THESIS

CHARACTERIZATION OF ERRORS AND PREDICTABILITY OF DISEASE USING THE

ADVIA 120 HEMATOLOGY ANALYZER LEUKOCYTE DIFFERENTIAL

Submitted by

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ABSTRACT

CHARACTERIZATION OF ERRORS AND PREDICTIBILITY OF DISEASE USING THE

ADVIA 120 HEMATOLOGY ANALYZER LEUKOCYTE DIFFERENTIAL

Automated instrumentation has allowed for enhanced speed, precision and cost-effective measures in the reporting of hematological data. These instruments have expanded the information attainable beyond simple numerical data through advanced techniques such as flow cytometry and cytochemical staining to produce specialized parameters and scatterplots as novel data delivery systems. The Advia 120 hematology analyzer leukocyte differentiation method produces several novel parameters, including two channel (perox and baso) cytograms and several indicators of intracellular myeloperoxidase activity. These parameters can be diagnostically useful in assessing the idiosyncratic species and mechanical issues that arise during routine operation, as well as provide discriminatory information related to variety of disease processes (inflammation, neoplasia, etc).

The purpose of this study was to identify specific cytogram misclassification patterns and errors that would have the greatest impact on clinical operation or interpretation of hematologic data. In addition, the analyzer phenomenon of pseudobasophilia was investigated for its potential use in raising clinical awareness of circulating atypical cells and their intrinsic properties. Lastly, the peroxidase indices MPXI, neut-x and neut-y were investigated for their diagnostic potential in identifying systemic inflammation and myeloid leukemias.

The results of this study detailed several cytogram patterns and their causes and misclassifications, of which recognition and understanding promote awareness to the existence

of certain hematological findings otherwise not documented in numerical data as well as to the limitations inherent in instrumentation. The analyzer phenomenon of pseudobasophilia was demonstrated in canine samples and was highly associated with the presence of atypical cells, commonly leukemia, in circulating blood. Lastly, the myeloperoxidase indices failed to demonstrate any benefit in differentiating hematologic changes associated with systemic inflammation and myeloid leukemias.

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CHAPTER 1: Literature Review

Automated hematology instrumentation and the leukocyte differential

Over the last few decades, hematology analyzers have evolved into fully automated instruments, continually enhanced by the progressive evolution of technology and software abilities. These advances and the general availability of the instruments have afforded many benefits to the practice of veterinary medicine by making procurement of hematological data faster, cheaper and more cost and labor efficient than the manual method of performing complete blood counts (CBCs). In addition, these instruments are capable of counting thousands of cellular events, thus lowering the inherent statistical variability of leukocyte differentials which gives manual differential analysis its well-known limitations resulting in poor precision and predictive value.[11,28,33,36] However, blood film review remains a mainstay of veterinary clinical pathology in spite of these inherent inaccuracies due to clinical gains made in the detection of cellular morphologies (left shifts, toxic changes, etc) and atypical cell populations (blast cells). However, such detailed evaluation requires rigorous training and sufficient time to execute, which is not always practical for every case. It is in the capacity as a screening tool that automated instrumentation may prove to be the most reliable and beneficial.

While earlier instruments were based on impedence principles alone,[3,19] newer analyzers are capable of providing more information from the incorporation of technology such as flow cytometric principles and cytochemistry. One method by which this new data is reported is through the use of cytograms. Cytograms are graphical displays that can help visualize changes within leukocyte populations due to disease or instrumentation error better than absolute

numbers or percentages of leukocytes can alone. Recognition of characteristic patterns associated with specific hematological conditions/findings or misclassification mechanisms can be useful in identifying cases which benefit from manual blood film review to characterize pathology.

The Advia 120 hematology analyzer (Siemens Medical Solutions Diagnostics, Tarrytown NY) is a system which incorporates flow cytometric principles and cytochemistry. It differentiates white blood cells via a two channel system, peroxidase (perox) and basophil (baso). The peroxidase method uses the reagent 4-chloro-1-napthol as a substrate that reacts with hydrogen peroxide within the azurophilic granules of white blood cells (typically neutrophils and eosinophils) to produce a dark precipitate. Then, employing light scatter principles to measure cell size (y-axis) and light absorption principles for peroxidase staining (x-axis), individual cellular events are recorded and plotted on a scattergram. Cluster analysis software sets gates, on a sample-by-sample basis, for identification and quantification of each cell population based on its position, area and density. Basophils, however, are not identified by this technique, so a second channel, the basophil/lobularity channel, is employed. In a heated cytochemical reaction chamber, blood cells are exposed to a reagent containing a surfactant and phthalic acid that strips the cytoplasm from all other white blood cells (except basophils) and lyses red blood cells and platelets. A laser light source then measures low-angle and high-angle light scatter to determine cell volume and nuclear density/lobularity, respectively. Cluster analysis software gates individual cell populations as previously described.

Several studies correlating the results from Advia and manual differential cell counts have been performed.[3,40,41,43] It should be emphasized, however, that statistical significance does

not always correlate with clinical relevance and it is from this perspective that many operators of automated instrumentation approach the issue of data interpretation. Investigations into the utility and limits of automated hematology analyzer leukocyte differentiation, with special attention to cytogram analysis and its role in clinical application, has not been performed.

Pseudobasophilia: incidence and meaning in the canine population

Basophils are among the rarest of circulating white blood cells, normally accounting for <0.5% of absolute total blood leukocytes in domestic animals[44] and enumeration via manual differential is often imprecise as a consequence of these low numbers and operator error.[1,31] To improve upon this for human CBCs, the Advia employs a second channel in leukocyte differentiation for specific isolation and enumeration of basophils. This method is based off of findings in human medicine that basophils appear resistant to the cytoplasmic stripping process previously described and as intact cells, appear larger than the bare nuclei of other WBCs, making them more easily identifiable. However, it has been documented that an array of automated analyzers, the Advia included, fail to detect basophils of domestic animals.[30,32,40,45]

Operators of the Advia, however, have noted the frequent occurrence of cellular events classified as "basophils" on the automated differential in the absence of microscopically detected basophils.[41] Pseudobasophilia is an analyzer phenomenon well documented in the human literature whereby abnormal cells in the blood are detected and classified as basophils; this phenomenon has been reported in numerous analyzer systems, including the Advia 120.[5,10,16,18,29] The cell types most frequently encountered include atypical lymphocytes, plasma cells or myeloma cells,[18,24,25,29] however single cases of nucleated red blood cells (nRBCs), granulocytic precursors, metastatic carcinoma cells, atypical cells of infectious mononucleosis, reactive

lymphocytes and the lymphocytes of infants have also been reported.[10] A handful of incidences

suggestive of this phenomenon have been reported in the veterinary literature[16,31] but, thus far,

have not been fully investigated.

Canine perox cytogram analysis and neutrophil myeloperoxidase indices

Myeloperoxidase (MPO) is an important enzyme located within the azurophilic granules

of polymorphonuclear leukocytes (neutrophils) and to a lesser extent, within the lysosomes of

monocytes. MPO serves as a catalyst in the reaction of $H_2O_2 + Cl^- \rightarrow HOCl^-$, the product of

which acts as a broad antimicrobial agent in the defense against a host of pathogens, including

bacteria, viruses and fungi. MPO is synthesized and packeted into primary granules at the

promyelocytic stage of granulopoiesis, and has been found in >30% of CD34+ cells within the

bone marrow in human studies.[30]

The Advia 120 hematology analyzer is unique in its reporting of various peroxidase

specific parameters which are summarized as follows:

-neut x: coordinate value for the mean light absorbance (peroxidase activity) of the gated

neutrophil population

-neut y: coordinate value for the mean cell size of the gated neutrophil population

-MPXI: a unitless value calculated by the Advia as the deviation of the mean of the x-axis

value for the gated neutrophil population from a stored archetypal population ([patient

sample-archetypal]/archetypal). It is important to note that even within the multispecies

software packages, this archetypal population is human.

MPXI serves as an estimate for MPO content per leukocyte, and thus a measurement of relative

intracellular MPO activity. These indices are routinely reported parameters and are therefore

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available for analysis on every patient that has a CBC performed. Studies in human medicine have investigated these indices, particularly MPXI, for a variety of different reasons, described below.

Neutrophilic degranulation and release of MPO from within the azurophilic granules occurs as part of the respiratory burst in response to a variety of stimuli. Since MPXI estimates the intracellular content of MPO, decreases in this parameter should therefore be expected with neutrophil degranulation and have been documented in several specific human conditions (ischemic events, septicemia, etc).[7,14,46] The application of this as a screening tool for diseases has proved useful, especially in cases of sepsis where the total WBC count or other variables do not change as expected. While there has been limited study in veterinary medicine as to the relation of this index to specific disease states[27,34,35,37] a broader study correlating the associated or expected hematological changes has not been attempted. Toxic changes occur at the metamyelocytic and myelocytic maturation stages in the bone marrow from increased granulopoiesis triggered by strong inflammatory conditions, such as those seen with bacterial infections and septicemia. Toxic changes can be evident within neutrophils long before characteristic leukogram changes are seen and may in fact be the only changes present to indicate inflammatory conditions.[21,38] The severity of the changes often reflects the severity of the disease. It can therefore be theorized then that the MPXI measurement may correlate with and serve as an indicator for the presence of toxic changes within neutrophils, with further potential as an marker for underlying systemic inflammation.

In addition, it has been hypothesized that for situations where cellular divisions within the bone marrow are skipped, the immature neutrophils which are released contain an increased concentration of MPO.[8,13,22,37] Such may be the case for left shifts, considered another hallmark of acute inflammation, where MPXI would be expected to increase. In the case of myeloid leukemias, the relative changes are not as straight forward. Human studies have used both cytogram analysis and peroxidase content as a means to differentiate myeloid from lymphoid leukemias, and myeloid subtypes from one another.[2,4,6,12,15,17,23,26,29,30,39,46] This has been minimally studied for dogs in veterinary medicine.[9,20,40] MPXI has been noted to increase with progressive maturation seen in human AMLs, along the order of M1<M4=M5<M2<M3 according to the FAB classification system.[9,12,20,39] This change is also reflected in the perox cytograms reported by the Advia, with the more mature leukemic cells falling within gated fields indicating peroxidase activity.[6,15,17,22,26,29] This seemingly makes sense as AML-M3 displays predominantly promyelocytic differentiation. MPO synthesis and packeting into granules has already occurred at this stage, but these cells have failed to undergo further cellular divisions typical of maturation, and thus contain a maximum content of MPO. Conversely, AML subtypes with minimal differentiation, M0 and M5a (monocytic) for example, show decreases in MPXI. The diagnosis of myeloid leukemia in dogs remains a challenging one. The development of a screening tool to select for potential cases worthy of further specialized analysis (typically flow cytometry) from the standard CBC analysis would be highly beneficial.

REFERENCES

1. Allison RW, Velguth KE. Appearance of granulated cells in blood films stained by automated aqueous versus methanolic Romanowsky methods. *Vet Clin Pathol* 2009; 31: 99-104.

2. Austin GE, Chan WC, Zhao W, et al. Myeloperoxidase gene expression in normal granulopoiesis and acute leukemias. *Leukemia and Lymphoma* 1994; 15:209-226.

3. Becker M, Moritz A, Giger U. Comparative clinical study of canine and feline total blood cell count results with several in-clinic and two commercial laboratory hematology analyzers. *Vet Clin Pathol* 2008; 37(4): 373-384.

4. Bendix-Hansen K, Nielsen HK. Myeloperoxidase-deficient polymorphonuclear leucocytes (IV): relation to FAB-classification in acute myeloid leukaemia. *Scand J Haemato* 1985; 35:174-77.

5. Bizarro N. Pseudobasophilia on the Technicon automated cell counters. *Clin Lab Haem.* 1996; 18:298-299.

6. Bruno A, Del Poeta G, Venditti A, et al. Diagnosis of acute myeloid leukemia and system Coulter VCS. *Haematologica* 1994; 79:420-28.

7. Buffon A, Biasucci LM, Liuzzo G, et al. Widespread coronary inflammation in unstable angina. *New England Journal of Medicine* 2002; 347: 5-12.

8. Carrick JB, Begg AP. Peripheral blood leukocytes. Veterinary Clinics of North America. Equine Practice. 2008; 24: 239-259.

9. Christopher MM, Metz AL, Klausner J, et al. Acute myelomonocytic leukemia with neurologic manifestations in the dog. *Vet Pathol* 1986; 23: 140-147.

10. Davies S, Bain BJ. Basophil counts on the Technicon H*1 automated counter. *Clin Lab Haem* 1996; 18:35-38.

11. Dutcher TF. Automated leukocyte differentials: A review and prospectus. *Lab Med* 1983; 14: 483-487.

12. Eivazi-Ziaei J. Myeloperoxidase index and subtypes of acute myeloid leukemia. *J Pak Med Assoc* 2009; 59(6):406-7.

13. Eivazi-Ziaei J, Dastgari S. Role of myeloperoxidase index in differentiation of megaloblastic and aplastic anaemia. *Indian Journal of Medical Sciences* 2004; 58:345-348.

14. El-Maallem H, Fletcher J. Effects of surgery on neutrophil granulocyte function. *Infection and Immunity* 1981; 32(1): 38-41.

15. Fernández-Castro M, Viloria A. Utility of the Technicon H-1 flags in the detection of peripheral blood blast cells of paediatric acute leukaemia patients. *Acta Haematol* 1995; 93:9-12.

16. Gelain ME, Rossi G, Giori L, et al. Identification of neoplastic cells in the blood using the Sysmex XT-2000iV: a preliminary step in the diagnosis of canine leukemia. *Vet Clin Pathol*. 2010; 39: 169-179.

17. Gibbs G. Peroxidase activity and nuclear density analysis (PANDA) in the diagnosis of haematological malignancy. *Br J Biomed Sci* 2005; 62(3): 142-44.

18. Gibbs G, Campbell G, Christie I. Pseudobasophilia and the Advia 120. *Hematology*. 2009; 14(3): 159-163.

19. Graham MD. The Coulter principle: foundation of an industry. *J Assoc Lab Automat* 2003; 8: 72-81.

20. Grindem CB, Stevens JB, Perman V. Cytochemical reactions in cells from leukemic dogs. *Vet Pathol* 1986; 23: 103-109.

