EVALUATION OF THE
A. MENARINI V-SIGHT HEMATOLOGY ANALYZER
FOR BOVINE BLOOD

DIPLOMARBEIT

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Mitbetreuender Assistent: Dr. Michael Iwersen
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List of abbreviations

\(a\) \quad \text{intercept (in Passing-Bablok regression)}

ADP  \quad \text{adenosine diphosphate}

Advia  \quad \text{Advia 2120i hematology analyzer (Siemens AG, Erlangen, Germany)}

\(b\) \quad \text{slope (in Passing-Bablok regression)}

BiAS  \quad \text{Biometrische Analyse von Stichproben software (version 10.0, epsilon-Verlag, Frankfurt, Germany)}

BVD  \quad \text{bovine virus diarrhea}

CBC  \quad \text{complete blood count}

Cusum  \quad \text{cumulative sum linearity test}

\(CV\) \quad \text{coefficient of variation}

d  \quad \text{mean of differences (in Bland-Altman analysis)}

DIC  \quad \text{disseminated intravascular coagulation}

EBFU  \quad \text{erythroid burst-forming units}

ECFU  \quad \text{erythroid colony-forming units}

EDTA  \quad \text{ethylenediaminetetraacetic acid}

EPO  \quad \text{erythropoietin}

fL  \quad \text{femtoliter}

FN  \quad \text{number of false-negative outcomes}

FP  \quad \text{number of false-positive outcomes}

g/dL  \quad \text{grams per deciliter}

Gran#  \quad \text{granulocyte count}

H\(_1\), H\(_2\), H\(_3\)  \quad \text{high concentration analyte tested thrice for carry-over calculation}

HCT  \quad \text{hematocrit}

HGB  \quad \text{hemoglobin concentration}

Hz  \quad \text{hertz}

L\(_1\), L\(_2\), L\(_3\)  \quad \text{low concentration analyte tested thrice for carry-over calculation}

LFG  \quad \text{Lehr- und Forschungsgut (Teaching and Research Farm)}

Lymph#  \quad \text{lymphocyte count}

MCH  \quad \text{mean corpuscular hemoglobin}
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>MCHC</td>
<td>mean corpuscular hemoglobin concentration</td>
</tr>
<tr>
<td>MCV</td>
<td>mean corpuscular volume</td>
</tr>
<tr>
<td>mmol/L</td>
<td>millimoles per liter</td>
</tr>
<tr>
<td>Mon#</td>
<td>monocyte count</td>
</tr>
<tr>
<td>MPV</td>
<td>mean platelet volume</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>n/a</td>
<td>not available</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>PASW</td>
<td>Predictive Analytics Software (version 17.02, IBM, Armonk, New York, United States)</td>
</tr>
<tr>
<td>PCT</td>
<td>thrombocrit</td>
</tr>
<tr>
<td>PDW</td>
<td>platelet distribution width</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>PLT</td>
<td>platelets</td>
</tr>
<tr>
<td>r</td>
<td>correlation coefficient</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>RDW</td>
<td>red blood cell distribution width</td>
</tr>
<tr>
<td>s</td>
<td>standard deviation of differences (in Bland-Altman analysis)</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>t*</td>
<td>test statistic</td>
</tr>
<tr>
<td>TN</td>
<td>number of true-negative outcomes</td>
</tr>
<tr>
<td>TP</td>
<td>number of true-positive outcomes</td>
</tr>
<tr>
<td>V CI/RI range</td>
<td>V-Sight 95% confidence interval (mean ± 1.96 standard deviations) divided by range of the reference interval</td>
</tr>
<tr>
<td>V/A range</td>
<td>V-Sight range divided by Advia range</td>
</tr>
<tr>
<td>V/A SD</td>
<td>V-Sight standard deviation divided by Advia standard deviation</td>
</tr>
<tr>
<td>V/RI range</td>
<td>V-Sight range divided by range of the reference interval</td>
</tr>
<tr>
<td>V-Sight</td>
<td>A. Menarini V-Sight hematology analyzer (A. Menarini, Vienna, Austria)</td>
</tr>
<tr>
<td>VA</td>
<td>volt ampere</td>
</tr>
<tr>
<td>VAC</td>
<td>voltage in alternating current</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cells</td>
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1. Introduction

Hematologic analysis is not only relevant for diagnosing disorders of the hematologic system, but is also helpful in the diagnosis, surveillance and prognosis of many other diseases. Today, automated hematology analyzers are essential instruments in veterinary laboratories. As smaller and more affordable automated cell counters have been launched, in-house analyzers have become increasingly popular in veterinary practices. They provide rapid results for complete blood counts (CBC) and allow the examiner to reduce time-consuming manual cell counting and to focus on pathological results. This permits a rapid assessment of an animal’s state of health and improves patient care.

The A. Menarini V-Sight device (A. Menarini Pharma GmbH, Vienna, Austria) is a fully automatic, in-house hematology analyzer providing up to 18 blood parameters for 16 animal species. It delivers a three-part differential white blood cell (WBC) count and three histograms plotting cell distribution widths. The measurement methods employed by the A. Menarini V-Sight hematology analyzer (V-Sight) are the impedance method for determining WBC, red blood cell (RBC), and platelet (PLT) data, and the colorimetric method for determining hemoglobin (HGB) (SHENZHEN MINDRAY BIO-MEDICAL ELECTRONICS CO. LTD., 2009). Up to this date, the V-Sight hematology system has been evaluated for blood from dogs, cats, and horses (SCHWENDENWEIN, 2010) but not from cattle.

The aim of this thesis is to evaluate the suitability of the A. Menarini V-Sight hematology analyzer for the analysis of bovine blood. Validation procedures of measurement systems like automated blood cell counters include in particular the assessment of accuracy and precision. Accuracy refers to the issue if a test method measures correctly what it is designed to measure. Precision answers the question if measurements are reproducible. Only if a hematology analyzer delivers accurate and precise results that are comparable to those obtained from established methods, it can contribute to high quality in hematological veterinary practice.
2. Literature overview

2.1 Hematology in cattle medicine

Hematology deals with analyzing the quantitative and qualitative properties of blood cells. Besides being relevant for diagnosing blood disorders, hematologic profiles are helpful in the diagnosis, progress control, and prognosis of many systemic and organ diseases (SCHWENDENWEIN, 2009). However, in cattle changes in the hematological profile, especially in the white blood picture, might not be as pronounced as in other species even during severe illness. Therefore care should be taken not to formulate a diagnosis or prognosis based on hematologic results alone (GRÜNDER, 2006).

For hematologic analysis, samples must be collected in tubes coated with anticoagulant agents. Anticoagulants prevent clotting and thus yield whole blood samples. Commonly used anticoagulants are ethylenediaminetetraacetic acid (EDTA) and heparin. Samples can be stored up to 24 hours at 4°C. Platelet counts should be conducted within 4 hours after venipuncture (SCHWENDENWEIN, 2009).

