia, from which 59 had regenerative anemia and 10 dogs were presented with non-regenerative anemia. The remaining 43 dogs were non-anemic. According to Schwarzt and Stansburz 1954 under normal conditions the peripheral blood of healthy humans is generally free of NRBC, which tends to be found in patients with severe diseases. In studies of NRBC occurrence in human medicine, erythroblasts are seen equally in hematological and non-hematological conditions (Tsuji et al 1999). The presence of NRBC in the absence of anemia is an unusual finding associated with poor outcome (Goggs 2014, Müller et al 2014). A cut-off point 18 NRBC/100 WBC

corresponded to a sensitivity and specificity of 91% and 88% for death (Aroch et al 2009) making the correct evaluation of NRBC a readily available test in critically ill patients.

Based on the number of NRBC, samples were most commonly represented just by one erythroblast. From all 141 samples, 43,3% were included in group 1 (1-4 NRBC). The median value for NRBC count in dogs with regenerative and non-regenerative anemia were 6 (range: 1 to 44) and 3 (range: 1 to 4), respectively. For the non-anemic group, the median value for NRBC count was 5 (range: 1 to 10). The median NRBC count in dogs with heatstroke was 24 (range: 0 to 124, Aroch et al 2009). None of dogs in our study suffered from this disease. However differential diagnoses for NRBC presence in peripheral blood include various conditions (Goggs 2014, Müller et al 2014). Patient with the highest number of NRBC (44) was diagnosed with regenerative immune-mediated hemolytic anemia. NRBC released into the peripheral blood are expected in states of regenerative anemia, when there is increase in erythropoiesis.

Dogs with regenerative anemia had statistically higher NRBC count than patients with non-regenerative anemia or non-anemic dogs. Patients from non-anemic group had significantly higher NRBC count than dogs with non-regenerative anemia. Significant difference between regenerative and non-regenerative anemia was also proved by Müller et al 2014 in critically ill dogs. From 13 patients in group 4 (>15 NRBC), 11 had regenerative anemia. In the study of Müller et al 2014 the highest mortality (80%) was seen in NRBC positive dogs with regenerative anemia which indicates the importance of NRBC evaluation.

The predominant morphological subtype was metarubricyte, representing 88% of all circulating NRBC. Almost identical number of metarubricytes (86%) was seen in the study of Aroch et al 2009. In patients with heatstroke they proved that the proportion of rubricytes compared to metarubricytes was higher in non-survivors. Thus, it is possible that the more severe injury, the higher is the proportion of rubricytes and lower is the proportion of matured metarubricytes of the total NRBC population.

In our study Yorkshire terrier was the most frequent breed seen with NRBC. The patients were of numerous different breeds (42) and crossbreeds (15), however there were 3 dachshunds and 2 miniature schnauzers which are stated to have a physiologically higher NRBC count (<7 NRBC/100WBC, Brockus 2011). These 5 patients were represented by 2, 7, 15 (dachshunds) and 1, 5 (miniature schnauzers) NRBC found on the blood smear which does not allow for conclusive result.

4.2. Evaluation of bias in total WBC count and different WBC population changes caused by NRBC in dogs

In veterinary medicine blood smear examination is the only way of detection for NRBC population. Due to the similarity of the NRBC with lymphocytes, especially the atypical or reactive ones, the intraobserver evaluation of these cells may be incorrect (Tsjui et al 1999, Rin et al 2017).

The hematology analyzers quantify all nucleated cells and therefore will in case of high NRBC misinterpret WBC and different WBC populations (Tantanate et al 2015, Hwang et al 2016). The Sysmex XT-2000iV analyzer is validated for RBC, PLT, WBC count and WBC differential count in dogs (Lilliehöök et al 2009a, b). In these studies, NRBC was revealed to appear in WBC-BASO and WBC-DIFF channel counts and merged into the lymphocyte and neutrophile area on the WBC DIFF scattergram. From 133 samples, 25 were flagged with WBC abnormal scattergram. From these 25 samples, 12 were with positive finding of more than 5 NRBC on the blood smear. This can indicate that the software (ACAS) was not able to correctly separate different WBC populations, which may be because of interference with NRBC.