21. Gossett KA, MacWilliams PS, Cleghorn B. Sequential morphological and quantitative changes in blood and bone marrow neutrophils in dogs with acute inflammation. *Can J Comp Med* 1985;49:291-297.

22. Gulley ML, Bentley SA, Ross DW. Neutrophil myeloperoxidase measurement uncovers masked megaloblastic anemia. *Blood* 1990; 76: 1004-1007

23. Hoyer JD, Fisher CP, Soppa VM, et al. Detection and classification of acute leukemia by the Coulter STKS hematology analyzer. *Am J Clin Pathol* 2006; 106: 352-8.

24. Hur M, Lee YK, Lee KM, et al. Pseudobasophilia as an erroneous white blood cell differential count with a discrepancy between automated cell counters: report of two cases. *Clin Lab Haem.* 2004; 26:287-290.

25. Jácomo RH, Lozano VF, Neto J, et al. What's the meaning of basophilia in Sysmex XE-2100? [Letter to the Editor]. *Arch Pathol Lab Med.* 2011; 135:415.

26. Kakkar N, Kaur R. Utility of white blood cell suspect flags and histogram pattern in the detection of acute leukemia by Advia-60 automated hematology analyzer. *Indian J Pathol Microbiol* 2004; 47(3): 322-6.

27. Klenner S, Richartz J, Bauer N, et al. Myeloperoxidase deficiency in dogs observed with the Advia 120. *Tierärztl Prax* 2010; 38(K): 139-146.

28. Koepke JA, Dotson MA, Shifman MA. A critical evaluation of the manual/visual differential leukocyte counting method. *Blood Cells* 1990; 93 (4 suppl):S11-S16.

29. Koui F, Chiba T. The clinicopathological evaluation of automated cytochemical hematology system (Technicon H1) in patients with leukemia and myelodysplastic syndrome. *Jpn J Clin Pathol.* 1992; 40(6): 586-594. [article in Japanese

30. Lanza F. Clinical manifestation of myeloperoxidase deficiency. *J Mol Med* 1998; 76:676-681.

31. Lilliehöök I, Tvedten HW. Errors in basophil enumeration with 3 veterinary hematology systems and observations on occurrence of basophils in dogs. *Vet Clin Pathol*. 2011;40:450-458.

32. Lilliehöök I, Tvedten HW. Validation of the Sysmex XT-2000iV hematology system for dogs, cats and horses. II. Differential leukocyte counts. *Vet Clin Pathol*. 2009;38:175-182.

33. Pierre RV. The routine differential leukocyte count vs. automated differential counts. *Blood Cells* 1985; 11:11-23.

34. Piviani M, Segura D, Monreal L, et al. Neutrophilic myeloperoxidase index and mean light absorbance in neonatal septic and nonseptic foals. *Vet Clin Pathol* 2011; 40(3): 340-44.

35. Queiroz-Junior CM, da Fonseca Pacheco CM, Fonseca AH, et al. Myeloperoxidase content is a marker of systemic inflammation in a chronic condition: the example given by the periodontal disease in rats. *Mediators of Inflammation* 2009; 1-7.

36. Rumke CL: Variability of results in differential cell counts on blood smears. *Triangle* 1960; 44:154-157.

37. Schwarz BC, van den Hoven R, Schwendenwein I. Diagnostic value of the neutrophils myeloperoxidase index in horses with systemic inflammation. *Vet J* 2012; 191:72-78.

38. Segev G, Klement E, Aroch I. Toxic neutrophils in cats: clinical and clinicopathologic features, and disease prevalence and outcome—a retrospective case control study. *J Vet Intern Med* 2006;20:20-31.

39. Tsakona CP, Kinsey SE, Goldstone AH, et al. Use of flow cytometry via the H*1 in FAB identification of acute leukemias. *Acta Haematol* 1992; 88:72-77.

40. Tvedten HW, Haines C. Canine automated differential leukocyte count: study using a hematology analyzer system. *Vet Clin Pathol.* 1994;23:90-96.

41. Tvedten HW, Lillehöök IE. Canine differential leukocyte counting with the CellaVision DM96Vision, Sysmex XT-2000iV, and Advia 120 hematology analyzers and a manual method. *Vet Clin Pathol* 2011; 40(3):324-339.

42. Villiers E, Baines S, Law AM, et al. Identification of acute myeloid leukemia in dogs using flow cytometry with myeloperoxidase, MAC387, and a canine neutrophils-specific antibody. *Vet Clin Pathol* 2006; 35(1):55-71

43. Welles EG, Hall AS, Carpenter DM. Canine complete blood counts: a comparison of four in-office instruments with the Advia 120 and manual differential counts. *Vet Clin Pathol* 2009; 38(1): 20-29.

44. Weiss DJ, Wardrop KJ. Schalm's Veterinary Hematology. 6th ed. Ames, IA: Wiley-Blackwell; 2010: 290-297.

45. Wenger-Riggenbach B, Hässig M, Hofmann-Lehmann R, et al. Evaluation of the LaserCyte: an in-house hematology analyzer for dogs and cats. *Comp Clin Pathol.* 2006;15:117-129.

46. Yonezawa K, Horie O, Yoshioka A, et al. Association between the neutrophils myeloperoxidase index and subsets of bacterial infections. *Int Jnl Lab Hem* 2010; 32: 598-605.

CHAPTER 2: Canine, feline and equine leukocyte cytogram analysis as a screening tool for the detection of idiosyncratic cell misclassifications and atypical cell populations in CBCs.

Introduction

The advent of automated hematology instrumentation has afforded many benefits: it is often faster, cheaper, more cost effective and more labor efficient than manual methods. It furthermore lowers the statistical variation in the quantification of leukocytes owing to its ability to count thousands of cellular events, in contrast to manual differentials, which typically include only 100 to 200-cell counts. It should, however, be strongly emphasized that not all hematological disorders and conditions can be detected using automated analysis of peripheral blood, specifically morphologic pathology and cell populations that do not belong in blood. While manual differentials have inherent inaccuracies, oftentimes in clinical settings these are largely outweighed by the gains made in the evaluation of cellular morphologies (left-shifts, toxic changes, etc) and atypical populations (blast cells). Routine blood film review is therefore an ideal practice although this may not be practical for every case. Here again, automated instrumentation may still provide useful information as a screening tool. In addition to the aforementioned benefits, technologic advances (use of flow cytometry, cytochemical staining, etc) have added a new dimension of data beyond the traditionally reported numerical values for CBCs. Most hematology analyzers provide graphical displays, in the form of histograms or cytograms, that help visualize changes within leukocyte populations and can reveal changes due to disease or instrumentation error better than absolute numbers or percentages of leukocytes can alone. Recognition of characteristic cytogram patterns, either indicative of specific hematologic conditions/findings or representative of misclassification types, can be extremely useful in

understanding the limitations of automated instrumentation, and in identifying cases in need of blood film review while alerting the technologist which specific abnormalities to look for.

The focus of this study, using hematologic data and cytogram analysis collected from canine, feline and equine cases over a one year time span from the Colorado State University Veterinary Teaching Hospital, was to highlight specific recognizable cytogram patterns using the Advia 120 hematology analyzer. We aim to demonstrate that while numerous benefits are found in the use of hematology analyzers, limitations exist, and careful consideration of their leukocyte differential results should be undertaken, especially in the context of clinical case management.

Materials and methods

Automated hematology analyzers employ various methodologies for leukocyte differentiation and typically employ either impedance, with or without laser light scattering, or flow cytochemistry. The Advia 120 hematology analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY) is an example of the latter. It differentiates white blood cells via a two channel system. The peroxidase method uses the reagent 4-chloro-1-naphthol as a substrate that reacts with hydrogen peroxide within the azurophilic granules of white blood cells (typically neutrophils and eosinophils) to produce a dark precipitate. Then, employing light scatter principles to measure cell size (y-axis) and light absorption principles proportional to peroxidase staining intensity (x-axis), individual cellular events are recorded and plotted on a scattergram. Cluster analysis software identifies each cell population based on its position, area and density which is then gated on a sample-by-sample basis to quantify each individual cell population. It

should be noted that feline eosinophils are quantified via the reticulocyte channel as they are peroxidase negative and thus are not evident as a distinct population on the perox cytogram.

Basophils, however, are not identified by the above described methods, so a second channel, the basophil/lobularity channel, is employed. In a heated cytochemical reaction chamber, blood cells are exposed to a reagent containing a surfactant and phthalic acid that strips the cytoplasm from all other white blood cells (except basophils) and lyses red blood cells and platelets. A laser light source then measures low-angle and high-angle light scatter to determine cell volume and nuclear density/lobularity, respectively. Cluster analysis software again identifies each cell population based on its position, area and density which is then enumerated and displayed graphically. Based on human data, basophils are thought to be resistant to the cytoplasmic stripping process and as intact cells, appear larger than the bare nuclei of other WBCs, making them more easily identifiable.

All CBCs from canine, feline and equine patients admitted to the CSU VTH were retrospectively reviewed from a one-year time period (1/1/2010-12/31/2010). Per laboratory protocol, manual differentials were performed on all samples. Raw data was obtained using the Advia 120 multispecies software and manually generated CBC data was retrieved from the CSU Chimera database and matched with the corresponding Advia data. During the study period, twice-daily internal quality control was performed using three levels of control material (TESTpoint, three levels, Siemens Healthcare, Bayswater, Victoria, Australia). In addition, external quality control programs were participated in quarterly (VLA Quality Assurance

Program, Blaine ME, USA). A total of 6,572 canine, 914 feline, and 751 equine cases were obtained.

Although method comparison was not the primary goal of this study, statistical analysis was performed between manual and Advia automated neutrophil, lymphocyte, monocyte, eosinophil and basophil concentrations. Results were compared using Passing-Bablok regression, Deming regression and Bland-Altman bias plots in order to confirm system performance within the expected accuracies as have been previously reported.[1,7] Recorded variables included correlation coefficients (r) and the SD of the residuals (Sx/y) from linear regression analysis, and the bias with 95% limits of agreement calculated by Bland-Altman plots.

Because statistically significant discrepancies do not always associate with clinical relevance, we also categorized our data according to traditional clinical interpretation of leukocyte concentrations using established reference intervals. Advia and manual differential results for each leukocyte type were categorized into one of three categories (low, normal, high) based on whether the values fell below, within, or above CSU Clinical Pathology Laboratory's previously established reference intervals for each method. The number of cases within each grouping was recorded in pivot tables. These tables were then used to determine how many cases had discrepant results.

From each of the categories which were shown to be discrepant, the top 5% or top 50 cases with the largest magnitude difference ($\geq$1000 cell difference) were selected for further cytogram evaluation. This method of case selection was chosen to highlight those

misclassifications or error patterns that would have the greatest impact on clinical data

interpretation.

Results

Table 2.1 shows the correlation coefficient and standard deviation of the residuals from

Passing-Bablok regression, in addition to the bias and 95% limits of agreement from Bland-

Altman calculation for each leukocyte type (neutrophil, lymphocyte, monocyte, eosinophil and

basophil) from each of the three species (dog, cat, horse) evaluated. Globally, instrument

performance was interpreted to be adequate.

Table 2.1: Method comparison statistics between Advia and manual absolute numbers of leukocytes for each of the three species.

Canine	Linear Regression		Bland-Altman	
	Correlation coefficient (r^2)	SD residuals (Sy/x)	Bias	95% limit of agreement
Neutrophils	0.95	1.62	-0.19	-3.41 to 3.04
Lymphocytes	0.83	2.49	0.45	-4.81 to 5.71
Monocytes	0.26	0.83	-0.02	-1.98 to 1.93
Eosinophils	0.61	0.27	-0.05	-0.59 to 0.49
Basophils	5.25×10^{-6}	0.07	0.1	-1.85 to 2.04

Feline	Linear Regression		Bland-Altman	
	Correlation coefficient (r²)	SD residuals (Sy/x)	Bias	95% limit of agreement
Neutrophils	0.97	1.35	-0.44	-3.13 to 2.26
Lymphocytes	0.73	0.81	0.48	-1.29 to 2.24
Monocytes	0.18	0.38	-0.03	-1.06 to 0.99
Eosinophils	0.95	0.28	0.08	-0.51 to .68
Basophils	0.004	0.09	-0.01	-0.2 to 0.17

Equine	Linear Regression		Bland-Altman	
	Correlation coefficient (r²)	SD residuals (Sy/x)	Bias	95% limit of agreement
Neutrophils	0.98	0.60	-0.07	-1.25 to 1.10
Lymphocytes	0.83	0.50	-0.12	-1.10 to 0.86
Monocytes	0.53	0.38	0.13	-0.75 to 1.01
Eosinophils	0.58	0.08	0.02	-0.15 to 0.19
Basophils	0.03	0.04	0.03	-0.1 to 0.15

Figure 2.1 depicts pivot tables for neutrophils, lymphocytes, monocytes and eosinophils from each of the species showing cross tabulation results from reference interval classification. Basophils were excluded from this analysis as they have been proven not to be enumerated by automated instrumentation.[4] The figure legend on page 25 contains construction details. Agreement was calculated from the number of cases where both the manual and Advia differential agreed on the response classification (blue shaded boxes). The blue hatched boxes indicate where there was disagreement, but regardless of which differential method was

accepted, impact on clinical interpretation would likely be inconsequential. Clinical agreement was calculated, including the blue hatched boxes, since misinterpreting eosinopenia or monocytopenia is unlikely to be clinically relevant.