As in all species, a certain amount of physiological variability is observed in hematologic profiles of cattle. Reference intervals for bovine hematologic parameters from four sources are summarized in Table 1. Variables that contribute to the width of reference intervals include age, sex, history, stress, diet, body condition, reproductive status, recent activity, hydration, ambient temperature, and altitude (WOOD and QUIROZ-ROCHA, 2010; KRIMER, 2011).
Many commonly used hematology reference intervals originate from research undertaken in the 1960s. GEORGE et al. (2010) compared reference intervals of healthy cows from 1957 to 2006 to account for changes in the genetic profile, environment, diet, management, and diseases of the modern dairy cow. Their main findings include that references for neutrophil counts have increased significantly over this period of time, whereas thresholds for lymphocyte, monocyte, and eosinophil counts as well as hemoglobin concentration have decreased significantly. As reasons for higher neutrophil numbers, genetic selection and decreased prevalence of bovine virus diarrhea (BVD) are suggested. The reduced eosinophil count may be due to decreased exposure to parasites, which is attributed to modern husbandry and parasite control programs. The study concludes that laboratories should update their reference ranges to reflect today’s cattle populations (GEORGE et al., 2010).
2.1.1 Erythrocytes

Red blood cells are biconcave discs and have a diameter of 5.5 μm on average in cattle. Their key function is the transport of oxygen, which is bound to hemoglobin. Hemoglobin is a tetramer composed of four heme units, which contain iron and four globin polypeptides (KRAFT, 2005; BROCKUS, 2011).

Erythropoiesis takes place in the bone marrow parenchyma and takes approximately five days. Pluripotent stem cells give rise to lymphoid and myeloid stem cells. The latter generate progenitor cells including erythroid burst-forming units (EBFU). EBFU differentiate into erythroid colony-forming units (ECFU). ECFU give rise to erythrocytes, via the sequence of rubriblasts, prorubricytes, rubricytes, metarubricytes, and reticulocytes. During maturation, the cell as well as the nucleus becomes smaller. The nucleus is dissolved in the metarubricyte. In cattle, reticulocytes mature in the bone marrow and virtually only erythrocytes are released into the circulation. Erythropoiesis is stimulated by erythropoietin (EPO), Interleukin-3, and colony-stimulating factors. EPO is produced in the kidneys. Bovine RBC have a relatively long lifespan of up to 160 days. Aged erythrocytes are removed from the bloodstream in the spleen, primarily via phagocytosis by macrophages (BROCKUS, 2011). In general, beef cattle breeds have higher RBC counts than dairy cattle and bulls have greater RBC numbers than cows (WOOD and QUIROZ-ROCHA, 2010).

2.1.1.1 Red blood cell parameters

A red blood cell count typically includes the total number of red blood cells (RBC), hematocrit (HCT), hemoglobin (HGB), erythrocyte indices, and occasionally red cell distribution width (RDW).

The total number of RBC is usually measured with automated cell counters. The measurement methods employed by hematology analyzers are discussed in Chapter 2.2.2. Total RBC can also be estimated via a hemocytometer. The hematocrit
indicates the percentage of cellular components of total blood volume. It is thus a relative measure and depends on the number and volume of erythrocytes in relation to plasma volume. The amount of other blood cells is small compared to RBC and is ignored. The method of choice for determining the HCT is centrifugation in a capillary tube. Automated cell counters calculate the HCT from RBC and mean corpuscular volume (MCV). Hemoglobin is typically determined using the cyanmethemoglobin technique or the hemoglobinhydroxylamine complex method as described in Chapter 2.2.2.3 (KRAFT, 2005; BROCKUS, 2011).

Erythrocyte indices include MCV, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). All indices deal with the size and hemoglobin content of red blood cells. MCV represents the average erythrocyte volume and is measured in femtoliters (fL). It is calculated by dividing hematocrit by erythrocyte number. In normocytic cells, MCV and MCHC are within the reference limit. An increased MCV is referred to as macrocytosis and a decreased MCV as microcytosis. MCH describes the HGB content of an average erythrocyte. It is obtained by dividing hemoglobin content by number of erythrocytes and is given in picograms (pg) per red blood cell. The term ‘normochromic’ refers to cells with HGB within the reference limit. Hypochromic cells exhibit HGB below the reference limit, and hyperchromic cells exhibit HGB above the reference limit. MCHC is the mean hemoglobin concentration per erythrocyte volume. It is measured in millimoles per liter (mmol/L) or grams per deciliter (g/dL) (SCHWENDENWEIN, 2009). Automated cell counters calculate the RBC indices from the respective parameters. MCV is measured directly.

The red cell distribution width is the coefficient of variation of the RBC volume distribution (BROCKUS, 2011). It can be presented as a diagram with the cell volume on the x-axis and the number of cells on the y-axis. The presence of macrocytosis or microcytosis results in abnormal peaks and a wider distribution (KRAFT, 2005).
2.1.1.2 Red blood cell abnormalities in cattle

The focus of this chapter is on abnormalities of RBC counts and indices that can be detected by automated hematology analyzers. The detailed discussion of erythrocyte morphology is beyond the scope of this thesis. However, abnormal cell morphology might result in systematic errors during automated cell counting. This will be explained in Chapter 2.2.2.1.

Common indications for red blood cell analysis are clinical anemia or hemorrhage. Reduced RBC concentration, hematocrit and/or hemoglobin are referred to as anemia. Anemia can be categorized into regenerative and non-regenerative anemia according to the bone marrow response. It can be further classified with regard to the cell size (normocytic, macrocytic, microcytic) and hemoglobin concentration (normochromic, hypochromic, hyperchromic) (KRAFT, 2005; BROCKUS, 2011). Causes for regenerative anemia are hemorrhage or hemolysis. With acute blood loss, the RBC parameters are initially within the reference ranges because cells and plasma are lost in the same proportion. Diminished RBC and HGB can be found only after several hours, when fluid in the blood vessels is replaced and dilutes the blood. In cattle, regeneration of erythrocytes begins after approximately two days. It takes weeks to be fully accomplished. In chronic hemorrhage, RBC, HCT, and HGB are decreased while reticulocytes as well as MCV are increased (KRAFT, 2005). In ruminants, only a moderate rise in reticulocytes is observed in responding anemia (BROCKUS, 2011). If regenerative capacity is depleted, chronic bleeding anemia can become non-regenerative. Hemolytic anemia is caused by autoimmune reactions, blood parasites, toxins, or hypo-osmolality (KRAFT, 2005). In cattle, common causes for hemolysis include unsuitable food, such as Brassica spp., rye grass, or onions (BAILY, 1978; MACWILLIAMS et al., 1982). Further factors include copper deficiency or chronic copper intoxication, hypophosphatemia (puerperal anemia) (SMART et al., 1981; MACWILLIAMS et al., 1982; MINERVINO et al., 2009), blood parasites (Babesia spp., Theileria spp., Leptospira spp.) (ZINTL et al., 2003; HOFMANN-LEHMANN et al., 2004), and water intoxication (BIANCA, 1970; GRÜNDER, 2006;
WOOD and QUIROZ-ROCHA, 2010; BROCKUS, 2011). MCH and MCHC are increased in hemolysis (SCHWENDENWEIN, 2009).

Non-regenerative anemia generally occurs in the following combinations: normochromic and normocytic, normochromic and macrocytic, or hypochromic and microcytic. As a consequence of normochromic and normocytic anemia, RBC, HCT and HGB are decreased. It is seen in non-specific disease, e.g. chronic inflammation. It is also observed in connection with EPO deficiency due to chronic renal disease or endocrine disorders, as well as bone marrow depression, certain drugs (e.g. estrogen, chloramphenicol, cytotoxic cancer medication), toxins (e.g. lead poisoning), and neoplasia. As a result of normochromic and macrocytic anemia, RBC, HCT and HGB are reduced while MCH and MCV are increased. This type of anemia occurs with vitamin B12, folic acid, or cobalt deficiency. As a consequence of hypochromic and microcytic anemia, RBC, HCT and HGB as well as all erythrocyte indices are diminished (KRAFT, 2005). In most cases, it is caused by iron deficiency as observed in calves raised solely on milk (GRÜNDEL, 2006; WOOD and QUIROZ-ROCHA, 2010). Microcytes are also observed in copper deficiency and portosystemic shunts (SMART et al., 1981; FORTIER et al., 1996; BROCKUS, 2011).