We evaluated bias in total WBC count and different WBC population changes caused by NRBC based on the recommended correction formula (Webb et at 2011). There was significant decrease in corrected total WBC count compared to original total WBC count in all groups of dogs, including group 1 considered as negative based on low number of NRBC on the blood smear (1-4 NRBC). Similar effect was observed in all WBC populations except basophils. Results in WBC count and different WBC populations after correction according to the amount of NRBC changed to or from reference values in almost 20% of cases (22/112). On the other hand, the median percentage of change in total WBC count caused by NRBC interference was lower than 5% meaning that corrected values are generally not of high clinical importance. However as showed the maximal percentage of change for WBC count over 30% in group 4, the interference between NRBC and WBC might be crucial in patients with high NRBC count, especially in leukopenic conditions. For basophilic population significant bias caused by NRBC interference was observed only in group 4. The reason for this result is probably the extremely low absolute number of basophils in peripheral blood.

4.3. Assessment of automatic analysis for detection of circulating NRBC in dogs

Several human automated hematology analyzers are able to quantify NRBC, whereas these are lacking in veterinary medicine and manual quantification on blood smear is the only approach. Microscopical evaluation is demanding and time consuming and requires a certain experience. In recent years, automated analyzers have distinguished occurrence of very low number of circulating NRBC that were not identified using manual quantification. This may be due to analysis of higher number of cells and thus higher sensitivity for an automated detection (Rin et al 2017). An automated quantification of NRBC would allow for cost effective, time saving and more accurate method in the hematology laboratory.

The Sysmex XT-2000iV analyzer uses fluorescence flow cytometry to detect a 5-part WBC differential count based on side fluorescence light and side scatter. According to Lilliehöök and Tvedten 2009a, NRBC appear to be included in both the WBC-BASO and the WBC-DIFF channel. In the WBC-DIFF channel, the NRBC are possibly located in the lymphocyte and neutrophile area (Lilliehöök and Tvedten 2009b). Our study investigated the possibility of quantifying NRBC using the WBC DIFF scattergram of Sysmex XT-2000iV, validated for hematology analysis in dogs and cats (Bauer et al 2011, Bauer et al 2012). Based on the morphology of NRBC and its similarities to small lymphocyte, these cells should be located in close proximity of each other in WBC DIFF scattergram. The predefined area included the location of small lymphocytes with a part of neutrophils and towards the area where RBC are located as ghost area. We hypothesized that samples from patients having leukocytosis would cause false positive results as there would be lymphocytes and neutrophils in the assumed area for NRBC. By using samples which had a high NRBC count but leukopenia as true positives, and comparing these with other samples, there should be a possibility of finding the location of these cells.

To identify circulating NRBC by Sysmex XT-2000iV in canine blood samples we tested 6 different software settings. When compared results from morphological NRBC evaluation, the accuracy was 63-66%. When reviewed data of all available hematological analyses, we observed that in patients with leukopenia, more false negative results occurred. Instead of decreasing the cut-off values, we decided to exclude all cases with possible interference (patients with leukopenia or flagged with WBC abnormal scattergram). A recalculated accuracy for morphologically identified NRBC was similar to 63-69%. Because within the DIFF-WBC channel more than 10000 of nucleated cells are evaluated compared to only 100 WBC in manual counting method, the automated result from predefined area would better correspond to

absolute numbers of circulating NRBC. Moreover, the numbers of NRBC from morphological evaluation are highly affected by WBC. Despite this, an excellent agreement between these two methods was found for Sysmex XN-module and Sysmex XE-2100 (Rin et al 2017).

In order to identify all morphologically positive samples (100% sensitivity), we selected as the true positive limit for estimated number of circulating NRBC value $\geq 0{,}3x10^9/l$. Using this limit, the accuracy improved to 71-81% for all cases and to 73-84% after exclusion of leukopenic samples and samples flagged with WBC abnormal scattergram. To confirm the best accuracy in a routine caseload at Small Animal Clinical Laboratory at VFU we reanalyzed 55 random canine blood samples). Settings DIFF1, DIFF4 and DIFF6 were excluded based on results from collected patient group. Settings DIFF2 and DIFF3 were excluded based on results from random group. The reason for exclusion was low sensitivity and low cut-off values. Using the algorithm formulated as: Patient has no leukopenia or is not flagged with WBC abnormal scattergram and the quantitative result is equal or higher than 0,06 in DIFF5 area, we obtained accuracy at the level of 80% for patient group and 84% for random group. These results are valid for patients having estimated number of circulating NRBC $\geq 0{,}3x10^9/l$ considered positive. The gate of DIFF5 is the most appropriate area for expected NRBC population according to Criswell et al 2014. The cut-off value 0,06 is close to the limit of detection ($0{,}067x10^9/l$) for NRBC evaluated by Sysmex XE-2100 (Rin et al 2017). According to this algorithm, from all false results, 66,6% were false negative in patient group and 33,3% were false negative in random group. There was no patient with leukocytosis among false negative cases. In patient group the highest number of NRBC detected on the blood smear that was missed by automatic analysis using proposed algorithm was 10 (patient N. 105). However, this patient would be selected for morphological examination due to moderate anemia and abnormal lymphocyte and neutrophile area on WBC-DIFF scattegram. The highest number of NRBC detected on the blood smear that would be missed by automatic analysis using proposed algorithm was 7 (patients N. 71 and 112).