Canine Neutrophils

RI= 2,600-11,000/µL

	Advia		
Manual	Low	Normal	High
Low	349	30	1
		(520)	(11,510)
Normal	74	4546	45
	(580)		(1,130)
High	4	131	1392
	(15,160)	(1,090)	

6286/6572
= 95.66% agreement

Canine Lymphocytes

RI= 1,000-4,800/µL

	Advia		
Manual	Low	Normal	High
Low	1206	1433	22
		(620)	(6,410)
Normal	87	3661	57
	(290)		(3,670)
High	0	15	91
		(1,210)	

4958/6572
= 75.44% agreement

Canine Monocytes

RI= 200-1,000/µL

Advia

	Low	Normal	High
Low	184	528 *(200)*	16 *(1,360)*
Normal	149 *(130)*	3956	318 *(620)*
High	2 *(1,170)*	630 *(550)*	789

Manual (row label)

4929/6572= **75.0%** agreement

Clinical agreement:5606/6572= **85.3%**

Canine Eosinophils

RI= 100-1,200/µL

Advia

	Low	Normal	High
Low	1542	786 *(140)*	0
Normal	665 *(140)*	3322	26 *(400)*
High	7 *(1,900)*	140 *(370)*	84

Manual (row label)

4948/6572= **75.28%** agreement

Clinical agreement: 6399/6572= 97.36%

Feline Neutrophils

RI= 2,000-12,000/µL

Advia

	Low	Normal	High
Low	27	3	0
		(110)	
Normal	29	663	6
	(580)		(3,330)
High	0	24	162
		(1,720)	

852/914

93.2= % agreement

Feline Lymphocytes

RI= 1,500-6,000/µL

Advia

	Low	Normal	High
Low	376	191	6
		(820)	(6,120)
Normal	24	228	12
	(650)		(2,370)
High	0	1	16
		(3.520)	

620/914

= 67.83% agreement

Feline Monocytes

RI= 0-800/µL

Advia

	Normal	High
Normal	790	29 *(660)*
High	58 *(660)*	37

827/914
= **90.5%** agreement

Feline Eosinophils

RI= 0-1,200/µL

Advia

	Normal	High
Normal	850	25 *(660)*
High	12 *(780)*	27

877/914
= **96.1%** agreement

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Equine Neutrophils

RI= 3,000-7,000/µL

Advia

Manual	Low	Normal	High
Low	117	11 _(280)_	0
Normal	17 _(460)_	397	4 _(610)_
High	1 _(6,190)_	15 _(690)_	189

703/751

93.6= % agreement

Equine Lymphocytes

RI= 1,500-4,000/µL

Advia

Manual	Low	Normal	High
Low	220	38 _(350)_	1 _(7,980)_
Normal	68 _(440)_	383	0
High	0	14 _(810)_	27

630/751

= 83.89% agreement

22

Equine Monocytes

RI= 0-600/µL

Advia

	Normal	High
Manual Normal	634	63 *(370)*
High	9 *(270)*	45

679/751
= **90.4%** agreement

Equine Eosinophils

RI= 0-800/µL

Advia

	Normal	High
Manual Normal	737	5 *(470)*
High	5 *(460)*	4

741/751
= **98.7%** agreement
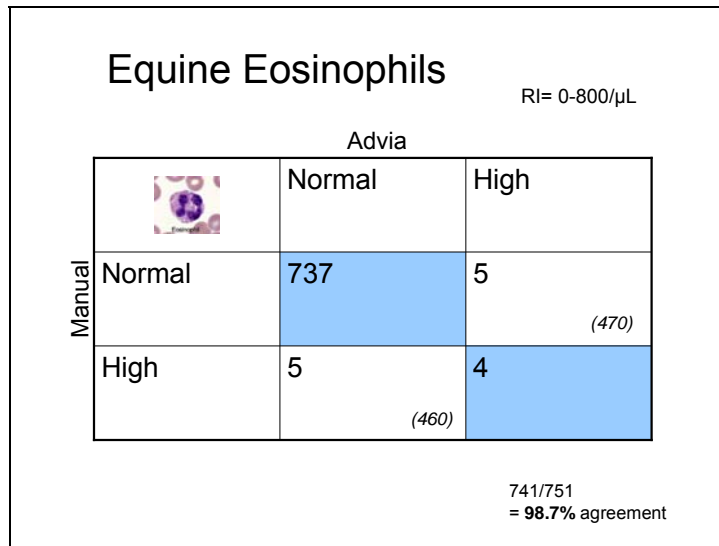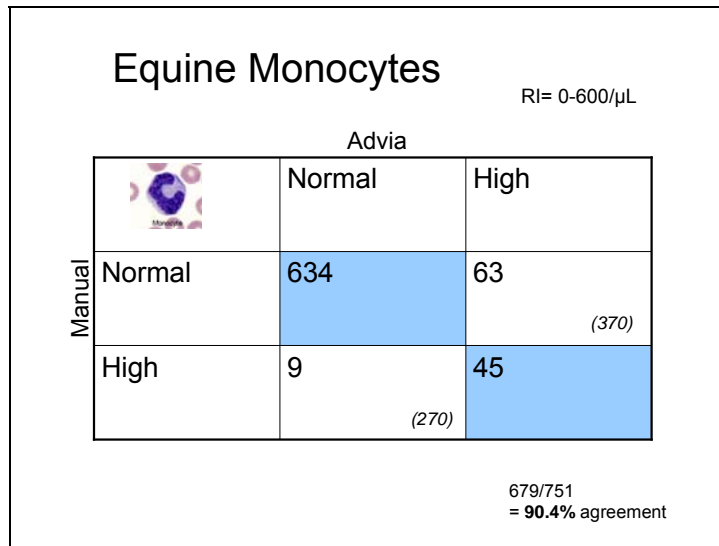
Figure 2.1: Advia categorization is displayed as columns, with the manual categorization in rows. Laboratory reference intervals for the leukocyte represented in the graph are noted in each upper right hand corner. Blue shaded boxes highlight the cases where both the manual and Advia differentials agreed on the classification (used to calculate agreement). Boxes with a diagonal shaded pattern represent discrepant classifications considered clinically inconsequential (added in for the clinical agreement calculation). Bolded numbers denote the number of cases which fall into each category. For those boxes in which classifications were discrepant, the median difference (given as absolute cell counts) between the two methodologies is given in parentheses.
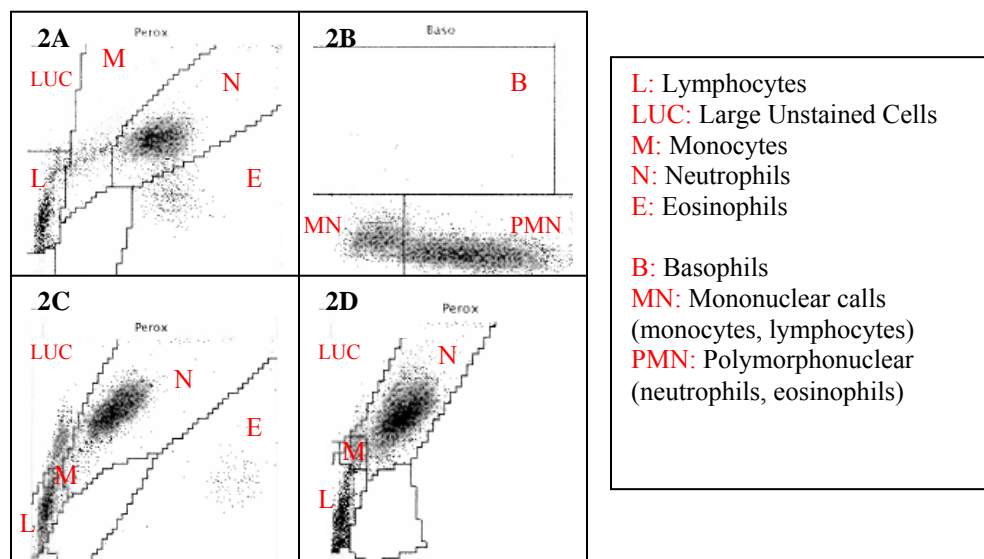
After determining which cases had discrepant results noted for the classification of

leukocyte numbers as determined by the Advia vs. manual methods, the cytograms of these cases

were evaluated to determine possible causes. Broad categorizations of the types of aberrant

findings noted on cytogram analysis are summarized in Table 2.2.

Table 2.2: Summary of common misclassifications by species

Species	Common misclassifications
Dog	Perox or baso channel mechanical error*, species mismatch (cat run as a dog, etc), neutrophil peroxidase content variability, nRBCs as lymphocytes, leukemia, left-shifts +/- toxic changes, eosinophil morphology and peroxidase content variations
Cat	Poor gating, left-shifts +/- toxic changes, platelet clumps, atypical cell populations (Heinz bodies, plasma cells, etc), debris contamination
Horse	Left-shifts +/- toxic changes, platelet clumps +/- fibrin strands

*Without internal flagging error

Examples and a more detailed description of the different types of misclassification

follow. For reference, normal perox and baso cytograms for canines are shown in Figures

2.2A&B and normal equine and feline perox cytograms are shown in Figure 2.2C&D,

respectively.

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L: Lymphocytes
LUC: Large Unstained Cells
M: Monocytes
N: Neutrophils
E: Eosinophils

B: Basophils
MN: Mononuclear calls
(monocytes, lymphocytes)
PMN: Polymorphonuclear
(neutrophils, eosinophils)

Figures 2.2A-D: (2A) Perox cytogram from a normal canine. (2B) Baso cytogram from a normal canine. (2C) Perox cytogram from a normal equine. (2D) Perox cytogram from a normal feline. Red letters indicate the gated cell populations.

Platelet clumping and other debris (Figure 2.3)

Description: One of the most frequently encountered misclassifications; platelet clumps, fibrin strands and sometimes unidentifiable "debris" detected within the perox channel are gated by the cluster analysis software as "noise". However, neutrophils and to a lesser extend lymphocytes are inappropriately added to the "noise" region and censored as well. Differential counts for these cell categories (one or both) are often falsely minimized. The Advia does have an error flag (PX-NW) designated to alert the operator to such interference possibilities, but this was not ubiquitously observed.
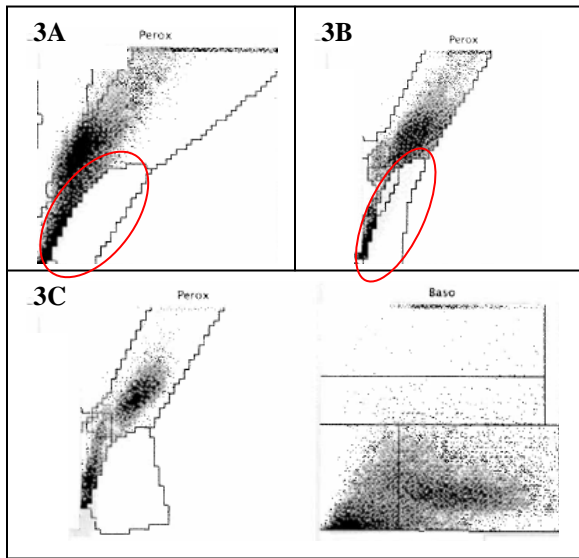
Figure 2.3: Examples of platelet clumping and debris on Advia cytograms. 3A) Platelet clumping and fibrin strands in horse blood. (red circle). (3B) Platelet clumping in a cat. Area denoted by both the red circle indicates the "noise" gate encompassing the lower portion of the neutrophil population. (3C) Amorphous debris (gel contamination) in a cat. Note the relatively normal perox cytogram in contrast to the randomized cellular events distributed in the baso channel.

Arbitrary gating in felines (Figure 2.4)

Description: There is often a density of cellular events between the lymphocyte and neutrophil gating areas. Immediate sample re-runs typically do not yield the same differential result.
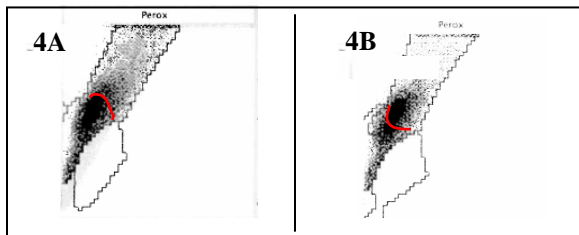
Figure 2.4: Example of arbitrary gating in a cat (4A) The location of the gate in the first run of this sample resulted in a lymphocyte count of 12,540/μL (4B) Immediate re-run of same sample with appropriate gating between lymphocytes and neutrophils. This lymphocyte count was closer to the manually derived count at 1,450/μL.

Perox or Baso channel mechanical errors (Figure 2.5)

Description: Unspecified mechanical malfunctions of either the perox or baso channel. These are plotted as either many randomized cellular events or with no events recorded at all. Typically, only a flag indicating a mismatch between the total white cells counts calculated by each method is raised.
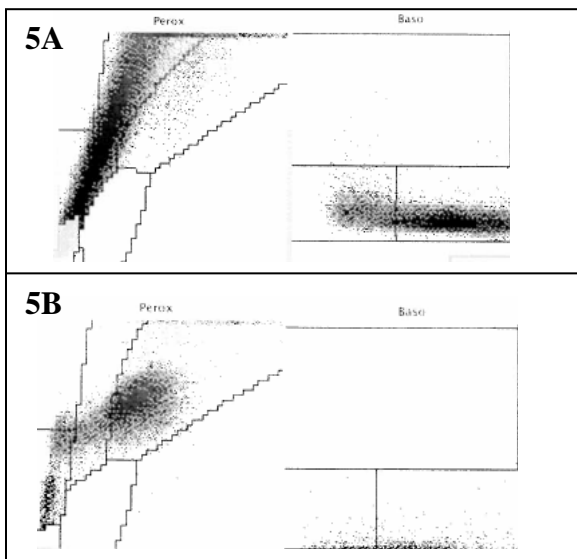

Figure 2.5: Example of unspecified mechanical malfunctions within the Perox channel (5A) and Baso channel (5B). In these cases, the perox channel error in 5A results in a high number of randomized events and the baso channel error in 5b results in the lack of recorded events. Note the normal cytogram for the opposite channel in each case.