The term polycythemia is used to describe increased RBC, HCT and/or HGB. Relative and absolute polycythemia are differentiated. Relative polycythemia is triggered by a reduction in plasma volume in dehydrated individuals. Absolute polycythemia is caused by an increased number of erythrocytes. It is observed with renal tumors and cysts, as a consequence of increased EPO production. It occurs further under hypoxic conditions, which can be of physiological (e.g. high altitude) or pathological (e.g. pulmonary or cardiac disorders) origin (KRAFT, 2005; BROCKUS, 2011). A bovine hereditary polycythemia is known in Jersey and Hereford cattle (GRÜNDEL, 2006).
2.1.2 Leukocytes

White blood cells or leukocytes play an important role in immune defense. They include different subpopulations: neutrophil, eosinophil, and basophil granulocytes, monocytes, and lymphocytes. Leukocytes are produced and mature in the bone marrow, and in case of lymphocytes in the lymphoid tissues. The stages of maturation comprise myeloblasts, myelocytes, metamyelocytes, band cells, and segmented cells (WEBB and LATIMER, 2011). In the blood vessels, WBC are transported to the tissues where they are required. The transport represents only a fraction of time in the lifespan of a WBC. Therefore, the number of leukocytes in the blood constitutes only a small percentage of the total population and undergoes wide fluctuation. In the vasculature, a marginal pool and a circulatory pool of neutrophils are differentiated. The marginal neutrophils are attached to the endothelial cells, but detach and join the circulatory pool if blood pressure rises and the blood flow velocity increases. Therefore, every change in blood pressure can result in a change of the amount of leukocytes present in the blood (KRAFT, 2005; WEBB and LATIMER, 2011).

In cattle, lymphocytes predominate, but lymphocyte proportions vary with age. The neonate has more granulocytes than lymphocytes. With approximately three months, the lymphocyte percentage has increased and constitutes up to 80% of the total circulating WBC population. In the adult, the lymphocyte concentration decreases progressively (WOOD and QUIROZ-ROCHA, 2010). Moreover, monocytes decline and eosinophils increase with increasing age (KRAFT, 2005).

Compared to other species, cattle have a small bone marrow reserve for granulocytes. This results initially in a neutropenic rather than a neutrophilic reaction in an early inflammatory process. Only after the speed of granulopoiesis is increased, neutrophilia and a left shift might be observed (GRÜNTER, 2006; WOOD and QUIROZ-ROCHA, 2010). ‘Left shift’ described the increased number of band
neutrophils in the circulation, which can occur after augmented tissue demand and
depletion of the storage reserve of segmented cells (WEBB and LATIMER, 2011).

2.1.2.1 White blood cell parameters

A complete white blood cell count is composed of the total number of leukocytes, the
relative differential blood count, and the absolute differential blood count. Total
leukocytes are usually measured in cells per microliter (μL) or liter blood. The
conventional differential is made by microscopic evaluation of a blood smear.
Hundred white blood cells are differentiated and then the absolute concentrations of
the respective populations are calculated from WBC. The relative differential WBC
count determines the percentage of each subpopulation. Usually, the following
subpopulations are differentiated: neutrophil, eosinophil, and basophil granulocytes,
lymphocytes, and monocytes. Furthermore, band and segmented neutrophils can be
distinguished. The absolute differential count is calculated from the total leukocyte
amount and the relative numbers. Abnormal morphology can be assessed by a
microscopic evaluation of a blood smear (KRAFT, 2005). WBC distribution width can
be assessed analogous to RDW.

2.1.2.2 White blood cell abnormalities in cattle

Analogous to Chapter 2.1.1.2, the focus of this chapter is on abnormalities of WBC
counts rather than on abnormal morphology.

Indications for a leukogram include general health assessments, monitoring of a
disease, or monitoring of therapeutic actions. However, it is seldom possible to come
to a definite diagnosis based solely on a white blood cell count (KRAFT, 2005).
Sequential leukograms can help to establish a prognosis. The return of the
leukogram within normal limits together with clinical improvement can be interpreted
as a favorable sign, whereas a rapid fall in leukocytes within or below the reference
interval without clinical improvement is regarded a grave prognostic sign. Guarded or
poor prognoses should be formulated with persisting neutropenia, a degenerative left shift (WEBB and LATIMER, 2011), or severe persistent leukocytosis. On the other hand, serious disease in cattle might not cause any significant changes in the blood at all. Therefore, care must be taken when a prognosis is formulated in a critically ill patient with an inconspicuous leukogram (GRÜNDER, 2006).

Leukopenia is caused by decreased production, increased tissue demand and consumption combined with marginalization. In general, it is observed in connection with viral infections, circulatory shock, peracute inflammation, cytotoxic substances, as well as hematopoietic stem cell disorders and bone marrow atrophy (KRAFT, 2005; WEBB and LATIMER, 2011). In cattle, stress-induced short-term leukopenia is often associated with metabolic disorders, liver disease, acetonemia, abomasal displacement, and severe infectious disease (e.g. mucosal disease, paratuberculosis, salmonellosis). Panleukopenia, a depression of all WBC subpopulations, is observed in viral disease (e.g. cattle plague, mucosal disease, infectious bovine rhinotracheitis), rickettsiosis, bacterial septicemia, and purulent splenitis (GRÜNDER, 2006).

The mechanisms underlying leukocytosis include increased release from the bone marrow, decreased emigration into the tissues, and a shift of cells from the marginal into the circulatory pool (WEBB and LATIMER, 2011). Physiologic leukocytosis is seen after excitation, fear, exercise, or parturition. Causes for pathological leukocytosis include infectious diseases, non-infective inflammation (traumatic injuries, necrosis, infarction, burns, thrombosis, etc.), endogenous or exogenous intoxication, absorption of proteins, endocrine conditions, central nervous disorders, and anaphylactic shock, leukemia, and leukocyte adhesion deficiencies (KRAFT, 2005; WEBB and LATIMER, 2011). In bovines, leukocytosis is commonly observed with acute endometritis or metritis, acute bacterial mastitis, and foreign body peritonitis (GRÜNDER, 2006; SHELDON et al., 2006).
The reasons for neutrophilia and neutropenia are very similar to those for leukocytosis and leukopenia, respectively (KRAFT, 2005). The most common causes for neutrophilia are acute inflammation and stress (RADOSTITS et al., 2006). Stress is a physiologic respond to a stimulus and belongs to the so-called physiologic leukocyte responses. Additionally, neutrophilia is observed with neoplasia, hemorrhage, and hemolysis (KRAFT, 2005; WEBB and LATIMER, 2011). Cattle neutrophil counts rarely exceed 30,000/μL. Neutrophils higher than 40,000/μL may be a sign of bovine leukocyte adhesion deficiency (BLAD) in Holstein Friesians (SHUSTER et al., 1992; NAGAHATA et al., 1993; WEBB and LATIMER, 2011). Neutropenia can be caused by protozoan, rickettsial, and fungal infections, as well as sepsis and idiosyncratic drug reactions (KRAFT, 2005; WEBB and LATIMER, 2011). A special case is corticosteroid-induced neutrophilia, which is usually accompanied by lymphopenia, eosinopenia, and occasionally monocytosis. It can be a consequence of endogenous corticosteroid release or therapeutic application. In cattle, the response may also be linked to abomasal replacement, ketosis, and dystocia (ZADNIK, 2003; WEBB and LATIMER, 2011; YILDIZ et al., 2011). However, steroid-induced neutrophilia is less explicit in cattle than in other species (WOOD and QUIROZ-ROCHA, 2010). During parturition, leukocytosis with neutrophilia, lymphopenia, and occasionally monocytosis can occur due to endogenous corticosteroid release and stress. In lactating cows the neutrophil concentration is lower compared to non-lactating ones (MEHRZAD et al., 2002; MEGLIA, 2004; KRAFT, 2005; WOOD and QUIROZ-ROCHA, 2010). Furthermore, neutrophil function is poor during the postpartum period, which might be linked to selenium, zinc, copper and vitamin E deficiency (CEBRA et al., 2003).