To evaluate the validity of formulated algorithm, further studies including healthy individuals are required.

5. Conclusion

We microscopically evaluated 144 blood samples with at least 1 NRBC / 100 WBC identified during peripheral blood smear evaluation regardless of other pathological findings over a time period of 18 months. The presence of NRBC in peripheral blood was observed in 141 canine and 3 feline samples. NRBC occurred in 3,2% of all canine samples delivered to the Small Animal Clinical Laboratory. Compared to dogs, NRBC were microscopically identified 10x less often in cats. The quantitative and morphological evaluation of NRBC population showed the majority of canine samples (83,0%) contained 10 or less NRBC, with the median of 5. The most common morphological subtype was a metarubricyte in both dogs and cats.
In the canine group, patients with regenerative anemia had statistically higher NRBC count than patients with non-regenerative anemia or non-anemic dogs. Dogs from non-anemic group had significantly higher NRBC count than patients with non-regenerative anemia.

We evaluated bias in total WBC count and different WBC population changes caused by NRBC based on correction formula. There was significant decrease in corrected total WBC count compared to original total WBC count in all groups of dogs. Similar effect was observed in all WBC populations except basophils. The median percentage of change in total WBC count caused by NRBC interference was lower than 5%. The highest interference was confirmed in the group with highest number of NRBC, with maximal value over 30%.

We used Sysmex XT-2000iV analyzer to identify circulating NRBC in canine blood samples. Using the algorithm formulated as: Patient has no leukopenia or is not flagged with WBC abnormal scattergram and the quantitative result is equal or higher than 0,06 in DIFF5 area, we obtained accuracy at the level of 80% for patient group and 84% for random group of canine blood samples. Results are valid for patients having estimated number of circulating NRBC $\geq 0{,}3 \times 10^9$/l considered positive.

6. Summary

Nucleated red blood cells are physiologically not found in the peripheral blood. Their presence is increasingly used as a negative prognostic marker in human medicine. Similar studies made on dogs have shown their usefulness in prediction of prognosis in certain pathological states. Nucleated red blood cells interfere with nucleated leukocytes in automatic quantification, causing an incorrect total and differential WBC count. Only some hematology analyzers are developed for accurate quantification of circulating NRBC in human medicine, whereas these are lacking for veterinary use. Manual quantification on blood smear remains the only way of detection for this specific cell population.

The main objectives of this study were to quantify and morphologically evaluate NRBC population in canine and feline patients, to evaluate the significance of bias in total WBC count and different WBC population changes by presence of NRBC and to assess the possibility for identification of circulating NRBC using hematology analyzer based on fluorescent flow cytometry.

In the study, canine and feline blood samples were microscopically evaluated based on the inclusion criteria with at least 1 NRBC / 100 WBC identified on the blood smear regardless of other pathological findings. According to the number of NRBC found, patients were divided into 4 groups (1-4 NRBC, 5-10 NRBC, 11-15 NRBC, >15 NRBC). The hematological analysis was performed using the Sysmex XT-2000iV analyzer for hemoglobin concentration, reticulocyte count and total WBC and differential WBC count. According to hemoglobin concentration and reticulocyte count, patients were classified as having anemia (regenerative or non-regenerative) or being non-anemic. Using the correction formula, we calculated corrected WBC count and estimated NRBC count. Based on fluorescence flow cytometry, we hypothesized the gate for NRBC population will be located close to the area of small lymphocytes. With the purpose to identify circulating NRBC population by Sysmex XT-2000iV analyzer, 6 different software settings were assessed in collected patient group and in a random canine group.