Leukemia (Figure 2.6)

Description: Unsurprisingly, leukemia patterns often do not follow typical blood leukocyte distributions. In the Advia, the majority of leukemic cells are gated into the LUC, or less often, the monocytic counting areas in the perox cytogram and fall into the basophil detection region within the baso cytogram. This is likely due to the fact that lymphocytic leukemias/lymphoproliferative disorders are predominant in dogs, with these cell populations

showing minimal to no peroxidase activity.[2,3] The properties which allow these cells to exceed

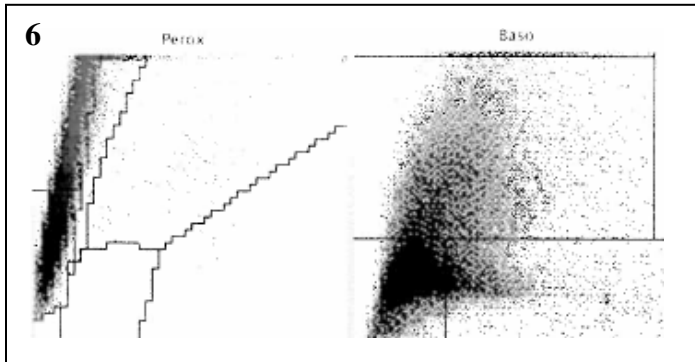the threshold gate defined for basophil region in the baso method are unknown.

Figure 2.6: Example of lymphocytic leukemia in a dog. Neoplastic cells are located in the lymphocyte/LUC (perox negative) gates in the perox channel and within the basophil region on the baso cytogram.

Nucleated red blood cells counted as lymphocytes (Figure 2.7)

Description: nRBCs are frequently misclassified as small lymphocytes by automated

differentiation. For the canine case depicted in figure 2.7, the total WBC concentration was 36.07

x 10^3/μL and lymphocytes, as determined by the Advia, comprised 19.7% (7.11 x 10^3/μL) of the

cells. Manual differential revealed a different picture, with only 1% (0.36 x 10^3/μL) lymphocytes

and 50% nRBCs. Note the increased small foci of increased cellular density towards the lower
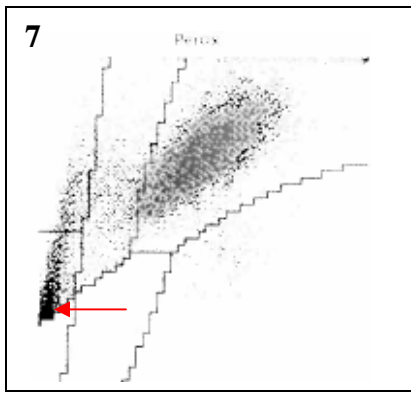
border of the gated lymphocytes.

Figure 2.7: Example of nRBCs misclassified as lymphocytes in a dog. nRBCs are enumerated within the lymphocyte gate (arrow).

Atypical cell populations other than leukemia (Figure 2.8)

Description: Occasionally, cells that do not typically circulate or cells that are not normally counted will be enumerated by the Advia. Where these aberrant cells are detected within the Perox and Baso channels will depend on respective cell properties. Plasma cells circulating in a cat (Figure 8A) were displayed in the lymphocyte/LUC region on the perox cytogram, and the basophil region of the baso cytogram, similar to atypical lymphocytes (as in figure 2.6). Heinz bodies in a cat (Figure 8B) are evident as small particles randomly distributed along the lower border of the baso channel.
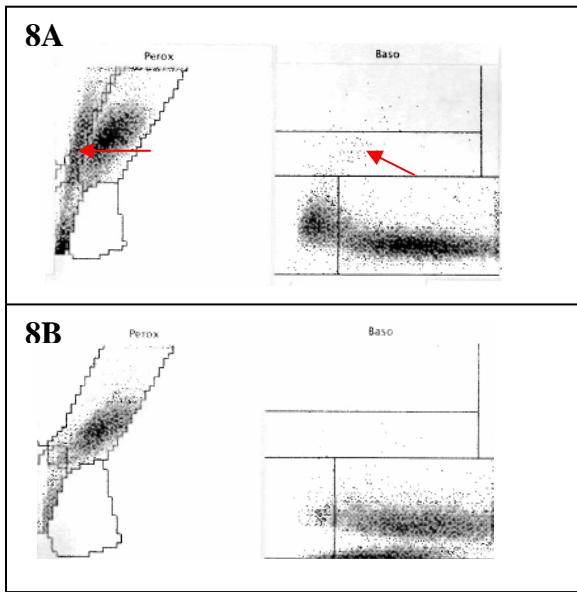
8A

8B

Figure 2.8: Examples of atypical cell populations other than leukemia. Plasma cells in a cat appear within the lymphocyte and LUC gates in the Perox cytogram and within the basophil region (arrows) (8A) Heinz bodies in a cat can be seen as a secondary cell population along the bottom of the baso cytogram (8B).

Variations within normal cell populations

Description: Variations within a cell population that is normally counted by the Advia can result in some of those cells falling outside of its normal gate and into adjacent gates. Because this is a common occurrence with neutrophils, those cells are more fully discussed below. Variation within eosinophil populations also resulted in misclassification of those cells. Figure 9 contains cytograms of degranulated and hyposegmented eosinophils in dogs where eosinophils were inappropriately gated within the neutrophil population.
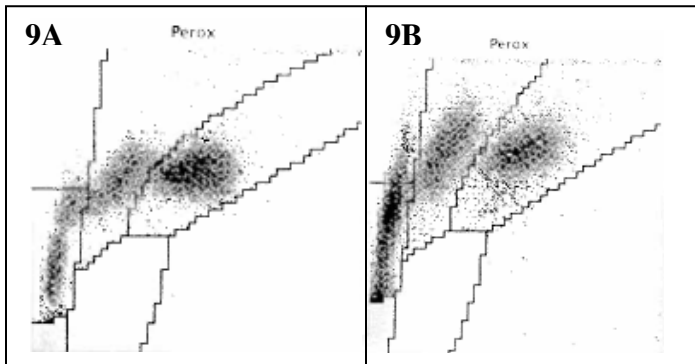
Figure 2.9: Examples of eosinophils being misclassified as neutrophils due to degranulation of the eosinophils (9A) or hyposegmentation of eosinophil nuclei (9B).

Left-shifts and variable peroxidase content in neutrophils (Figure 2.10)

Description: Left-shifts within the neutrophil population ± toxic changes were the most common reason for misclassification of neutrophils. The neutrophil population typically moves to the left along the x-axis (peroxidase activity) and into the monocyte gate (Figure 10A). Additionally, numerous cytograms displayed a similar move to the left, (Figure 10B) when there was no increase in band neutrophil numbers, although toxic changes were occasionally noted.
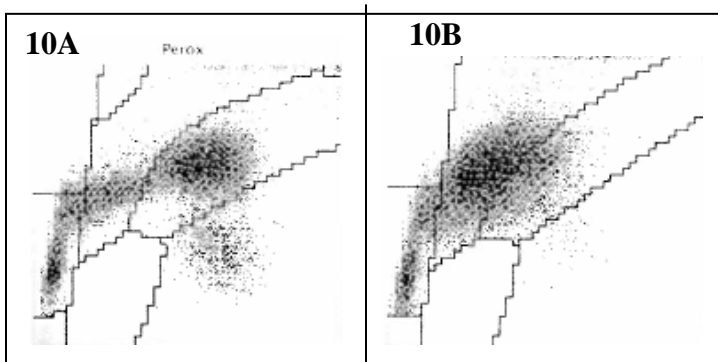

Figure 2.10: Examples of neutrophil misclassification associated with a peripheral neutrophil left shift (10A) and peroxidase content variability without a peripheral neutrophil left shift (10B) in dogs. The move to the left along the x-axis suggests a decrease in peroxidase activity.

Species mismatches (Figure 2.11)

Description: The case in figure 2.11 was a cat sample entered to run as a dog. Note the vertical

distribution of the leukocytes that is typical of feline cytograms in contrast to the more angled gating
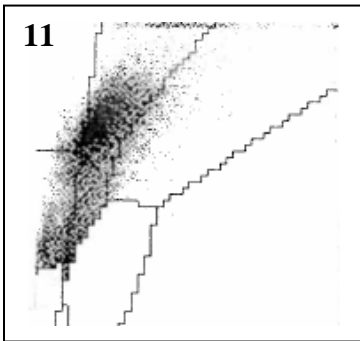
regions established for canines.

Figure 2.11. Example of a case where there was a species mismatch (cat run as dog). Note the more vertical
distribution typical of feline perox cytogram and lack of proper gating.

Discussion

Overall, equine and feline cytogram analysis revealed the fewest recognizable pattern

deviations. Cytogram analysis of these species presents a unique challenge. Cats and horses contain

on average <10% and <50%, respectively, of the peroxidase activity seen in dog and human

neutrophils.[6] Because of this and possibly because the gains displayed on the x-axis (indicating

peroxidase activity) remain standardized even in the multispecies software, the scattergrams for these

species are greatly condensed. Such density of cellular events and lack of differentiation between

neutrophils and lymphocytes makes the identification of specific cytogram misclassification patterns

almost impossible. For felines, the most frequently encountered issue causing discrepancies between

absolute leukocyte counts was arbitrary gating between the respective neutrophil and lymphocyte

populations (Figure 2.4). This was frequently detected when the Advia differential calculated absolute numbers of lymphocytes to be greater than neutrophils. The reason for this phenomenon remains unclear. No atypical cell populations or morphology variations were identified via light microscopy to offer an explanation. It is recommended that anytime the leukocyte distribution pattern displays an atypical result (ie absolute lymphocyte concentration > neutrophils) for this species, a re-run of the sample is indicated. If the differential still fails to correct, manual blood film review should be performed to confirm the presence of cell types and their proportions.

The similarity of canine neutrophil myeloperoxidase (MPO) content to human[6] and sheer volume of canine CBC's performed in our laboratory helped illustrate a wider variety of aberrant cytogram patterns for this species, although the following would not be expected to be exclusive to dogs. Numerous species mismatches, cats entered as dogs etc, were noted on cytogram review (Figure 11). While this is obviously a human error and in no way attributable to any instrumentation or software failure, a simple visual inspection of the cytogram can quickly save time and effort in identifying samples as erroneous before results are reported or interpretation is attempted by a clinician.

Instrumentation error was noted to occur in both the perox and baso channels. While most every hematology analyzer, the Advia included, has a system of error flags to alert the operator to such events, which analytical parameters are flagged is somewhat variable and not necessarily specific to the instrument error. For example, in the cases represented in figure 5 in which one channel had an obvious misread of the sample, the only warning flag raised was to indicate a mismatch between the total white blood cell counts performed independently in each channel. While most alert instrument operators would scrutinize this mismatch, it could also easily be overlooked or disregarded, further highlighting the subtly of errors that warrant further investigation.

While artifact and instrument error are reasons for incongruent results, these should be fairly easily detected and corrected. On the other hand, the majority of cases that had mismatches between the Advia and manual absolute leukocyte concentrations were due to cellular abnormalities within the sample, the cause of which may be less obvious based on instrument print-outs.

Upon reviewing the canine lymphocyte pivot table (Figure 2.1), it becomes clear that the Advia has a tendency to overestimate the number of lymphocytes that are determined to be present by manual blood film review. What is interesting is that the most extreme overestimations were most commonly due to misclassification of nucleated red blood cells (nRBCs) as lymphocytes. Clinical impression of the Advia data of the case depicted in figure 7 would have included differentials for a moderate lymphocytosis, including but not limited to: reactive lymphocytosis, ehrlichiosis, and chronic lymphocytic leukemia. These are significantly different clinical diagnoses than the ones suggested by the actual findings of a lymphopenia and marked rubricytosis (the dog in question had IMHA) and highlights the importance of reliable CBC data.

Across all species, the most common cytogram misclassification pattern was associated with variation within the neutrophil population which often included left shifts (± toxic changes). There were also cytograms which exhibited variable neutrophil peroxidase content without evidence of a left shift on the blood film. Both of these conditions produced a cytogram pattern that was skewed to the left. Patterns similar to these have been identified previously in dogs[4] although the presence of a left shift was not investigated for every case in that study. For the 46 cases in our study in which a left-skewed cytogram was noted in the absence of granulocytic precursors (bands neutrophils, metamyelocytes and/or promyelocytes), the full medical record was reviewed. Twenty-one of those cases possessed only a solitary CBC and thus a global picture of events could not be constructed. However, of the remaining 25 cases, left-shifts ± toxic changes were identified on CBCs run only 1-2

days prior to the appearance of the peroxidase-shifted cytograms. This seems to suggest that myeloperoxidase activity and content varies with maturity of neutrophils; band neutrophils may have decreased activity of MPO. Another consideration for why neutrophils might move to the left on the cytogram is that the remaining mature neutrophils contain decreased MPO activity, potentially from degranulation and MPO release or adaptations during development. In all of the above cases, these immature or peroxidase-deficient neutrophils were gated into the monocytic or less often, LUC categories. It is therefore suggested that any increase in monocyte concentration or LUC count warrants a manual blood film review to confirm the lineage of cell types present and assess their morphology. Furthermore, even in the absence of immature neutrophils upon blood film review, when the neutrophil cloud is skewed to the left on the cytogram consideration should be given for an underlying inflammatory condition.

It should be noted that since all 8,237 CBCs from canine, equine and felines patients were not able to be reviewed, a global statistic could not be calculated for the overall prevalence rate for each of the aberrant cytogram patterns. However, such a statistic would likely be limited in its potential benefit, since prevalence of these aforementioned conditions would likely vary between different clinical populations and instrumentation used.

In conclusion, leukocyte cytogram patterns from automated analyzers can provide quick assessments of sample quality as well as provide clinically useful information for otherwise unsuspected hematological conditions. We have described common errors reported by the Advia 120 and made recommendations as to further steps to take when those errors are recognized.

REFERENCES

1. Becker M, Mortiz A, Giger U. Comparative clinical study of canine and feline total blood cell count results with seven in-clinic and two commercial laboratory analyzers. *Vet Clin Pathol*. 2008; 37:373-384.

2. Christopher MM, Metz AL, Klausner D, et al. Acute myelomonocytic leukemia with neurologic manifestations in the dog. *Vet Pathol.* 1986; 23: 140-147.

3. Grindem CB, Stevens JB, Perman V. Cytochemical reactions in cells from leukemic dogs. *Vet Pathol* 1986; 23:103-109.

4. Lilliehöök I, Tvedten HW. Errors in basophil enumeration with 3 veterinary hematology systems and observations on occurrence of basophils in dogs. *Vet Clin Pathol*. 2011;40:450-458.