Conditions commonly associated with eosinophilia include hypersensitivity reactions and parasitic infection (RADOSTITS et al., 2006). Additional causes are neoplasia, certain infections, hypoadrenocorticism, and drug reactions. Eosinophils may also be present in localized lesions such as eosinophilic myositis (WEBB and LATIMER, 2011). Eosinopenia might occur in the early phase of infectious diseases, during stress, Cushing’s syndrome, uremia, acute hemolysis, and after corticosteroid
therapy (KRAFT, 2005). QUIROZ-ROCHA et al. (2010) report a decrease in eosinophils as the only hematological value that changed after calving.

Basophilia has been linked to hyperlipidemia and occasionally to parasitic infections, allergies, and ulcerations (KRAFT, 2005).

Lymphocytosis can occur in the healing phase of infectious diseases, during chronic antigenic stimulation, neoplasia and hypoadrenocorticism (KRAFT, 2005; WEBB and LATIMER, 2011). Infection with bovine leukemia virus results in a persistent lymphocytosis (KENYON and PIPER, 1977; DA et al., 1993). Up to 30% of affected cattle exhibit a leukemic blood profile with WBC counts exceeding 100,000 cells/μL and abnormal lymphocyte morphology (WEBB and LATIMER, 2011). Reasons for lymphopenia include acute stress, acute infection, immune suppression, chronic renal insufficiency, Cushing’s syndrome, and application of corticosteroids (KRAFT, 2005). It is also induced by loss of lymph and disruption of lymph node architecture (e.g. inflammation, neoplasia) (WEBB and LATIMER, 2011).

Monocytosis has been observed during acute stress and in the healing phase of acute as well as chronic infections. It is further caused by hemolysis, hemorrhage, exudative inflammation, suppuration, necrosis, ulceration, Cushing’s syndrome, and corticosteroid therapy (COSTA et al., 2002; KRAFT, 2005; GRÜNDER, 2006). Monocytosis was also found to be a successful marker of bacteremia and bacterial endocarditis. Monocytopenia has so far not been proven to have much clinical relevance (WEBB and LATIMER, 2011). In cattle suffering from the inherited Chédiak-Higashi syndrome, phagocytic capacity of neutrophils and monocytes is impaired (RADOSTITS et al., 2006).

2.1.3 Thrombocytes

Platelets are anuclear cytoplasmic fragments of megakaryocytes. Their average diameter is 3-5 μm. Thrombocyte production is regulated by thrombopoietin and
cytokines. Thrombopoietin is produced constitutively in a steady state, but can surge in severe thrombocytopenia or inflammation. Up to 40% of platelets are sequestered in the spleen and enter circulation in response to epinephrine release (BOUDREAUX, 2011). Bovine platelets survive up to 10 days in peripheral blood (WOOD and QUIROZ-ROCHA, 2010).

Thrombocytes play an important role in hemostasis. In intact blood vessels, platelets circulate predominantly in the marginal pool. Following vascular injury or other procoagulant stimuli, they adhere to exposed subendothelial collagen and aggregate with other platelets to form a plug. This process is referred to as primary hemostasis (KRAFT, 2005). Activated platelets undergo a translocation in membrane proteins, which enables binding of coagulation factors and generation of thrombin. Platelets are equipped with cytoplasmic granules that contain coagulation and growth factors as well as cytokines. Examples include fibrinogen, factor V, von Willebrand factor (VWF), platelet-derived growth factor, adenosine diphosphate (ADP), and serotonin. Release of these factors triggers further platelet activation and thrombus growth. Coagulation ultimately yields in the generation of fibrin, which ensures an effective and stable hemostasis (BOUDREAUX, 2011).

2.1.3.1 Platelet parameters

The following parameters can be estimated: total number of platelets (PLT), mean platelet volume (MPV), thrombocrit (PCT), and platelet distribution width (PDW) (BOUDREAUX, 2011).

The number of platelets can be determined directly, indirectly, automatically, or by quantitative buffy coat analysis. The direct method refers to manual counting of thrombocytes in a hemocytometer. The indirect method relies on estimating the number of platelets in relation to erythrocytes. It is prone to estimation errors. Automatic counts are commonly conducted by applying the impedance method. In quantitative buffy coat analysis, blood components are stained and quantified based
on their unique fluorescence (see Chapter 2.2.2.3) (KRAFT, 2005; BOUDREAUX, 2011).

MPV represents the average platelet size and is measured in fL. It can be determined by impedance or optical counters. The thrombocrit indicates the percentage of platelets per blood volume. PDW reflects range and variation of platelet volume (BOUDREAUX, 2011).

2.1.3.2 Platelet abnormalities in cattle

A count of platelets is indicated with an increased bleeding tendency. This includes clinical signs as petechia, suggillation, hematuria, epistaxis, melena, hematemesis, and hyphema (SCHWENDENWEIN, 2009).

The total number of platelets is influenced by the amount of production, consumption, sequestration, and loss (BOUDREAUX, 2011). An increased platelet count is referred to as thrombocytosis and is observed in connection with chronic blood loss or inflammation (SCHWENDENWEIN, 2009). Physiologically, it occurs as a consequence of epinephrine-induced splenic contraction. Thrombocytosis might result in an increased risk for thrombosis (BOUDREAUX, 2008; BOUDREAUX, 2011). A reduction in platelets is called thrombocytopenia and is found in many diseases. Examples include bone marrow hypoplasia, disseminated intravascular coagulation (DIC), trauma, idiopathic thrombocytopenic purpura, and distribution disorders (e.g. cardiac insufficiency, splenomegaly) (KRAFT, 2005).

Enlarged platelets are associated with accelerated production (WOOD and QUIROZ-ROCHA, 2010). Decreased MPV is observed in bone marrow failure and immune-mediated thrombocytopenia (BOUDREAUX, 2011).

Platelet dysfunctions are inherited or acquired and can be categorized in extrinsic and intrinsic disorders. A well-known extrinsic dysfunction is von Willebrand disease,
where a defect VWF protein results in impaired platelet adhesion (BOUDREAUX, 2011). A hereditary factor XI deficiency is observed in Holstein Friesians (GRÜNDER, 2006). Intrinsic platelet dysfunctions are caused by defects in the thrombocytes themselves. Examples relevant in cattle include Chédiak-Higashi syndrome (observed e.g. in Hereford cattle) and bovine thrombopathia. Acquired disorders result in hypo- or hyperresponsive platelets. Causes for hyporesponsiveness include drugs (e.g. cyclooxygenase inhibitors, β-lactam antibiotics, calcium channel blockers), uremia, DIC, and liver disease. Hyperresponse is triggered by the nephrotic syndrome and infective agents (BOUDREAUX, 2011).