We microscopically evaluated 144 blood samples (141 canine and 3 feline). Compared to dogs (3,2%), in cats NRBC were microscopically identified 10x less often. The quantitative and morphological evaluation of NRBC population showed the majority of canine samples (83,0%) contained 10 or less NRBC, with the median of 5. The most common morphological subtype was a metarubricyte in both, dogs and cats.

In canine group patients with regenerative anemia had statistically higher NRBC count than patients with non-regenerative anemia or non-anemic dogs. Dogs from non-anemic group had significantly higher NRBC count than patients with non-regenerative anemia.

We evaluated bias in total WBC count and different WBC population changes caused by NRBC based on correction formula. There was significant decrease in corrected total WBC count compared to original total WBC count in all groups of dogs. However, the median percentage of change in total WBC count caused by NRBC interference was lower than 5%. The highest interference was confirmed in group 4 with maximal value over 30%.

We used Sysmex XT-2000iV analyzer to identify circulating NRBC in canine blood samples. Using the algorithm formulated as: Patient has no leukopenia or is not flagged with WBC abnormal scattergram and the quantitative result is equal or higher than 0,06 in DIFF5 area, we obtained accuracy at the level of 80% for patient group and 84% for random group of canine blood samples. Results are valid for patients having estimated number of circulating NRBC $\geq 0{,}3 \times 10^9/l$ considered positive. However, further studies including healthy individuals are required.

REFERENCES

Akhtar, S., Mahure, S. (2015). Nuance of Nucleated Rbcs (Normoblastemia) in Peripheral Blood film. *Pamacea Journal of Medical Sciences*, January-April 2015: 5(1)7-13

Angel, S.E., Geothe, J. (2016). Normoblastemia in Neonates and Infants. *IMA Kerala Medical Journal*. 2016 Mar 30; 9(1):32-35

Aroch, I., Segev, G., Loeb, E., and Bruchim, Y. (2009). Peripheral Nucleated Red Blood Cells as a Prognostic Indicator in Heatstroke in Dogs. *J Vet Intern Med* 2009; 23:544–551

Ballegeer, E.A., Forrest, L.J., Dickinson, R.M. (2007). Correlation of ultrasonographic appearance of lesions and cytologic and histologic diagnoses in splenic aspirates from dogs and cats: 32 cases (2002–2005). *J Am Vet Med Assoc*. 2007; 230:690–696.

Bauer, N., Nakagawa, J., Dunker, C., Failing, K., Moritz, A. (2011). Evaluation of the automated hematology analyzer Sysmex XT-2000iV compared to the ADVIA 2120 for its use in dogs, cats, and horses: part I—precision, linearity, and accuracy of complete blood cell count. *Journal of Veterinary Diagnostic Investigation* 23(6) 1168–1180.

Bauer, N., Nakagawa, J., Dunker, C., Failing, K., Moritz, A. (2012). Evaluation of the automated hematology analyzer Sysmex XT-2000iV compared to the ADVIA 2120 for its use in dogs, cats, and horses. Part II: Accuracy of leukocyte differential and reticulocyte count, impact of anticoagulant and sample aging. *Journal of Veterinary Diagnostic Investigation* 24(1) 74–89.

Bertazzolo, W., Dell'Orco, M., Bonfant, I.U. (2005). Canine angiosarcoma: cytologic, histologic, and immunohistochemical correlations. *Vet Clin Pathol.* 2005; 34:28–34

Bienzle, D., Kwiecien, M.J., Parent, M.J. (1995). Extramedullary Hematopoiesis in the Choroid Plexus of Five Dogs. *Vet Pathol*. 1995; 32:437-440

Boskabadi, H., Zakerihamidi, M., Hadi Sadeghian, M., Avan, A., Ghayour-Mobarhan, M., and Ferns, G.A. (2017). Nucleated red blood cells count as a prognostic biomarker in predicting the complications of asphyxia in neonates. *J Matern Fetal Neonatal Med*, 2017; 30(21): 2551–2556

Boskabadi, H., Omidian, M., Tavallai, S., Mohammadi, S., Parizadeh, M., Mobarhan, M.G., Ferns, G.A. (2015). Serum Hsp70 Antigen: Early diagnostic marker in perinatal asphyxia. *Iran J Pediatr,* 2015; 25e381

Brockus, C.W. (2011) Erythrocytes. *Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology*. Latimer, K.S. 2011, 3-44.