5. Klenner S, Richartz J, Bauer N, et al. Myeloperoxidase deficiency in dogs observed with the Advia 120. *Tierärztliche Praxis Kleintiere*. 2010: 39-146.

6. Rausch PG, Moore TG. Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. *Blood*. 1975; 46:913-919.

7. Welles EG, Hall AS, Carpenter DM. Canine complete blood counts: a comparison of four in-office instruments with Advia 120 and manual differential counts. *Vet Clin Pathol* 2009; 38: 20-29.

CHAPTER 3: Pseudobasophilia in the dog: its occurrence and predictability of disease using the Advia 120 hematology analyzer

Introduction

Basophils are among the rarest of circulating white blood cells, accounting for <0.5% of absolute total blood leukocytes in domestic mammals.[11] Basophil counts determined by manual differential are imprecise as a consequence of these low numbers. Automated instrumentation, in particular the Advia 120 and its predecessor, the Technicon H-1 series, have attempted to lower this statistical variation by developing a specialized methodology for the classification and enumeration of basophils.

The Advia 120 hematology analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY) building upon the Technicon H-1 series, differentiates white blood cells via a two channel system. The peroxidase method uses a combination of light scatter (cell volume) and light absorption (peroxidase staining intensity) to numerically and graphically differentiate normal blood leukocytes. Basophils are not identified via this technique, thus a second basophil/lobularity channel is employed. In a heated cytochemical reaction chamber, blood cells are exposed to a reagent containing a surfactant and phthalic acid that strips the cytoplasm from all other white blood cells (except basophils) and lyses red blood cells and platelets. A laser light source then measures low-angle and high-angle light scatter to determine cell volume and nuclear density/lobularity, respectively. Cluster analysis software identifies each cell population based on its position, area and density which is then enumerated and displayed graphically. Based on human data, basophils are thought to be resistant to the cytoplasmic stripping process

and since intact cells appear larger than the bare nuclei of the other WBC, this makes them more easily identifiable.

However, several studies have documented that automated analyzers, the Advia and the Technicon H-1 included, fail to detect the basophils of domestic mammals.[8-10,12] Nevertheless, operators of the Advia 120 will note the frequent occurrence of cellular events classified as "basophils" on the automated differential in the absence of microscopically detected basophils (See Figures 3.1 & 3.2). This raises the question of what the Advia is detecting, if not true basophils.
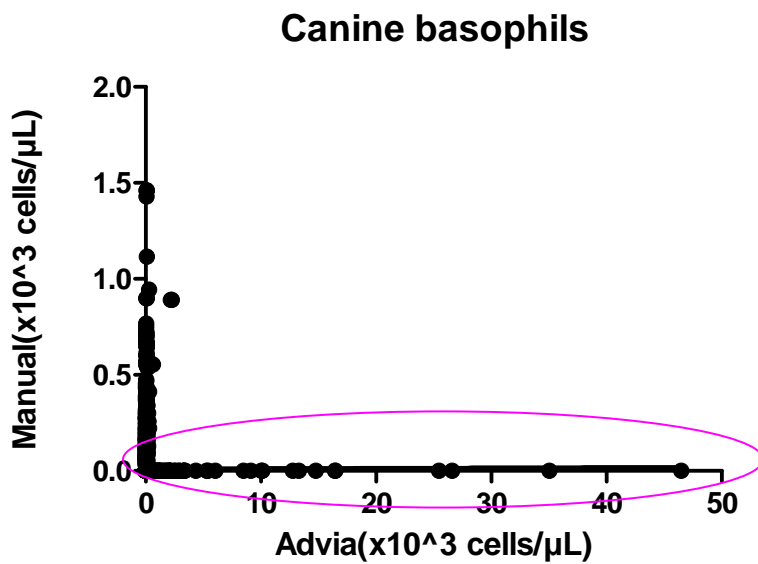
Canine basophils

Figure 3.1: Linear regression model of manual (y-axis) and Advia automated (x-axis) counts of absolute numbers of basophils. Note the lack of association between the two methods (r^2= 5.254e-006) and the numerous "basophil" events detected by the Advia (circled).
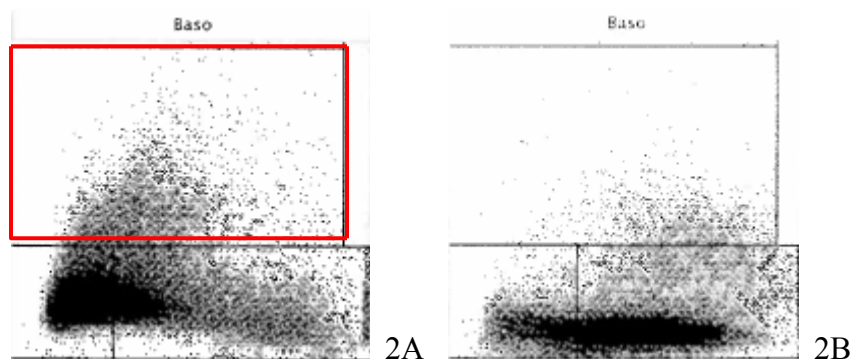
Figure 3.2: Baso channel cytograms of canine blood. Note the numerous cellular events falling within the large upper rectangle that is the basophil region (highlighted in red in 2A). Figure 2A is case of CD21+ leukemia. Figure 2B is a case of immune-mediated hemolytic anemia that contained both granulocytic precursors and an immature erythroid series.

Pseudobasophilia is an analyzer phenomenon well documented in the human literature whereby abnormal cells in the blood are detected and classified as basophils. This phenomenon has been reported in numerous automated analyzer systems, including the Advia 120.[2,4-7] The cell types most frequently associated with pseudobasophilia include atypical lymphocytes, plasma cells or myeloma cells[4-7], however single cases of nucleated red blood cells (nRBCs), granulocytic precursors, metastatic carcinoma cells, atypical cells of infectious mononucleosis, reactive lymphocytes and the lymphocytes of infants have also been reported.[2]

A handful of incidences suggestive of the pseudobasophilia phenomenon have been reported in the veterinary literature[3,8] but thus far, have not been fully investigated. Based on observations at the Colorado State University Clinical Pathology Laboratory (CSU CPL), we hypothesized that the phenomenon of pseudobasophilia would be evident in our canine CBCs and would be associated with the atypical cells frequently found in patients with leukemia. Given the relatively high volume of leukemic samples analyzed in our laboratory, additional aims of the study were to determine if a particular immunophenotypic or morphologic property was

associated with the manifestation of pseudobasophilia, as well as to determine a diagnostically useful cut-off value to serve as a predictor of disease.

Materials and Methods

A search was performed to collect all CBCs completed by the CSU CPL for canine patients admitted to the CSU VTH during a one-year period (1/1/2010-12/31/2010). Over the same time interval, all canine cases evaluated by the CSU Clinical Immunology Laboratory (CSU CIL) with a flow cytometric diagnosis of leukemia and a concurrent CBC performed by the CSU CPL were reviewed. Only two cases overlapped, with the remaining leukemia cases coming from outside submission sources.

CBCs were analyzed on the Advia 120 hematology analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY) within four hours of receiving the sample. Per laboratory protocol, manual 100-cell differentials were performed on all samples by the clinical pathology technical staff or pathologists on duty. During the study period, twice-daily internal quality control was performed using three levels of control material (TESTpoint, three levels, Siemens Healthcare, Bayswater, Victoria, Australia). In addition, external quality control programs were participated in quarterly (VLA Quality Assurance Program, Blaine ME, USA).

The leukemic cases obtained from the CSU CIL were divided into CD21+ (B-cell), CD8+ (T-cell), CD34+ (Acute/progenitor cell), and myelomonocytic-suspect (based on CD14+, null cell, or cytologically determined differentiation) leukemia subcategories. One hundred twenty-five of the blood films were re-reviewed by a single author (Ryseff) blinded to

immunophenotypic classification. Distinguishing morphologic features of neoplastic cells were recorded for each leukemic case. Descriptive criteria included: approximate cellular size in relation to neutrophils (greater than, equal to, or smaller), percentage of blasts as defined by presence of a nucleolus, presence of granules (either primary or LGL-like), nuclear configuration (indented, cloverleaf, convoluted, etc), chromatin pattern, decreased N:C ratio with more abundant cytoplasm, and cytoplasmic features (vacuolated, royal blue, presence of a perinuclear clear zone, etc). In cases where blood films could not be located, the manual differential and associated comments from the original CBC were used.

The non-leukemia cases were selected from VTH patients. From all CBCs obtained, samples were excluded if there was a confirmed diagnosis of leukemia via flow cytometry or if there was a clinical impression/diagnosis of leukemia and/or lymphoproliferative disorder with circulating blast cells noted on blood film review.

Statistical analysis

Manual and automated absolute basophil concentrations were evaluated and compared using linear regression, Deming regression, and Bland-Altman plots. Reported variables include correlation coefficients (r) and the SD of the residuals (Sx/y) from linear regression analyses, the intercept and slope with 95% confidence intervals calculated by Deming regression, and biases with 95% limits of agreement calculated by Bland-Altman plots. Accuracy of the automated basophil count was determined by comparing results to the manual differential, which was defined as the reference method in this study.

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Data was evaluated for normality by visual inspection of frequency histograms. Basophil distribution by either method did not appear normally distributed; therefore median values were used for description. The Mann-Whitney *U*-test was used to compare Advia-derived absolute "basophil" numbers (ie. pseudobasophilia) between the groups of dogs with confirmed leukemia and those that were non-leukemic. Kruskal-Wallis analysis with post-hoc Dunn's multiple comparison testing was performed to compare subcategories of leukemia (CD21+, CD8+, CD34+, myelomonocytic-suspects). Receiver operating characteristic (ROC) curve analysis was used to investigate the sensitivity and specificity of pseudobasophilia as determined by the Advia for detecting dogs with leukemia.

All statistical analyses were performed and figures produced using commercially available statistical software (GraphPad Prism, v 5.0, GraphPad Software Inc., La Jolla, CA, USA). Criterion for statistical significance was set at a *P*-value of < 0.05.

Results

A total of 6384 cases met the inclusion criteria for the non-leukemia group. There were 61 cases of CD21+, 36 cases of CD8+, 23 cases of CD34+ and 6 cases of myelomonocytic-suspect leukemias identified, for a total of 126 leukemic cases.

Linear regression and difference plots for absolute basophil numbers between manual differential counts and the Advia were constructed and failed to show any correlation or agreement between the two methods (See Figure 3.1 and 3.3).

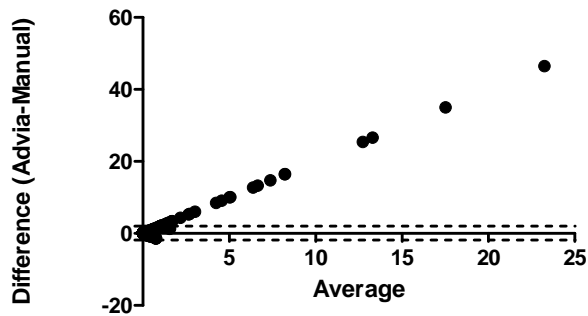**Bland-Altman of Canine basophils:Difference vs average**

Figure 3.3: Bland-Altman difference plots comparing absolute basophil numbers obtained by the manual method and the Advia 120. Black dashed lines represent the 95% limits of agreement.

A "High" basophil flag was recorded on samples that exceeded the previously established laboratory reference interval of 0-0.1 x 10^3/μL. Of the 126 leukemic samples, 114 were noted to have a "High" basophil flag raised on the Advia differential count, for a prevalence rate of 90.5%. Similarly, 749 of the 6384 non-leukemic samples had a value over the reference interval reported, for a prevalence rate of 11.8%.

There was a statistically significant difference ($p<0.0001$) in the median basophil concentration as reported by the Advia between leukemic and non-leukemic canines. (See Figure 3.4). The median and range of the leukemia group were 0.04 (0.0-3.41), and for the non-leukemic group were 0.63 (0.02-57.55). Statistically significant differences in Advia basophil counts between immunophenotypic-subgroups of leukemia (CD21+, CD8+, CD34+, myelomonocytic-suspects) were not found. In addition, there was no correlation between any of the described morphologic characteristics of neoplastic cells and the presence, or absence, of pseudobasophilia.
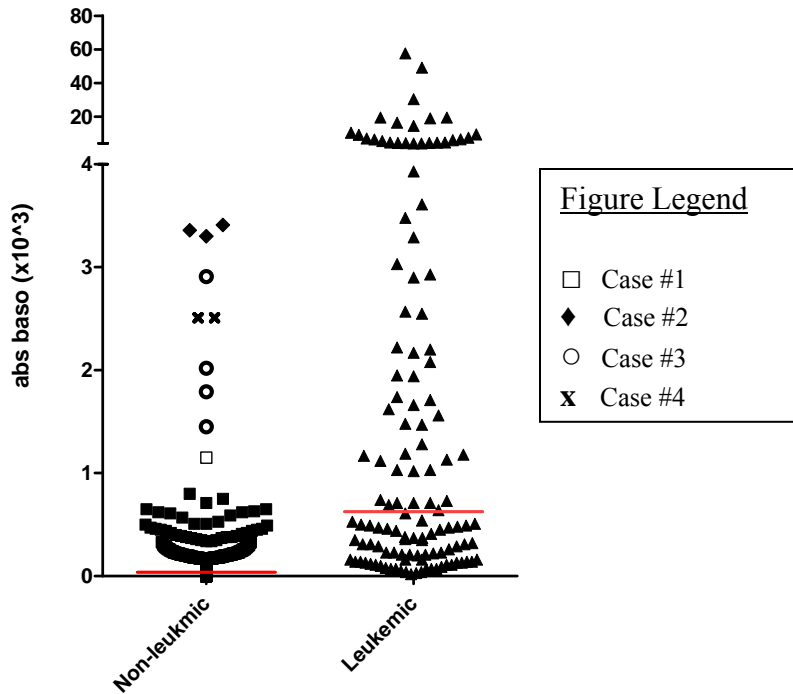
Figure 3.4: Absolute "basophil" numbers as defined by the Advia for dogs with leukemia and non-leukemic dogs. Horizontal bar represents the medians. A significantly higher "basophil" count was found in dogs with leukemia. See discussion section regarding cases #1-4.