2.2 Automated hematology systems

2.2.1 Automated hematology analyzers in veterinary medicine

Experiments with automatic counting of blood cells started in the 1930s (PINKERTON et al., 1970). In the 1950s and 1960s, Coulter Electronics, Inc. introduced an automatic cell counter based on electrical impedance. At approximately the same time, Technicon Instruments launched an instrument that used dark field optical scanning. In the 1970s, Ortho Diagnostic Systems designed a cell counter based on laser light scatter. Today's analyzers are based on electrical impedance, optical, and centrifugation based systems. Light scatter analysis is the principle in flow cytometry and is an optical system. Cytochemical staining is a refinement for optical systems. Differential lysing can be applied in impedance systems. Centrifugation based systems refer to quantitative buffy coat analysis (MORITZ and BECKER, 2010).

While the first automated cell counters were developed for human medicine, their use in veterinary medicine has increased steadily. Due to the species-specific variations of animal blood, adaptations must be made in most cases. These include primarily calibration of threshold values (MORITZ, 2002). Performance of automated blood cell
counters is considered to be very good for most consolidated parameters, such as WBC and RBC counts or HGB. Performance regarding the measurement of other parameters like components of the leukocyte differential count or platelet counts is regarded to be less satisfactory. This is especially true for small populations, e.g. monocytes, basophils, and eosinophils. Therefore, it is essential to verify results by blood film evaluation (BUTTARELLO and PLEBANI, 2008; MORITZ and BECKER, 2010).

In recent years, smaller and less expensive instruments have been introduced. Today, in-house analyzers are widely distributed in veterinary practices (WENGERRIGGENBACH et al., 2006; MORITZ and BECKER, 2010). They employ quantitative buffy coat analysis, impedance, or laser flow cytometry. Quantitative buffy coat analyzers are economical as well as easy to use and to maintain, but provide only a two-part differential WBC count (lymphocytes/monocytes combined and granulocytes). Impedance counters deliver a three-part differential (granulocytes, lymphocytes, and monocytes). They are moderately priced and deliver fast results, but require the use of reagent fluids, which increases maintenance efforts. Laser flow cytometers provide a five-part differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) and are considered to be most accurate, but are generally more expensive (METZGER, 2006).

2.2.2 General principles and methods

2.2.2.1 Electronic impedance

Impedance counters are based on differences in conductivity between cells and the solution they are suspended in. The suspension is directed through a small aperture between two electrodes. As a cell passes through the aperture, a change in electrical resistance occurs and is measured. The change in resistance is proportional to cell size. Lower and upper thresholds are defined and only pulses that lie between these
thresholds are allocated to a specified cell species (MORITZ, 2002; MORITZ and BECKER, 2010).

Since cells are classified based on size alone by the impedance method, certain abnormalities may result in counting errors. Falsely high counts for leukocytes can result from abnormally large or clumped platelets, nucleated red blood cells, insufficient lysates of erythrocytes or platelets, or excessive Heinz bodies. Clumping of leukocytes can cause falsely low counts of WBC. In addition, small clumps of thrombocytes may be counted as RBC (METZGER, 2006; WEBB and LATIMER, 2011). Blood smears may be examined microscopically to rule out false results due to aggregation or abnormal cell morphology (BOUDREAUUX, 2011).

2.2.2.2 Laser flow cytometry

With the light scatter method, particles can be differentiated as they pass through a beam of light. In modern hematology analyzers, lasers are used as light emission source. Cells are suspended in a sheath fluid and pass through an aperture one by one in a process called hydrodynamic focusing. As cells are illuminated by the light source, they produce absorption and scattered light, which is detected by photodetectors (MORITZ and BECKER, 2010; PICOT et al., 2012). The angle of the scattered light corresponds with certain cell properties: Forward scatter light reflects cell volume and side (90°) scatter internal complexity or granularity. Based on size and granularity, cells can be distinguished and grouped (WEISS and WILKERSON, 2010; PICOT et al., 2012). Light scatter can be measured by two methods. In the darkfield method, the photodetector is kept dark and light is only registered when cells are present. In the absorbance method, the photodetector is illuminated continuously and measures absorbance of light as cells pass through (MORITZ, 2002; MORITZ and BECKER, 2010). Light signals are converted into voltage pulses and analyzed by a computer. In addition, fluorescence emissions can be measured (WEISS and WILKERSON, 2010; PICOT et al., 2012). Results are generally presented as a dot plot exhibiting cell size against absorption (MORITZ, 2002).
Specific applications of flow cytometry include determination of RBC count, indices, and histograms, as well as reticulocyte count and maturation index, erythrocyte-bound immunoglobulin, detailed WBC differentials including immature forms and atypical cells, platelet maturity, anti-platelet antibody, and activated platelets (BUTTARELLO and PLEBANI, 2008; WEISS and WILKERSOHN, 2010).

### 2.2.2.3 Other methods

Other methods employed by automated cell counters include centrifugal or quantitative buffy coat analysis, cytochemical staining, and differential lysing (MORITZ and BECKER, 2010).

In the centrifugal analysis, cells are differentiated based on density and staining. A whole blood sample is centrifuged in a microhematocrit tube. This results in separation of the blood into plasma, the buffy coat including WBC and platelets, and RBC. The cells in the buffy coat stain with acridine orange and emit fluorescent light, which can be measured (METZGER, 2006; MORITZ and BECKER, 2010).

Cytochemical staining involves mainly peroxidase and basophil/lobularity staining and is relevant for WBC differential counts. Neutrophils, eosinophils, and monocytes contain the enzyme peroxidase and can be stained based on its activity. In the basophil/lobularity reaction, all WBC except basophils are stripped of their cytoplasm. Thereafter they are categorized as mononuclear or polymorphnuclear by light scatter. Lysis of RBC is employed for HGB analysis and before WBC counts (MORITZ and BECKER, 2010).

Hemoglobin is generally measured using the cyanmethemoglobin technique or the hemoglobinhydroxylamine complex method. After the respective substance is added, it forms colored complexes with HGB, which can be measured colorimetrically by automated counters (KRAFT, 2005; BROCKUS, 2011).
2.3 Method validation

SHINTON et al. (1982) recommend undertaking a general, safety, and efficiency assessment as the first step.

Moreover, the evaluation of a measurement method typically aims to answer the following questions:

1) Are the test results comparable to established methods? This is addressed by ‘comparability’ or ‘agreement’.
2) Do the test results reflect the true value of the variable? This question is answered by ‘accuracy’.
3) Are the test results precise and repeatable? The answer to this is obtained by calculating ‘precision’ or ‘repeatability’.
4) Are the test results linear for different concentrations? This is answered by ‘linearity’.
5) Are the test results influenced by previous results? ‘Carry-over’ addresses this issue.
6) Does the test correctly identify diseased and non-diseased patients? This is addressed by ‘sensitivity’ and ‘specificity’.

2.3.1 General, safety, and efficiency assessment

At the preliminary stage, general information about the instrument should be gathered, including maintenance and guarantee arrangements, space and service requirements, instruction book, and training requirements. At the technical stage, besides the scientific performance, safety and efficiency should be evaluated. The safety assessment includes electrical, mechanical, microbiological, chemical and radiation issues. The efficiency assessment takes throughput, clinical usefulness, mechanical and electrical reliability, acceptability in practice and costs into account (SHINTON et al., 1982).
2.3.2 Comparability, agreement, and accuracy

SHINTON et al. (1982) define comparability as ‘the ability of the instrument to produce results which agree satisfactorily with the results selected as the reference results’. BLAND and ALTMAN (1999) use the term ‘agreement’ accordingly. They go one step further and propose that ‘the key to method comparison is to quantify disagreements between individual measurements’ (BLAND and ALTMAN, 1999). The mean difference between two methods is referred to as ‘bias’ and describes the systematic error. The smaller the bias the better the agreement (SHINTON et al., 1982; BLAND and ALTMAN, 1986; HANNEMAN, 2008).