Bruchim, Y., Klement, E., Saragusty, J., Finkeilstein, E., Kass, P., and Aroch, I. (2006). Heatstroke in dogs: A retrospective study of 54 cases (1999–2004). *J Vet Intern Med* 2006; 20:38–46.

Bruegel, M., Nagel, D., Funk, M., Fuhrman, P., Zander, J., Teupser, D. (2015). Comparison of five automated hematology analyzers in a university hospital setting. *Clin Chem Lab Med*. 2015; aop

Christopher, M.M. (2003). Cytology of the spleen. *Vet Clin North Am Small Anim Pract.* 2003; 33:135–152.

Clifford, G.O. (1966). The clinical significance of leukoerythroblastic anemia. *Med Clin North Am.* 1966; 50:779–790.

Constantino B.T, Cogionis B. (2000). Nucleated RBCs – Significance in the Peripheral Blood Film. *Laboratory Medicine,* Volume 31, No 4.

Danise, P., Maconi, M., Barella, F., Di Palma, A., Avino, D., Rovetti, A., Gioia, M., Amendola, G. (2012). Evaluation of nucleated red blood cells in peripheral blood of hematological diseases. *Clin Chem Lab Med* 2012;50(2):357–360

Das, D.K., Maiti, A.K., Chakraborty, C. (2017). Automated identification of normoblast cell from human peripheral blood smear images. 2017 *Royal Microscopical Society*, 00, 1–11

DeNicola, D.B. (2011). Advances in Hematology Analyzers. *IDEXX Laboratories*, Inc., Westbrook, ME USA.

EClinPath Cornell University College of Veterinary Medicine | Online February 2018 | Nucleated RBC, Hematology

Erslev, A.J, Beutler, E. (1995). Production and destruction of erythrocytes. In: *Williams Hematology, 5th ed*. Beutler, E., Lichtman, M.A., Coller, B.S., Kipps, T.J., eds. pp 425-441, McGraw- Hill, New York, NY, 1995.

Faunt, K.K., Turk, J.R., Cohn, L.A., Dodam, J.R., Johnson, K.H. (1998). Isolated right-ventricular hypertrophy associated with severe pulmonary vascular apolipoprotein A1-derived amyloidosis in a dog. *Journal of the American Animal Hospital Association*: January/February 1998, Vol. 34, No. 1, pp. 35-37.

Frey, B., Duke, T., Horton, S. B., (1999). Nucleated red blood cells after cardiopulmonary bypass in infants and children: is there a relationship to the systemic inflammatory response syndrome? *Perfusion* 1999; 14: 173–180

Goggs, R., (2014). Normoblasts: not always normal. November 22, 2014. *Veterinary Record*

Grandi, F., Colodel, M.M., Monteiro, L.N. (2010). Extramedullary hematopoiesis in a case of benign mixed mammary tumor in a female dog: cytological and histopathological assessment. *BMC Vet Res.* 2010; 6:45.

Grindem, C.B., Haddad, J.L., Tyler, R.D., Cowell, R.L, (2014). The Bone Marrow. In *Cowell and Tyler's Diagnostic Cytology and Hematology of the Dog and Cat.* Cowell, R. L., Valenciano, A. C., 2014 2014: 489-526. Elsevier, St. Louise, MO.

Hammer, A.S., Wellman, M. (1999). Leukoerytroblastemia and Normoblastemia in the Cat. *J Am Anim Hosp Assoc* 1999;35:471–3.

Harvey, J. W. (2001). *Atlas of Veterinary Hematology; Blood and bone marrow of domestic animals.* 2001, pp 21-44, 93-124, 125-161 Saunder, Elsevier, Philadelphia, PA.

Hermansen, M.C. (2001). Nucleated red cells in the fetus and newborn. *Arch Dis Child Fetal Neonatal Ed*, 2001; 84: F211-F215.