The following results were obtained from ROC curve analysis in the evaluation of absolute "basophil" numbers as defined by the Advia, comparing canine leukemic and non-leukemic samples. The AUC was determined to be 0.95 (p<0.0001) which is considered excellent. (See Figure 3.5) The cut-off value of >1.05 x 10^3/μL "basophils" detected on the Advia differential was considered optimal; this value yielded an 88.7% sensitivity and 88.3% specificity for the detection of leukemic samples.
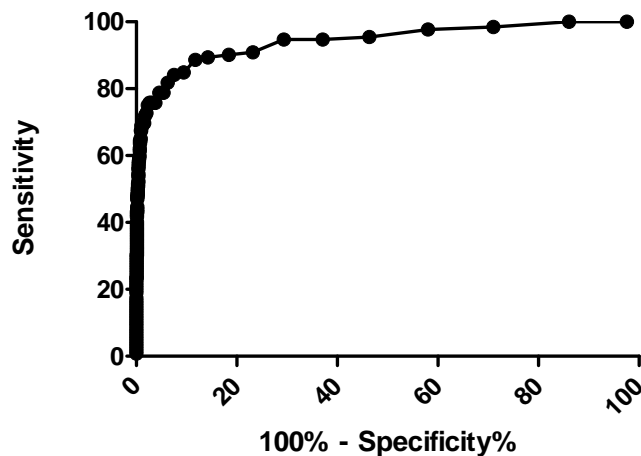
Figure 3.5: ROC analysis for absolute numbers of "basophils" as determined by the Advia in canine leukemic and non-leukemic samples.

Discussion

It is important to remember that the cells being quantified by the Advia are not in fact basophils, but a host of other cell types, which is indicated by the term pseudobasophilia. It is unclear what properties of a cell result in its ability to exceed the threshold gate defined for basophils. Using pseudobasophilia as a screen for leukemia has the benefit of using data that is a normal by-product of CBC analysis and requires no additional specialized sample processing, reagents, software analysis or cost. ROC curve analysis was used to determine that a cut-off value of >1.05 x 10^3/μL "basophils" as defined by the Advia automated differential is both a highly sensitive and highly specific indicator for the detection of leukemia in canine CBC analysis. This study confirms the findings documented in other investigations[3,8] that suggest atypical lymphocytes are the most common aberrant cell population encountered within the basophil region on canine Advia cytogram.

The leukemias that were associated with pseudobasophilia failed to show any correlation between Advia basophil counts and cellular immunophenotypic or morphologic properties. Similarly, it is not known why some leukemias failed to produce a pseudobasophilia. Further study could include prospective analysis of pseudobasophilic samples in which the sample is collected as it is being run through the Advia, immediately following the cytochemical reaction in the basophil chamber, as has been previously performed on human samples.[4] This may provide information as to whether the cells being counted in the basophil region are resistant to lysis or display any other unique properties.

In the non-leukemic group, only 10 samples out of 6384 were noted to have a pseudobasophilic count above the $1.05 \times 10^3/\mu L$ cut-off value determined by ROC curve analysis. These 10 samples actually represented 4 individual canines, with multiple CBC submissions over the course of 1-3 days following hospital admission (See Figure 3.4). Case #1 in figure 4 was documented in hospital records as a suspected rodenticide toxicity presenting with a non-traumatic hemoperitoneum. However, CBC blood film review revealed numerous atypical blasts cells. Unfortunately, the dog was euthanized and no further diagnostics were performed. Cases #2, 3 and 4 in figure 4 were diagnosed as immune-mediated hemolytic anemia, hepatocellular carcinoma, and pyometra, respectively. Every CBC submission for all three of these cases not only possessed a marked neutrophilic left-shift (with band neutrophils, metamyelocytes and promyelocytes) but also numerous nRBC's with an immature erythroid series noted. There is a single report of both nRBCs and early granulocytic precursors inducing pseudobasophilia in humans.[2] It is interesting to note that in all three canine cases, the cells falling within the basophil region of the cytogram were found more toward the right side of that

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region, directly over the polymorphonuclear area of the cytogram (Figure 2B), in contrast to leukemic samples (Figure 2A), where the cellular events are detected more toward the left side of the basophil region (where one would expect to see minimal nuclear lobularity in predominantly lymphocytic leukemias). This would seemingly suggest that the granulocytic precursors, rather than the erythroid line, were the cells mainly responsible for the pseudobasophilia observed in these cases. Unfortunately, as this was a retrospective study, confirmation of the cellular lineage responsible could not be determined. However, this again may be an interesting finding to pursue in a prospective study as previously described.

Temperature is a crucial element in the cytoplasmic stripping process and it is thought that variations in temperature can induce false instances of pseudobasophilia. With the multispecies software, elevated basophil counts have been reported in cats and horses with incomplete cytoplasmic lysis of leukocytes other than basophils in association with aging of the sample from the time of collection (>12-24 hrs). However, increasing the baso reaction chamber temperature to 34ºC minimizes the chances of this occurrence (Siemens Healthcare Diagnostics Inc, Customer Bulletin). Temperature related incomplete cytoplasmic lysis of leukocytes has not been reported to occur in canine samples and since our laboratory utilizes the 34ºC correctional technique, this issue was not considered to be a factor in our findings. In addition, human samples run at temperatures less than 20-25ºC (room temperature) increased the prevalence of pseudobasophilia due to incomplete lysis of leukocytes.[1,4] In our laboratory, all samples are allowed time to equilibrate to room temperature before analysis, making this issue unlikely to have interfered with out findings.

Another potential contributing factor for the occurrence of pseudobasophilia is that of coincidence, whereby multiple particles (clumps of cells or bare nuclei) enter the flow cell simultaneously, giving the appearance of a single, large cellular event. This phenomenon is more likely to occur in samples with high cellular concentrations, such as the frequently encountered marked leukocytoses of leukemic patients. However, the Advia limit of linearity includes cell counts up to 400,000 leukocytes/µL for human samples and, while the limits of linearity for leukocyte counts have only been tested and guaranteed for samples up to 100,000 leukocytes/µL in the multispecies software package, coincidence is not expected to be a significant factor in our study. Any sample falling within the limit of linearity has compensatory mechanisms in place to prevent interference from coincidence. One hundred five of our 126 canine leukemic samples fell within the limit of linearity. A potential technique for the confirmation or rejection of coincidence would be to identify samples containing extremely high leukocyte counts that result in pseudobasophilia and perform dilutions. If coincidence is indeed a contributing factor, then serial dilutions would be expected to resolve the pseudobasophilia. If, however, the pseudobasophilia persists in spite of the dilutions, it would confirm that coincidence is not a contributing factor to this observed phenomenon.

Although this report of pseudobasophilia within canine species utilized only the Advia 120, human literature has reported this phenomenon to occur in a wide array of automated hematology instruments.[1,2,4-7] In addition, a study in the veterinary literature using the Sysmex XT-2000iV reported higher percentages of lysis-resistant region events in leukemic groups.[3] It is highly likely then that this phenomenon is not confined to one particular system and may occur with the use of other methodologies. Further investigations into the presence of pseudobasophilia

in other hematology instruments and its associated cellular types and properties would be interesting.

We have shown that a pseudobasophilia in dogs of >1,050 cells/µL is associated with atypical cell populations, frequently of neoplastic origin. Corroboration of atypical baso channel cytograms with pseudobasophilia should prompt manual blood film review with these findings in mind. Additional diagnostics will be necessary for further characterization of atypical cell types present.

REFERENCES

1. Bizarro N. Pseudobasophilia on the Technicon automated cell counters. *Clin Lab Haem.* 1996; 18:298-299.

2. Davies S, Bain BJ. Basophil counts on the Technicon H*1 automated counter. *Clin Lab Haem.* 1996; 18:35-38.

3. Gelain ME, Rossi G, Giori L, et al. Identification of neoplastic cells in the blood using the Sysmex XT-2000iV : a preliminary step in the diagnosis of canine leukemia. *Vet Clin Pathol.* 2010; 39:169-179.

4. Gibbs G, Campbell G, Christie I. Pseudobasophilia and the Advia 120. *Hematology.* 2009;14(3):159-163.

5. Hur M, Lee YK, Lee KM et al. Pseudobasophilia as an erroneous white blood cell differential count with a discrepancy between automated cell counters: report of two cases. *Clin Lab Haem.* 2004; 26:287-290.

6. Jácomo RH, Lozano VF, Neto J, et al. What's the meaning of basophilia in Sysmex XE-2100? [Letter to the Editor]. *Arch Pathol Lab Med.* 2011; 135:415.

7. Koui F, Chiba T. The clinicopathological evaluation of automated cytochemical hematology system (Technicon H1) in patients with leukemia and myelodysplastic syndrome. *Jpn J Clin Pathol.* 1992; 40(6): 586-594. [article in Japanese

8. Lilliehöök I, Tvedten HW. Errors in basophil enumeration with 3 veterinary hematology systems and observations on occurrence of basophils in dogs. *Vet Clin Pathol*. 2011;40:450-458.

9. Lilliehöök I, Tvedten HW. Validation of the Sysmex XT-2000iV hematology system for dogs, cats and horses. II. Differential leukocyte counts. *Vet Clin Pathol*. 2009;38:175-182.

10. Tvedten HW, Haines C. Canine automated differential leukocyte count: study using a hematology analyzer system. *Vet Clin Pathol*. 1994;23:90-96.

11. Weiss DJ, Wardrop KJ. Schalm's Veterinary Hematology. 6th ed. Ames, IA: Wiley-Blackwell; 2010: 290-297.

12. Wenger-Riggenbach B, Hässig M, Hofmann-Lehmann R, et al. Evaluation of the LaserCyte: an in-house hematology analyzer for dogs and cats. *Comp Clin Pathol*. 2006;15:117-129.

CHAPTER 4: Canine perox cytogram analysis and neutrophil myeloperoxidase indices in the identification of inflammation and myeloid leukemias.

Introduction

Myeloperoxidase (MPO) is an important enzyme located within the azurophilic granules of polymorphonuclear leukocytes (specifically neutrophils) and to a lesser extent, within the lysosomes of monocytes. MPO serves as a catalyst in the reaction of $H_2O_2 + Cl^- \rightarrow HOCl^-$, the product of which acts as a broad antimicrobial agent in the defense against a host of pathogens, including bacteria, viruses and fungi. MPO is synthesized and packeted into primary granules at the promyelocytic stage of granulopoeisis, and has been found in >30% of CD34+ cells within the bone marrow in human studies.[18]

The Advia 120 hematology analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY) employs a peroxidase channel method of leukocyte differentiation, whereby cells are plotted on a scattergram according to their light scatter (cell size, y-axis) and light absorption (peroxidase activity, x-axis) principles. Cluster analysis software then gates individual cell populations depending on their area, position and density. In addition, the Advia also reports a variety of specific peroxidase indices:

> *-neut x: coordinate value for the mean light absorbance (peroxidase activity) of the gated neutrophil population*
>
> *-neut y: coordinate value for the mean cell size of the gated neutrophil population*

-MPXI: a unitless value calculated by the Advia as the deviation of the mean of the x-axis value for the gated neutrophil population from a stored archetypal population ([patient sample-archetypal]/archetypal). It is important to note that even within the multispecies software packages, this archetypal population is human.

MPXI serves as an estimate for MPO content per leukocyte, and thus a measurement of relative intracellular MPO activity.

The perox cytogram and indices are routinely reported parameters by the Advia, and are therefore available for analysis on every patient that has a CBC performed. Studies in human medicine have investigated these indices, particularly MPXI, for a variety of different reasons as described below.

Neutrophilic degranulation and release of MPO from within the azurophilic granules occurs as part of the respiratory burst in response to a variety of stimuli. Since MPXI measures the intracellular content of MPO, decreases in this parameter should therefore be expected. This finding has been documented in several specific human conditions (ischemic events, septicemia, etc).[3,9,24] The application of MPXI as a screening tool for diseases has proved useful, especially in cases of sepsis where the total WBC or other leukocyte variables do not change as expected. While limited study in veterinary medicine as to the relation of this index to specific disease states has not yielded similar results[16,19-21] a broader study attempting to correlate the associated or expected hematological changes has also not been attempted. Toxic changes occur at the metamyelocytic and myelocytic maturation stages in the bone marrow from increased granulopoiesis triggered by strong inflammatory conditions, such as those seen with bacterial

infections and septicemia. Toxic changes can be evident within neutrophils long before characteristic leukogram changes are seen and may in fact be the only changes present to indicate inflammatory conditions. The severity of the changes often reflects the severity of the disease. We hypothesize that the perox indices, particularly MPXI, should show significant changes when applied to other hematological changes implicative of severe systemic inflammation and thus serve as potential markers for those morphologic changes and possible disease states.

In addition, it has been hypothesized that for situations where cellular divisions within the bone marrow are skipped, the immature neutrophils which are released contain an increased concentration of MPO.[5,8,13,21] Such would be the case for left shifts, considered another hallmark of acute inflammation, where MPXI would be expected to increase. In the case of myeloid leukemias, the relative changes are not as straight forward. Human studies have used both cytogram analysis and peroxidase content as a means to differentiate myeloid from lymphoid leukemias, and myeloid subtypes from one another.[1-3,7,10,11,14,15,17,18,22,24] This has been minimally studied for dogs in veterinary medicine.[6,12,23] MPXI has been noted to increase as progressive maturation seen in AMLs, along the order of M1<M4=M5<M2<M3 according to the FAB classification system.[6,7,12,22] This change is also reflected in the perox cytograms reported by the Advia, with the more mature leukemic cells falling within gated fields noted to display peroxidase activity.[3,10,11,14,15,17] This seemingly makes sense as AML-M3 displays predominantly promyelocytic differentiation. MPO synthesis and packeting into granules has already occurred at this stage, but these cells have failed to undergo further cellular divisions typical of maturation, and thus contain a maximum content of MPO. Conversely, AML subtypes

with minimal differentiation, M0 and M5a for example, show decreases in MPXI. The diagnosis of myeloid leukemia in dogs remains a challenging one. The development of a screening tool to select for potential cases worthy of further specialized analysis (typically flow cytometry) built into standard CBC analysis would be highly beneficial. An additional aim of this study was to better characterize peroxidase activity indices and cytogram patterns for different types of leukemias as a potential screening tool for myeloid differentiation in dogs.