‘Accuracy’ addresses the issue if test results reflect the true value of a variable. A numerical approach to accuracy derived from sensitivity and specificity will be introduced in Chapter 2.3.6. Some authors argue that the true value might be different from the value obtained by comparison with an established technique and cannot be determined definitively in hematology (SHINTON et al., 1982; BLAND and ALTMAN, 1986; HANNEMAN, 2008). Other authors use the term ‘accuracy’ or ‘inaccuracy’ interchangeably with ‘agreement’ or ‘bias’ (LUNDORFF JENSEN and KJELGAARD-HANSEN, 2011; KRIMER, 2011). Under the assumption that the reference method delivers true results ‘agreement’ equates ‘accuracy’ (KOEPKE, et al. 1992).

In this thesis, the second definition will be used, because the reference method has been evaluated extensively (see Chapter 3.3) and is considered to deliver results that are close enough to true values for clinical purposes to allow for the equation of ‘comparability’ with ‘accuracy’. This means that if the V-Sight provides test results that agree with the reference method and are sufficiently precise, they will be regarded as correct.
2.3.3 Precision and repeatability

Precision is a measure of repeatability and is defined as ‘the degree to which the same method produces the same results on repeated measurements’. It is also described as ‘the degree to which values cluster around the mean of the distribution of values’ (HANNEMAN, 2008). Precision reflects random error and is independent from agreement (KRAFT, 2005; HANNEMAN, 2008).

Two types of precision can be assessed: For within-run or intra-assay precision, one sample is measured several times. For between-run or inter-assay precision, several samples are measured several times (SHINTON et al., 1982; MORITZ, 2002; KRIMER, 2011). Between-run precision tests generally run over five to ten days (MORITZ, 2002; LUNDORFF JENSEN and KJELGAARD-HANSEN, 2011).

Common indicators of precision are variance, standard deviation (SD) and confidence intervals around a mean. The smaller the result, the more precise is the method (KRAFT, 2005; HANNEMAN, 2008). Another measure of repeatability is the coefficient of variation (\( CV \)). It is calculated as standard deviation \( \times 100\% / \) mean. Usually, a \( CV \) of smaller than 5% is considered acceptable (CAMPBELL et al., 2007; LUNDORFF JENSEN and KJELGAARD-HANSEN, 2011).

2.3.4 Linearity

Linearity is concerned with the effect of dilution. It describes the condition that a test delivers results, which are directly proportional to the actual concentration of a substance in the sample. Most measurement methods are linear between a certain lower and upper limit, which should include at least the physiological range (SHINTON et al., 1982; KRIMER, 2011). However, some variables should not be affected by dilution, e.g. the red cell indices (SHINTON et al., 1982). Linearity is measured by preparing series of dilutions of samples and measuring analyte
concentrations in each dilution (SHINTON et al., 1982; LUNDORFF JENSEN and KJELGAARD-HANSEN, 2011).

2.3.5 Carry-over assessment

Carry-over tests if a test result is influenced by the result of the sample run previously. It is generally calculated according to the method of BROUGHTON et al. (1969) as explained by SHINTON et al. (1982). It is conducted by testing a sample with a high concentration of a certain analyte three times (H\(_1\), H\(_2\), H\(_3\)), followed immediately by testing a sample with a low concentration thrice (L\(_1\), L\(_2\), L\(_3\)). Carry over in % is calculated as \((L_1 - L_3) / (H_1 - L_3) \times 100\%\) (SHINTON et al., 1982).

2.3.6 Sensitivity and specificity

In order to assess test diagnostic efficiency with regard to detect a certain symptom or abnormality, the terms sensitivity and specificity are used. For this purpose, test results are divided into negative and positive, depending on whether they lie within specified limits or not. Sensitivity provides information about the probability of a test to recognize a pathologic outcome or a disease (KRAFT, 2005; LUNDORFF JENSEN and KJELGAARD-HANSEN, 2011). Diagnostic and analytic sensitivity can be distinguished. Diagnostic sensitivity is the percentage of diseased individuals who are recognized as positive for the disorder by the test method. The diagnosis is made by a gold standard method. Diagnostic sensitivity does not solely depend on technical measures but also on the frequency of the detected abnormality in affected individuals. Analytic sensitivity describes the assay method and the smallest amount of an analyte that can be detected (SAAH and HOOVER, 1997; KRIMER, 2011).

Specificity informs about the probability of a test to correctly identify non-diseased patients. Specificity is calculated as the number of negatively tested non-diseased patients divided by the total number of non-diseased patients (KRAFT, 2005; LUNDORFF JENSEN and KJELGAARD-HANSEN, 2011). The optimum is a
sensitivity and specificity of 100%, respectively. However, in reality this is hardly ever achieved (KRAFT, 2005).

The results of a test can be presented in form of a matrix (see Table 2), which accounts for the true situation (patient diseased or non-diseased) and the test outcome (positive or negative).

Table 2: Matrix of test outcomes versus true situation

<table>
<thead>
<tr>
<th>Test positive</th>
<th>Patient diseased</th>
<th>Patient non-diseased</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (true-positive)</td>
<td>FP (false-positive)</td>
<td></td>
</tr>
<tr>
<td>Test negative</td>
<td>FN (false-negative)</td>
<td>TN (true-negative)</td>
</tr>
</tbody>
</table>

Four outcomes are possible: Correct outcomes include the cases that the patient is either diseased and the test positive (situation TP) or non-diseased with a negative test outcome (situation TN). These outcomes are referred to as true-positive (TP) and true-negative (TN), respectively. Incorrect outcomes occur if the patient is either diseased but the test is negative or if the patient is not diseased but the test outcome is positive. These results are denoted as false-negative (FN) and false-positive (FP) (YOUDEN, 1950; BLAND and ALTMAN, 1994; BEAGLEHOLE et al., 1997).

From this matrix, sensitivity can be calculated as TP/(TP+FN) and specificity as TN/(FP+TN). Accuracy can be expressed as (TP+TN)/(TP+FP+FN+TN) and gives the amount of correctly identified outcomes in total (YOUDEN, 1950; BLAND and ALTMAN, 1994; BEAGLEHOLE et al., 1997). YOUDEN (1950) developed an index (Youden index) that takes into account sensitivity and specificity and reflects the number of all correctly identified outcomes. It is calculated as sensitivity plus specificity minus one or TP/(TP+FN) + TN/(FP+TN) -1. The value of the Youden index can range from zero to one. A value close to one indicates a valuable diagnostic test, whereas a value close to zero implies a worthless test (YOUDEN, 1950).
Furthermore, predictive values and prevalence can be derived from the matrix. The positive predictive value gives the percentage of individuals having a positive test result, which are truly affected. The negative predictive value provides the percentage of individuals having a negative test result, which are truly not affected. Prevalence indicates the total percentage of diseased patients within the investigated population. The predictive values are dependent from the prevalence of the disease. The positive predictive value is calculated as TP/(TP+FP), the negative predictive value as TN/(FN+TN) and prevalence as (TP+FN)/(TP+FP+FN+TN) (BLAND and ALTMAN, 1994a; BEAGLEHOLE et al., 1997).

2.4 Statistics for method comparison studies

In method comparison studies, a new analytical method is compared with an established one. If there is sufficient agreement between the two methods, the new method can be used instead of the old one and might even replace it (BLAND and ALTMAN, 1986; HANNEMAN, 2008).