Hwang, D.H., et al (2016). Automated Nucleated RBC Measurement Using the Sysmex XE-5000 Hematology Analyzer. *Am J Clin Pathol* March 2016; 145:375-384

Irizarry-Rovira, A., Stephens, J., Christian, J., Kjemtrup, A., DeNicola, D.B., Widmer, W.R., Conrad, P., (2001). Babesia gibsoni Infection in a Dog from Indiana. *Vet Clin Pathol.* 2001; 30:180-188)

Johns, J.L., Christopher, M.M. (2012). Extramedullary Hematopoiesis: A New Look at the Underlying Stem Cell Niche, Theories of Development, and Occurrence in Animals. *Veterinary Pathology* 49(3) 508-523

Kim, C.H. (2010). Homeostasis and pathogenic extrameduallary hematopoiesis. *Journal of Blood Medicine* 2010:1 13–19

Lillenhöök, I., Tvedten, H. (2009)a. Validation of the Sysmex XT-2000iV hematology system for dogs, cats, and horses. I. Erythrocytes, platelets, and total leukocyte counts. *Vet Clin Pathol* 38/2 (2009) 163–174.

Lillenhöök, I., Tvedten, H. (2009)b. Validation of the Sysmex XT-2000iV hematology system for dogs, cats, and horses. II. Differential leukocyte counts. *Vet Clin Pathol* 38/2 (2009) 175–182.

Mastrorilli, C., Welles, E.G., Hux, B., Christopherson, P.W. (2013). Botryoid nuclei in the peripheral blood of a dog with heatstroke. *Vet Clin Pathol* 42/2 (2013) 145–149

Metcalf, D. (1993). Hematopoietic regulators: redundancy or subtlety. *Blood* 82:3515-3523, 1993.

Moretti, P., Giordano, A., Stefanello, D., Ferrari, R., Castellano, S., Paltrinieri, S. (2015). Nucleated erythrocytes in blood smears of dogs undergoing chemotherapy. *Veterinary and Comparative Oncology,* 15, 1, 215–225

Morgan, L.W., and McConnell, J. (1999). Cobalamin deficiency associated with erythroblastic anemia and methylmalonic aciduria in a border collie. *Journal of the American Animal Hospital Association*, September/October 1999, Vol. 35, No. 5, pp. 392-395.

Müller, M., Dörfelt, R., Hamacher, L., Wess, G. (2014). Association of nucleated red blood cells with mortality in critically ill dogs. November 22, 2014. *Veterinary Record*.

Novacco, M., Martini, V., Grande, C., Comazzi, S. (2015). Analytic errors in Sysmex-generated hematology results in blood from a dog with chronic lymphocytic leukemia. *Vet Clin Pathol* 44/3 (2015) 337–341.

O'Malley, D.P. (2007). Benign extramedullary myeloid proliferations. *Mod Pathol*. 2007; 20:405–415.

O'Malley, D.P., Kim, Y.S., Perkins, S.L., (2005). Morphologic and immunohistochemical evaluation of splenic hematopoietic proliferations in neoplastic and benign disorders. *Mod Pathol.* 2005; 18: 1550–1561.

Quesenberry, P.J. (1995). Hemopoietic stem cells, progenitor cells, and cytokines. In: *Williams Hematology, 5th ed.* Beutler E, Lichtman MA, Coller BS, Kipps TJ, eds. pp 211-228, McGraw-Hill, NewYork, NY, 1995.

Pinto da Cunha, N., Giordano, A., Caniatti, M., Paltinieri, S. (2009). Analytical validation of the Sysmex XT-2000iV for cell counts in canine and feline effusions and concordance with cytologic diagnosis. *Vet Clin Pathol* 38/2 (2009) 230–241

Rai R., Tripathi G., Singh D. (2014). Nucleated RBC count as predictor of neurological outcome in perinatal asphyxia. *Indian Pediatr* 2014;51: 231–2.

Reagan, W.J. (1993). A review of myelofibrosis in dogs. *Toxicol Pathol*. 1993; 21:164–169.