Material and methods

A search was performed to collect all CBCs completed by the CSU Clinical Pathology Laboratory (CSU CPL) for canine patients admitted to the CSU VTH during a one-year period (1/1/2010-12/31/2010). Over the same time interval, all canine cases evaluated by the CSU Clinical Immunology Laboratory (CSU CIL) with a flow cytometric diagnosis of leukemia and a concurrent CBC performed by the CSU CPL were reviewed. Only two cases overlapped, with the remaining leukemia cases coming from outside submission sources.

CBCs were run on the Advia 120 hematology analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY) within four hours of receiving. Per laboratory protocol, manual 100-cell differentials were performed on all samples by the clinical pathology technical staff or pathologists on duty. During the study period, twice-daily internal quality control was performed using three levels of control material (TESTpoint, three levels, Siemens Healthcare, Bayswater, Victoria, Australia). In addition, external quality control programs were participated in quarterly (VLA Quality Assurance Program, Blaine ME, USA).

Neutrophilic left shifts were defined by the presence of ≥400 band neutrophils/μL and/or

any metamyelocytes or promyelocytes noted. Toxic changes included cytoplasmic vacuolization,

cytoplasmic basophilia, Döhle bodies or toxic granules, or any combination thereof. These were

classified subjectively according to the percent neutrophil population affected (0%- none, <33%-

slight, 33-66%- moderate, >66%- marked). The leukemic cases obtained from the CSU CIL were

divided into CD21+ (B-cell), CD8+ (T-cell), CD34+ (Acute/progenitor cell), and

myelomonocytic-suspect (based on CD14+, null cell, or cytologically determined differentiation)

leukemia subcategories.

Briefly, MPXI reference intervals for humans have been reported as -10 to 10. Before the

Advia 120 MPXI results could be compared, a laboratory reference interval specific for dogs was

established. Inclusion criteria were defined as > 1 year of age, and clinically healthy as

determined by a lack of known disease, on no medications other than routine preventatives with

no abnormalities noted on CBC and biochemistry panel. The 68 dogs used to establish a

reference interval included 29 castrated male and 39 spayed female dogs. Thirty-two of the dogs

were mixed-breed and 33 pure breed (1-2 dogs per type), with 3 unreported. Their ages ranged

from 1-19 years with a mean of 6.7 years. Data was visualized for normal distribution and the

central 95% of the values taken to establish a reference interval.

It should be noted that when using the multispecies software, the neut x,y coordinates are

occasionally reported as 0,0. This is due to a software default when atypical morphologies or

scattergram patterns are detected by the instrument (personal communication, Siemens

Diagnostics technical support). One hundred nineteen of our 6634 cases had neut x,y coordinates

reported as 0,0 and were subsequently censored during statistical analysis. Since this comprised only 1.8% of our total cases, statistical analysis on the remaining cases was still considered interpretable.

Statistical analysis

Data was evaluated for normality by visual inspection of frequency histograms. None of the peroxidase indices (MPXI, neut x, neut y) appeared normally distributed; therefore median values were used for description. The Mann-Whitney *U*-test was used to compare neut x, neut y and MPXI between the groups having left shifts vs. not and toxic changes vs. not. Kruskal-Wallis with post-hoc Dunn's multiple comparison testing was performed to compare neut x, neut y and MPXI between the different categories of toxic changes (marked, moderate, slight and none) and subcategories of leukemia (CD21+, CD8+, CD34+, myelomonocytic-suspects). All statistical analyses were performed and figures produced using commercially available statistical software (GraphPad Prism, v 5.0, GraphPad Software Inc., La Jolla, CA, USA). Criterion for statistical significance was set at a *P*-value of < 0.05.

Results

The reference interval for MPXI was determined to be 8.1-22.2 [unitless]. A total of 6,634 cases met the study inclusion criteria.

Toxic changes: Of the 6,634 cases in the study, 399 were noted to have some degree of toxic change, with 30 cases having marked changes, 114 cases with moderate changes and 255 cases with slight changes noted. No statistical significance was detected in the neut-x coordinate between cases classified as having toxic changes within neutrophils and those that did not,

although significance was detected in the neut-y coordinate ($p < .0001$), with a mean and standard deviation of 33.06 ± 3.56 for those with toxic changes and 32.4 ± 1.22 for all those without (Figure 4.1). There was a statistically significant difference in MPXI ($p < .0001$) between cases with toxic changes (18.44 ± 8.44) and those without (15.33 ± 6.39). In addition, although none of the mild, moderate, or marked categories of toxic change were different from each other (17.27 ± 8.53, 20.24 ± 9.49, and 21.54 ± 8.51, respectively), all had a significantly higher MPXI than the group with no toxic change. However, wide overlap was evident among values both with the reference interval and between groups. (Figure 4.2)
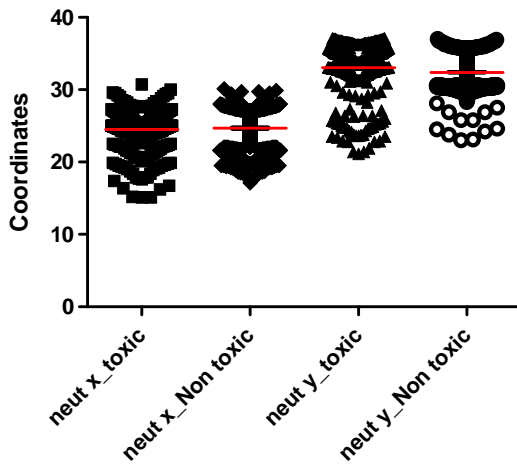

Figure 4.1. Neut x and neut y coordinates of cases with toxic changes noted and those without. Red horizontal lines represent the mean. A statistically higher neut y coordinate was found for cases with toxic changes compared to those that did not.
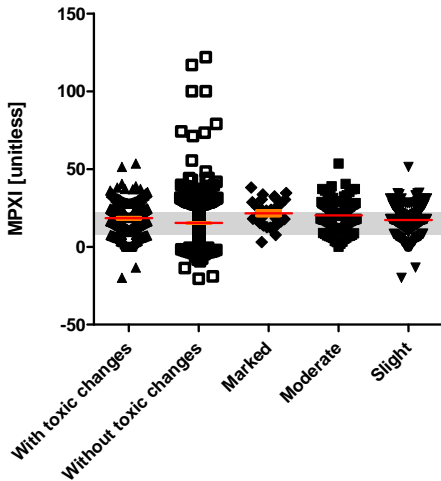
Figure 4.2. MPXI of all cases with toxic changes (n=399) and those without (n=6235). Cases with toxic changes were also subdivided into groups where changes were noted to be marked (n=30), moderate (n=114) or slight (n=255). Red horizontal lines represent the mean. The shaded grey area denotes the reference interval. Statistically significant differences were noted between cases with toxic changes and those without whether all or subdivided cases with toxicity were evaluated.

Left shift: Of the study cases, 446 had a neutrophilic left-shift identified, and 6,188 did not. MPXI values were found to be statistically significantly different ($p < .0001$) between those cases with a left shift (mean and standard deviation 17.86 ± 7.67) and those without (15.35 ± 6.50) (Figure 3). Statistical significance ($p < .0001$) was also found in the neut-y coordinate between the groups (with left shift = 32.97 ± 2.89 and without = 32.40 ± 1.29) (Figure 4.4). However, there was large overlap in the distribution of results between groups and, although the MPXI was significantly different, group medians both fell within the reference interval. No significance was found between the neut-x coordinates (Figure 4.4).
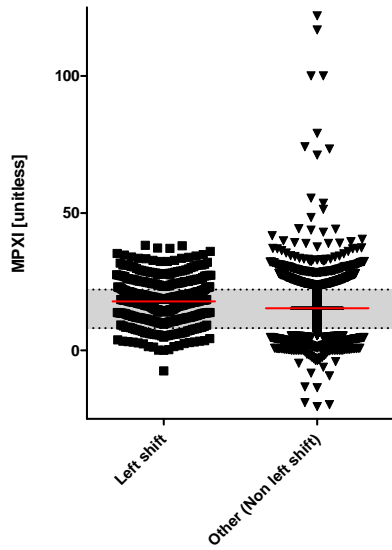
Figure 4.3. MPXI of cases with a neutrophilic left shift and those without. Red horizontal lines represent the mean. A significant difference was detected between the medians at a p-value of <0.0001 The shaded grey area denotes the reference interval.
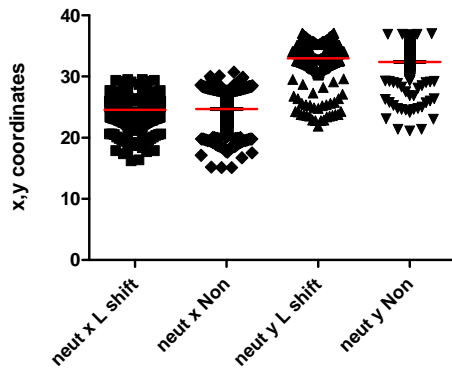

Figure 4.4. Neut x and neut y coordinates of cases with a neutrophilic left shift and those without. Red horizontal lines represent the mean. The neut y coordinate for cases with a left shift is statistically higher than the coordinate for cases without.

Leukemia groupings and subtypes: One hundred and twenty-six leukemic cases met the

inclusion criteria, with 61 cases of CD21+ leukemia, 36 cases of CD8+ leukemia, 18 cases of

CD34+ leukemia, and 11 cases of myelomonocytic-suspect leukemia. MPXI analysis for the immunophenotypic subgroups of leukemia yielded a significant statistical difference ($p < .0001$) between the medians, but failed to maintain that significance when the groups were compared to each other in a post-hoc test. The MPXI mean and standard deviations were 14.92 ± 4.89 for CD21+ (B-cell), 13.91 ± 5.66 for CD8+ (T-cell), 18.54 ± 9.47 for CD34+ (Acute/progenitor cell), and 18.89 ± 6.31 for cases with a strong immunophenotypic suggestion of myeloid differentiation. Again, large overlap existed between the groups, with the majority of cases falling within the established reference interval (Figure 4.5).
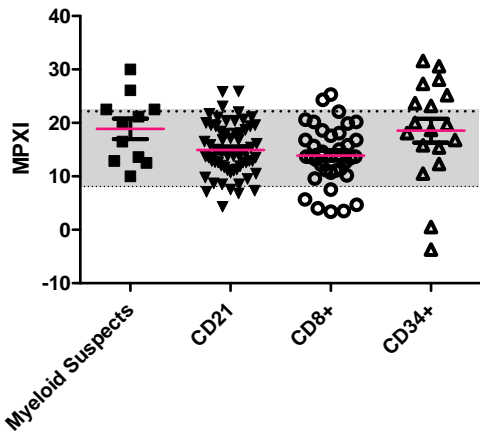

Figure 4.5. MPXI of leukemic cases, organized within immunophenotypic subgroupings. Pink horizontal lines represent the mean. No significant statistical difference was found. The shaded grey area denotes the reference interval.

Analysis of the peroxidase channel cytograms was then employed to identify patterns that could potentially help detect variations in myeloid differentiation or otherwise help distinguish different types of leukemia. For all 126 leukemic samples, perox cytograms were analyzed to look for distinct patterns while the observer was blinded to the immunophenotypic diagnosis. Overall, three patterns became evident, as depicted in figure 4.6. Pattern 1 was defined as a lack of peroxidase activity, with the majority of cells falling into the lymphocyte and/or LUC gating

area on the scattergram. One hundred twenty of the 126 cases fit this pattern. Pattern 2 was

defined as minimal peroxidase activity, with the cell population shifting slightly to the right and

into the monocyte gate. Three cases with this pattern were noted. Lastly, pattern 3 had the most

intense peroxidase content; cell populations were angled towards the upper right area of the

scatterplot. Only 3 cases fit this pattern type; these were strongly suspected to be of myeloid

origin. One of these cases was CD14+ and two were CD34+. Pattern 2 was associated with two

potential myeloid suspects; one of these cases was CD 14+ and one was CD34+/4+. The other

case with pattern 2 was a CD21+ B-cell leukemia. Pattern 1 encompassed the vast majority of

the cases. When cases from patterns 2 and 3 were grouped together and compared to cases with

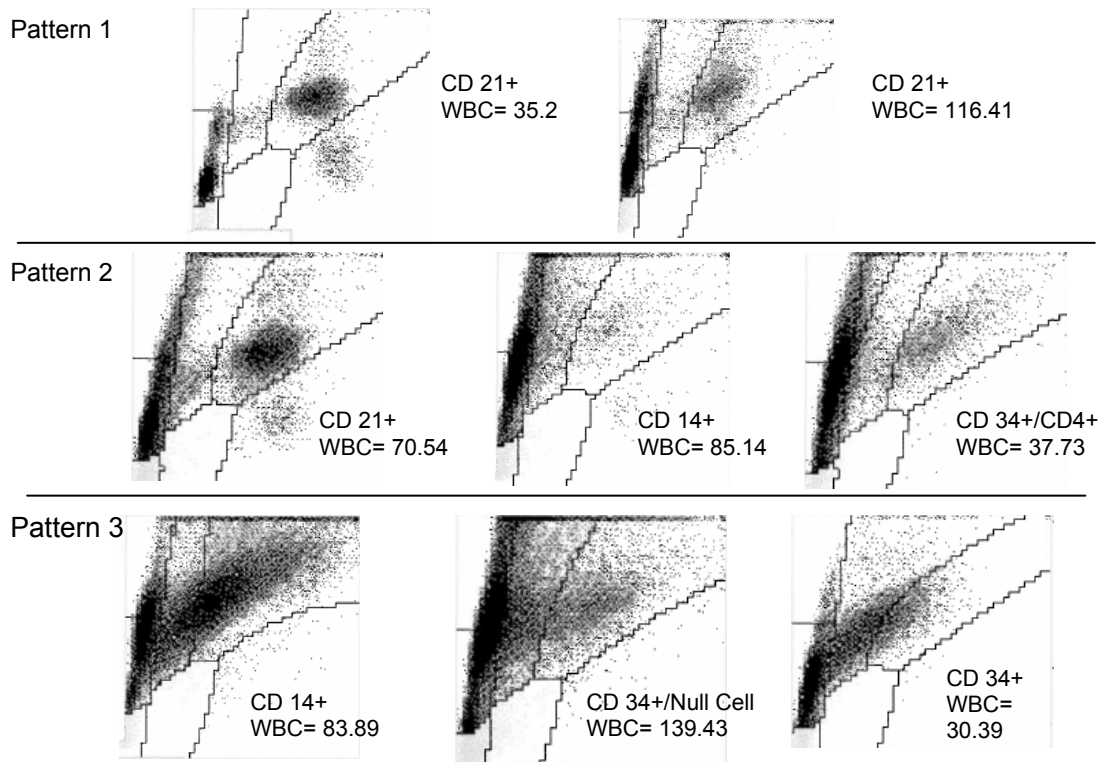pattern 1 there was no difference in MPXI (Figure 4.7).