Paired observations are often analyzed using scatter plots, correlation, and regression. However, the usefulness and validity of some of these methods in comparison studies is limited (BLAND and ALTMAN, 1986; CAMPBELL et al., 2007; HANNEMAN, 2008). Therefore, some statistical methods were adapted or specifically designed for the purpose of testing comparability between two methods. The most widely used approaches are the Bland-Altman analysis (ALTMAN and BLAND, 1983; BLAND and ALTMAN, 1986; BLAND and ALTMAN, 1995; BLAND and ALTMAN, 1999) and the Passing-Bablok regression (PASSING and BABLOK, 1983; BABLOK, 1984; BABLOK et al., 1988).

With regard to the sampling process, LUNDORFF JENSEN and KJELGAARD-HANSEN (2011) suggest using 30 to 100 samples that cover the analytical range for comparison studies. Samples for validation studies should include concentrations of
parameters in high, low, and intermediate ranges and ideally cover pathological outcomes as well (SHINTON et al., 1982).

2.4.1 Scatter plots and correlation

A scatter plot is a simple diagram suitable for comparison of two measurement methods. Usually, the results of the established method are plotted against the x-axis and the results of the tested method against the y-axis. It helps to estimate strength and type of association between the two methods and to detect outliers (BLAND and ALTMAN, 1986; HANNEMAN, 2008; WEISS, 2010). The strength of association can be assessed visually by how close the points are relative to each other, or quantitatively by the correlation coefficient ($r$). The association type can be described mathematically by the regression equation (WEISS, 2010). The line of equality or identity line represents the hypothetical optimum situation of the two measurement methods yielding exactly the same results ($x = y$). The closer the results lie to the identity line the better is the agreement (BLAND and ALTMAN, 1986; HANNEMAN, 2008; LUNDORFF JENSEN and KJELGAARD-HANSEN, 2011).

Correlation analysis is widely used in method comparison studies, but is not recommended by many statisticians. Usually, $r$ is calculated and the null hypothesis of no association between the two methods is formulated (ALTMAN and BLAND, 1983; BLAND and ALTMAN, 1986; CAMPBELL et al., 2007). The assumptions underlying a correlation analysis are that the variables are quantitative, the association is almost linear, and the observations are independent. If $r$ is close to 0, association between the two variables is weak. If $r$ is approaching 1, there is a strong positive correlation, and with $r$ near -1, correlation is negative. The interpretation of $r$ should be undertaken carefully, because a formal correlation is not necessarily based on a causal relationship. This might give rise to nonsense or spurious correlation. Examples for nonsense correlation include correlation due to selection of sub-samples, outliers, two inhomogeneous groups, or a third factor (WEISS, 2010). The correlation coefficient in method comparison analysis is often high. For example,
WENGER-RIGGENBACH et al. (2006) reported correlation coefficients of higher than 0.93 for RBC parameters, WBC, and neutrophils in their evaluation of the LaserCyte for dogs and cats. HARRIS et al. (2005) calculated correlation coefficients of over 0.95 for most parameters when comparing two Advia hematology analyzers. However, a high $r$ suggests only a strong linear association and not necessarily good agreement. Further, correlation depends on the range of measurements, with a wide range resulting in a stronger correlation (ALTMAN and BLAND, 1983; BLAND and ALTMAN, 1986; CAMPBELL et al., 2007). Therefore, correlation alone is not a suitable method for method comparison and the correlation coefficient should be interpreted with care.

### 2.4.2 Bland-Altman analysis

BLAND and ALTMAN (1983) describe an approach to method comparison that is based on simple calculations and graphical aids. It accounts for both agreement and repeatability. They suggest a diagram that plots the difference between the test method and the standard method against their mean. This graph allows to assess agreement, discover bias, spot outliers, and identify trends (ALTMAN and BLAND, 1983). The bias is given by the mean difference ($d$) between the two methods. Precision is estimated by the standard deviation ($s$ or SD) of the differences (BLAND and ALTMAN, 1986; HANNEMAN, 2008). Assuming a normal distribution, 95% of differences are estimated to lie in a confidence interval calculated as $d \pm 1.96s$, the so-called ‘limits of agreement’. Even if the differences are not normally distributed, this approach is likely to be successful. Otherwise, a logarithmic transformation can be conducted prior to further analysis. In the Bland-Altman plot, bias and limits of agreement are represented by horizontal lines (ALTMAN and BLAND, 1983; BLAND and ALTMAN, 1986; BLAND and ALTMAN, 1999). An example of a Bland-Altman plot is given in Figure 1.
Since the bias and limits of agreement result from a sample, they are only estimates of the values applying to the whole population. To assess their precision, their 95% confidence can be calculated using the t distribution to find the test statistic ($t^*$) for the respective degrees of freedom. The 95% confidence interval of the bias is then calculated as $d \pm (t^* \times \text{standard error})$. The confidence intervals for the upper and lower limit of agreement are calculated accordingly (BLAND and ALTMAN, 1986).

If the limits of agreement are small enough to be tolerable from a clinical point of view, the methods may be used interchangeably. If a constant bias is observed, $d$ can be subtracted from the new method to achieve agreement (ALTMAN and BLAND, 1983; BLAND and ALTMAN, 1986).

2.4.3 Regression

LUNDORFF JENSEN and KJELGAARD-HANSEN (2011) suggest that a correlation coefficient $r > 0.975$ for data encompassing a small range and $r > 0.99$ for data
encompassing a wide range indicate that simple linear regression delivers useful information. Otherwise, more data should be collected or a different type of regression, such as Passing-Bablok regression should be used. In regression analysis, the intercept of the regression equation is a measure of systematic error and should not differ significantly from 0 whereas the slope is a measure of proportional error and should not differ significantly from 1 (LUNDORFF JENSEN and KJELGAARD-HANSEN, 2011).

PASSING and BABLOK (1983) describe a linear regression analysis for method comparison. It requires no special assumptions regarding the distribution of the samples and the measurement errors. The linear relationship between two measurements is tested and confidence intervals for the intercept \( a \) and the slope \( b \) of the regression line are calculated. The result does not depend on the assignment of the methods to the x- or y-axis. Testing for linearity involves to inspect how the regression equation \( y = a + bx \) fits the data. The intercept \( a \) is a measure of the systematic error between both methods. The hypothesis that \( a = 0 \) is tested and is accepted if the confidence interval contains the value 0. The slope \( b \) reflects the proportional differences between the two methods. The hypothesis that \( b = 1 \) is tested and accepted if the confidence interval contains the value 1. If both hypotheses \( a = 0 \) and \( b = 1 \) are accepted, the methods are regarded as identical. (PASSING and BABLOK, 1983). The assumption of linearity of the data is tested by the cumulative sum linearity (Cusum) test. Residual plots are drawn with method A on the x-axis and method B minus \( F(x) \) on the y-axis. They reveal outliers and deviation from linearity (BILIĆ-ZULLE L., 2011). An example of a Passing-Bablok regression plot is given in Figure 2.
Some authors regard regression as a non-suitable method for method comparison because it ultimately predicts the measurement value obtained by one method from that obtained by another method. However, prediction is not the aim of method comparison (ALTMAN and BLAND, 1983).

Figure 2: Example of a Passing-Bablok regression plot
3. Material and methods

3.1 Blood samples

Blood samples were obtained from 75 dairy cows located at the Teaching and Research Farm (Lehr- und Forschungsgut, LFG) of the Veterinary University of Vienna in Kremesberg, Austria. The use of animals for sampling purpose was discussed and approved by the institutional ethics committee in accordance with Good Scientific Practice guidelines and national legislation.

The sample population consists of Simmental, Brown Swiss, and Holstein Friesian cows of different ages and in different stages of lactation. Cows are housed in large groups in free-range stables with access to outside paddocks. They are fed automatically with silages, hay, and concentrates several times per day and receive water ad libitum. Lactating cows are milked twice daily with milking machines.