Rin, G. et al. (2017)- Performance evaluation of the automated nucleated red blood cell count of five commercial hematological analyzers. *Int J Lab Hem*. 2017; 39:663-670

Schaer, C., Schmugge. M., and Frey, B. (2014). Prognostic Value of Nucleated Red Blood Cells in Critically Ill Children. *Swiss Medical Weekly* 144, W13944

Schwarzt, S.O., Stansbury, F. (1954). Significance of nucleated red blood cells in peripheral blood; analysis of 1,496 cases. *Journal of the American Medical Association* 154, 1339–1340

Shah, R., Reddy, S., Horst, H.M., Stassinopoulos, J., Jordan, J., Rubinfeld, I. (2012). Getting back to zero with nucleated red blood cells: following trends is not necessarily a bad thing. *The American Journal of Surgery* (2012) 203, 343–346

Simon, D., Nolte, I., Eberle, N., Abbrederis, N., Killich, M., and Hirschberger, J. (2006). Treatment of dogs with lymphoma using a 12-week, maintenance free combination chemotherapy control protocol. *Journal of Veterinary Internal Medicine* 2006; 20: 948 – 954.

Stachon, A., Segbers,E., Holland-Letz, T., Kempf, R., Hering, S., Krieg, M. (2007). Nucleated red blood cells in blood of medical intensive care patients indicate increased mortality risk: a prospective cohort study of in-hospital mortality. *Clinical* care, 2007

Stachon, A., Bolulu, O., Holland-Letz, T. (2005). Association between nucleated red blood cells in blood and the levels of erythropoietin, interleukin 3, interleukin 6, and interleukin 12p70. *Shock* 2005;24: 34 –9.

Stachon, A., Sondermann, N., Imohl, M. (2002). Nucleated red blood cells indicate high risk of in-hospital mortality. *J Lab Clin Med* 2002;140: 407–12.

Stacy, N.I., Harvey, J.W. (2017). Bone marrow aspirate evaluation. *Vet Clin Small Anim* 47 (2017) 31–52

Sysmex Xtra Online | March 2018 | Hematology – Multispecies, XN-V

Tantanate, C., Klinbua, C. (2015). Performance evaluation of the automated nucleated red blood cell enumeration on Sysmex XN analyser. 2014 John Wiley & Sons Ltd, *Int. Jnl. Lab. Hem.* 2015, 37, 341–345

Tolle, D.V., Fritz, T.E., Norris, W.P. (1977). Radiation-induced erythroleukemia in the beagle dog. *Am J Pathol* 1977, 87:499-510

Tsuji, T., Sakata, T., Hamaguchi, Y., Wang, F., Houwen, B. (1999). New rapid flow cytometric method for the enumeration of nucleated red bloods. *Cytometry* 37 :291–301.

Valenciano, A.C., Cowell, R.L., Rizzi, T.E., Tyler, R.D., (2014). Red blood cells. *Atlas of Canine and Feline Peripheral blood smears.* 2014, pp 24-112. Elsevier, St. Louise, MO.

Wang, F-S., Itose, Y., Tsuji, T., Hamaguchi, Y., Hirai, K., Sakata, T. (2003). Development and clinical application of nucleated red blood cell counting and staging on the automated haematology analyser XE-2100TM. *Clin. Lab. Haem.* 2003, 25, 17–23

Webb, J.L., Latimer, K.S. Leukocytes (2011). *Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology.* Latimer, K.S. 2011, 45-82.

Wozniak, E.J., Barr, B.C., Thomford, J.W., (1997). Clinical, anatomic, and immunopathologic characterization of Babesia gibsoni infection in the domestic dog (Canis familiaris*). J Parasitol.* 1997; 83:692–699.

Wright, D.E., Wagers, A.J., Gulati, A.P., (2001). Physiological migration of hematopoietic stem and progenitor cells. *Science.* 2001;294: 1933–1936.

Zabolotzky, S.M., Walker, D.B., (2014). Peripheral Blood Smear. In *Cowell and Tyler's Diagnostic Cytology and Hematology of the Dog and Cat.* Cowell, R. L., Valenciano, A. C., 2014: 457-488.

ACKNOWLEDGEMENTS

I would like to sincerely thank everyone who has helped and supported me during my work on this diploma thesis.

First and foremost, I would like to acknowledge to my supervisor MVDr. Kristína Řeháková, PhD. Thank you for introducing me to this topic and the opportunity to work under your guidance. I am very grateful for all the time and effort you have spent with me on this study. Your patience and knowledge has been greatly appreciated.

I would like to thank the technicians at the Small Animal Clinical Laboratory of the University of Veterinary and Pharmaceutical Sciences Brno for their contribution in collecting and preparing samples used in this study.

In addition, I would like to thank MVDr Václav Ceplecha for assistance on statistical analysis.

Thank you to my family, friends and classmates for great support and encouragement throughout this process.