Figure 4.6. Advia 120 perox channel cytograms demonstrating three distinct patterns of varying myeloperoxidase

activity in leukemic blood from dogs: pattern 1- no detectable MPO, pattern 2- minimal MPO, pattern 3- moderate

to abundant amounts of MPO. Each cytogram is labeled with the leukemic cell phenotype and the total WBC count (x 10^3/ul) for that case.
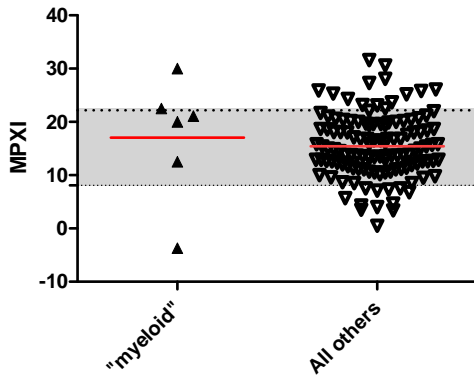

Figure 4.7. MPXI of canine leukemic cases that are grouped as either strong myeloid leukemia suspects (based on perox cytogram analysis) or all others. Red horizontal lines represent the mean. No significant statistical difference was found. The shaded grey area denotes the reference interval.

Discussion

To the author's knowledge, this is the first study which has examined the various peroxidase indices as reported by the Advia 120 and their relationship to inflammatory processes as well as their potential diagnostic value in differentiating myeloid from lymphoid leukemias.

A mild relative increase in the MPXI index was noted in cases where a left-shift was evident. This seems to validate the assumption that the average MPO activity within these neutrophils was increased, possibly reflecting skipped divisions within the bone marrow and release of the immature forms into circulation. MPXI though, failed to demonstrate any correlation with the absolute number of band neutrophils in circulation, and thus did not appear to be an indicator of severity of disease (Figure 4.8). However, the wide range of values obtained within all of the groups within this study, as well as their considerable overlap between each

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other and with the reference interval, likely limits the diagnostic utility of this index. A significant difference was also seen in the y-axis coordinate between the cases with a neutrophilic left shift and those without a left shift. This difference makes sense as immature granulocytes are of slightly larger size than mature forms. However, differences between the two groups were not disparate enough to be of diagnostic utility based on current instrument settings.
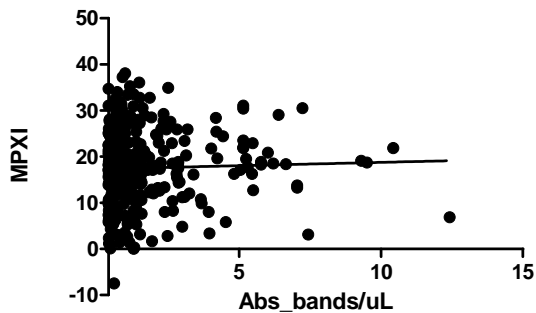

Figure 4.8. Linear regression model of absolute numbers of canine band neutrophils/uL and MPXI. $r^2=0.001$

While MPXI was higher in cases with toxic change vs. without, these differences are also not disparate enough to be diagnostically useful. The mechanism of toxic change formation is not thought to have a direct influence on MPO activity within neutrophils, however, human data has noted a decrease in MPXI in specific disease subsets (sepsis, etc) where toxic changes and/or left shifts would be anticipated. Correlation between myeloperoxidase activity indicators and these hematologic conditions has not been attempted. While increases in MPXI where noted when left-shifts and toxic changes were present in our study, large overlap between the values and reference intervals made its diagnostic utility as a screening tool for manual blood film review for these morphologic variations questionable. Furthermore, limitations of this study include its retrospective nature and inability to fully define specific disease states and conditions associated with the hematologic changes identified. Toxic changes and left-shifts are not specific

findings for any one condition or stimuli, and can be evident in multiple different disease processes. The mixing of numerous pathogeneses with differences in neutrophil activation and behavior (intravascular vs extravascular degranulation, chronic vs acute inflammation, etc) likely limited the degree of change detected within the perox indices.

MPXI and the other peroxidase indices failed to identify significant enough differences to be a useful screening tool for the identification of myeloid vs lymphoid leukemias or a means of subclassifying leukemias according to stages of myeloid differentiation. One possible explanation is that the studied peroxidase indices measurements are solely calculated from cells that fall within the gated region identified as neutrophils. As the pattern distributions depicted in figure 6 demonstrate, the majority of leukemic cells showed minimal, if any, peroxidase activity on cytogram analysis that would place them within the neutrophil region. These populations of neoplastic cells would not be taken into account during the calculations of the peroxidase indices and would therefore be inconsequential to their results.

More likely explanations as to why the peroxidase indices failed to detect significant changes within any of the studied populations are two-fold. The more minor issue is the retention of a stored human population as the archetypal reference for MPXI, even in the multispecies software. While the development of a species specific reference interval using that parameter should eliminate any negative influence, this cannot be guaranteed as the intrinsic software calculations are not known. It would be helpful to have the MPO activity, itself, reported as well as its deviation from the mean of a species specific archetypical population to further evaluate the accuracy and predictability of the MPXI index.

The more significant issue though, likely lies within the larger diversity seen between species, breeds, and individuals in veterinary medicine than is typically seen in human populations. Greater variation in the results from bioanalytical assays is often seen within veterinary species than in humans and this likely holds true for peroxidase indices as well. In fact, high inter-subject variability has already been noted to be present in previous veterinary studies[8,11-12] especially in MPXI. Thus, it seems that determining a reference interval for peroxidase indices in the traditional manner that encompasses many different breeds and ages is not likely to be useful. Refinement of the reference interval is needed, either by establishment of individual baseline values, or potentially according to breed type.

In conclusion, the peroxidase indices as reported by the Advia 120 have limited diagnostic potential within their currently reported values. However, establishment of more specific individual or breed specific reference intervals may prove to be more diagnostically useful.

REFERENCES

1. Austin GE, Chan WC, Zhao W, et al. Myeloperoxidase gene expression in normal granulopoiesis and acute leukemias. *Leukemia and Lymphoma* 1994; 15:209-226.

2. Bendix-Hansen K, Nielsen HK. Myeloperoxidase-deficient polymorphonuclear leucocytes (IV): relation to FAB-classification in acute myeloid leukaemia. *Scand J Haemat* 1985; 35:174-77.

3. Bruno A, Del Poeta G, Venditti A, et al. Diagnosis of acute myeloid leukemia and system Coulter VCS. *Haematologica* 1994; 79:420-28.

4. Buffon A, Biasucci LM, Liuzzo G, et al. Widespread coronary inflammation in unstable angina. *New England Journal of Medicine* 2002; 347: 5-12.

5. Carrick JB, Begg AP. Peripheral blood leukocytes. Veterinary Clinics of North America. Equine Practice. 2008; 24: 239-259.

6. Christopher MM, Metz AL, Klausner J, et al. Acute myelomonocytic leukemia with neurologic manifestations in the dog. *Vet Pathol* 1986; 23: 140-147.

7. Eivazi-Ziaei J. Myeloperoxidase index and subtypes of acute myeloid leukemia. *J Pak Med Assoc* 2009; 59(6):406-7.

8. Eivazi-Ziaei J, Dastgari S. Role of myeloperoxidase index in differentiation of megaloblastic and aplastic anaemia. *Indian Journal of Medical Sciences* 2004; 58:345-348.

9. El-Maallem H, Fletcher J. Effects of surgery on neutrophil granulocyte function. *Infection and Immunity* 1981; 32(1): 38-41.

10. Fernández-Castro M, Viloria A. Utility of the Technicon H-1 flags in the detection of peripheral blood blast cells of paediatric acute leukaemia patients. *Acta Haematol* 1995; 93:9-12.

11. Gibbs G. Peroxidase activity and nuclear density analysis (PANDA) in the diagnosis of haematological malignancy. *Br J Biomed Sci* 2005; 62(3): 142-44.

12. Grindem CB, Stevens JB, Perman V. Cytochemical reactions in cells from leukemic dogs. *Vet Pathol* 1986; 23: 103-109.

13. Gulley ML, Bentley SA, Ross DW. Neutrophil myeloperoxidase measurement uncovers masked megaloblastic anemia. *Blood* 1990; 76: 1004-1007

14. Hoyer JD, Fisher CP, Soppa VM, et al. Detection and classification of acute leukemia by the Coulter STKS hematology analyzer. *Am J Clin Pathol* 2006; 106: 352-8.

15. Kakkar N, Kaur R. Utility of white blood cell suspect flags and histogram pattern in the detection of acute leukemia by Advia-60 automated hematology analyzer. *Indian J Pathol Microbiol* 2004; 47(3): 322-6.

16. Klenner S, Richartz J, Bauer N, et al. Myeloperoxidase deficiency in dogs observed with the Advia 120. *Tierärztl Prax* 2010; 38(K): 139-146.

17. Koui F, Chiba T. The clinicopathological evaluation of automated cytochemical hematology system (Technicon H1) in patients with leukemia and myelodysplastic syndrome. *Jpn J Clin Pathol.* 1992; 40(6): 586-594. [article in Japanese]

18. Lanza F. Clinical manifestation of myeloperoxidase deficiency. *J Mol Med* 1998; 76:676-681.

19. Piviani M, Segura D, Monreal L, et al. Neutrophilic myeloperoxidase index and mean light absorbance in neonatal septic and nonseptic foals. *Vet Clin Pathol* 2011; 40(3): 340-44.

20. Queiroz-Junior CM, da Fonseca Pacheco CM, Fonseca AH, et al. Myeloperoxidase content is a marker of systemic inflammation in a chronic condition: the example given by the periodontal disease in rats. *Mediators of Inflammation* 2009; 1-7.

21. Schwarz BC, van den Hoven R, Schwendenwein I. Diagnostic value of the neutrophils myeloperoxidase index in horses with systemic inflammation. *Vet J* 2012; 191:72-78.

22. Tsakona CP, Kinsey SE, Goldstone AH, et al. Use of flow cytometry via the H*1 in FAB identification of acute leukemias. *Acta Haematol* 1992; 88:72-77.

23. Villiers E, Baines S, Law AM, et al. Identification of acute myeloid leukemia in dogs using flow cytometry with myeloperoxidase, MAC387, and a canine neutrophils-specific antibody. *Vet Clin Pathol* 2006; 35(1):55-71.

24. Yonezawa K, Horie O, Yoshioka A, et al. Association between the neutrophils myeloperoxidase index and subsets of bacterial infections. *Int Jnl Lab Hem* 2010; 32: 598-605.

CHAPTER 5: Concluding Remarks

The first portion of this study focused on the depiction of repeatable cytogram patterns representative of misclassification schemes and atypical cellular morphologies that, when present, can have a significant influence on clinical data interpretation. Awareness and recognition of these patterns are encouraged and recommended in order to optimize reported CBC data and its interpretation for case management.

In the second portion of this study, we demonstrated that the analyzer phenomenon of pseudobasophilia can occur during canine sample analysis. Using ROC curve analysis, we demonstrated that in samples with automated "basophil" counts >1.05 x 10^3/μL, the likelihood of leukemia is high, with a sensitivity of 88.7% and a specificity of 88.3%. Given the high prevalence of leukemia in cases with a pseudobasophilia >1.05 x 10^3/μL, a detailed blood film review is recommended for all samples that demonstrate this property which may then be followed up with flow cytometry for immunophenotyping.

The third portion of the study investigated the various peroxidase indices (MPXI, neut x, neut y) as reported by the Advia 120, and their associations with other hematological findings suggestive of systemic inflammation. We first established a normal reference interval for MPXI in dogs using 68 healthy individuals as controls.
The peroxidase indices were then compared between two groups of dogs that either had or did not have a neutrophilic left shift. Similar evaluations were performed between four groups of dogs that had variable degrees of toxic change within the neutrophil population. Grouping of dogs was based solely on hematologic data, and included a vast array of unspecified conditions and diseases. Left-shifts and toxic changes were predicted to influence the peroxidase indices. We found that although there were some minor statistically significant differences between

groups, none of the changes were sizable enough to provide diagnostic utility. In addition, these same parameters in conjunction with perox cytogram analysis were used to attempt to differentiate and potentially classify myeloid leukemia and its subtypes. No significant differences were detected in the peroxidase parameters, either between immunophenotypic groups or between cases categorized according to cytogram patterns.

Interpreting results from the peroxidase indices may be complicated by a couple of factors. One, MPXI calculations are based on deviation from a human archetypal population, even in multispecies software, and two, that high inter-subject variability is present in veterinary species, possibly related to breed, and thus establishment of broad reference intervals for individual animal use may be futile. Further studies to investigate this high inter-individual variation within the veterinary species may help establish more specific, and more diagnostically useful, recommendations as to the interpretation of peroxidase parameters.