In total, 97 blood samples were drawn at four dates; three of which within approximate one-week intervals in August/September (n = 75) and one approximately two months later (n = 22). The sampling took place in the morning hours. For the purpose of blood collection, the animals were confined in the feed fence. Blood was collected by vascular puncture of the coccygeal vein or artery with 20G single use drawing needles (0.90 x 38 mm, Greiner Bio-One, Kremsmünster, Austria) into 9 ml K3-EDTA coated vacuum tubes (Vacuette®, Greiner Bio-One, Kremsmünster, Austria).

75 samples were analyzed with the A. Menarini V-Sight analyzer at the LFG within 2 hours after collection. The samples had room temperature and were mixed thoroughly by hand before analysis. Afterwards they were transported in a cooled box to the Central Diagnostic Unit (Zentrallabor) of the Veterinary University of Vienna. In the Central Diagnostic Unit, the samples were analyzed within 8 hours after collection with the Siemens Advia 2120i (Siemens AG, Erlangen, Germany).
hematology analyzer (see Chapter 3.3). The 22 samples collected at the fourth date were analyzed with the V-Sight only for intra-assay precision and carry-over assessment.

3.2 The A. Menarini V-Sight hematology analyzer

The A. Menarini V-Sight (see Figure 3) is a fully automated, compact hematology analyzer suitable for in-house use. It is produced by Mindray Medical International Ltd. (Mindray), Shenzhen, China. Mindray is a developer, manufacturer, and marketer of medical devices (MINDRAY MEDICAL INTERNATIONAL LIMITED, 2010). The V-Sight is distributed in Europe by A. Menarini Diagnostics International. The head company of the Menarini group is A. Menarini Diagnostics S.r.l, Florence, Italy (A. MENARINI INDUSTRIE FARMACEUTICHE RIUNITE SRL, 2013). Menarini’s subsidiary in Austria is A. Menarini Pharma GmbH, Vienna (A. MENARINI PHARMA GMBH, 2013).

![Figure 3: The A. Menarini V-Sight hematology analyzer (Source: A. MENARINI DIAGNOSTICS INTERNATIONAL, 2012)](image)

The V-Sight weights up to 23 kg and measures 38.6 cm in depth, 32.2 cm in width, and 43.7 cm in height. Space requirements include the space occupied by the analyzer itself as well as space allocation for reagents and service procedures (at least 28 cm at each side), ventilation, and cables (10 cm behind the analyzer). Power
requirements include 100 to 240 of voltage in alternating current (VAC), a frequency of 50 to 60 hertz (Hz), a power of 180 volt ampere (VA) and a 250V T4A fuse. Operating and storage temperature, humidity, and pressure are specified in the manual (SHENZHEN MINDRAY BIO-MEDICAL ELECTRONICS CO. LTD., 2009).

The V-Sight provides 18 blood parameters, including a three-part differential WBC count and three histograms for pre-programmed species (dog, cat, horse, cow, rat, rabbit, and monkey), as well as 12 parameters and three histograms for the species pig, sheep, and buffalo. Furthermore, three user-defined animal species can be analyzed. The 18 parameters delivered by the analyzer, their abbreviations, units, and reference ranges for cattle are listed in Table 3. The A. Menarini V-Sight can analyze up to 25 samples per hour. It is equipped with a keypad, a display, and a printer. Up to 1,000 sample results can be stored for further reference (SHENZHEN MINDRAY BIO-MEDICAL ELECTRONICS CO. LTD., 2009; MENARINI DIAGNOSTICS INTERNATIONAL, 2012).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Abbreviation</th>
<th>Unit</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes</td>
<td>WBC</td>
<td>$10^9$/L</td>
<td>5.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Total lymphocytes</td>
<td>Lymph#</td>
<td>$10^9$/L</td>
<td>1.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Total monocytes</td>
<td>Mon#</td>
<td>$10^9$/L</td>
<td>0.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Total granulocytes</td>
<td>Gran#</td>
<td>$10^9$/L</td>
<td>2.3</td>
<td>9.1</td>
</tr>
<tr>
<td>Lymphocyte percentage</td>
<td>Lymph%</td>
<td>%</td>
<td>20.0</td>
<td>60.3</td>
</tr>
<tr>
<td>Monocyte percentage</td>
<td>Mon%</td>
<td>%</td>
<td>4.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Granulocyte percentage</td>
<td>Gran%</td>
<td>%</td>
<td>30.0</td>
<td>65.0</td>
</tr>
<tr>
<td>Total erythrocytes</td>
<td>RBC</td>
<td>$10^{12}$/L</td>
<td>5.00</td>
<td>10.10</td>
</tr>
<tr>
<td>Hemoglobin concentration</td>
<td>HGB</td>
<td>g/L</td>
<td>90</td>
<td>139</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>HCT</td>
<td>%</td>
<td>28.0</td>
<td>46.0</td>
</tr>
<tr>
<td>Mean corpuscular volume</td>
<td>MCV</td>
<td>fl</td>
<td>38.0</td>
<td>53.0</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin</td>
<td>MCH</td>
<td>pg</td>
<td>13.0</td>
<td>19.0</td>
</tr>
<tr>
<td>MCH concentration</td>
<td>MCHC</td>
<td>g/L</td>
<td>300</td>
<td>370</td>
</tr>
<tr>
<td>Red cell distribution width</td>
<td>RDW</td>
<td>%</td>
<td>14.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Total platelets</td>
<td>PLT</td>
<td>$10^9$/L</td>
<td>120</td>
<td>600</td>
</tr>
<tr>
<td>Mean platelet volume</td>
<td>MPV</td>
<td>fl</td>
<td>5.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Platelet distribution width</td>
<td>PDW</td>
<td>%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thrombocrit</td>
<td>PCT</td>
<td>%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Among these parameters, total leukocytes, erythrocytes, platelets, and hemoglobin concentration are directly measured. The histograms are derived from raw-data,
which are generated by direct measurement. The percentages for the leukocyte subpopulations and MCV, RDW, MPV, and PDW are derived from the histograms. The total values for lymphocytes, monocytes, and granulocytes are calculated. The analyzer is programmed with default reference ranges. It will flag results that lie above (H = high) or below (L = low) the reference limits. The histograms show cell distributions for RBC, WBC, and PLT (SHENZHEN MINDRAY BIO-MEDICAL ELECTRONICS CO. LTD., 2009). The x-axis represents the cell volume and the y-axis cell number. An example of a printout of the V-Sight is shown in Figure 4.

Figure 4: Sample printout of the V-Sight

Each analysis cycle consists of sample aspiration, dilution, mixing, and measurement. With whole blood samples, 13 μL are aspirated. The V-Sight uses specific reagent solutions, as specified by Shenzhen Mindray Bio-Medical Electronics Co. Ltd. The reagents include a diluent, rinse, lyse, probe cleaner, and E-Z cleaner solution. The diluent is designed to dilute the blood samples and provides an environment similar to blood plasma. It separates the blood cells, which facilitates the entry of single cells in the aperture for cell counting. The rinse is formulated to flush the tubes and to form a meniscus during measurement cycles. The lyse breaks down the RBC walls, releases the hemoglobin, and converts it to a complex. Erythrocytes must be destroyed before WBC counting, because RBC outnumber WBC by an approximate factor of 1,000 and might interfere with the counting process. Volumetric metering is employed to obtain an exact sample volume, which is essential for accurate cell counting. The metering unit consists of a tube with two optical sensors. The distance between the sensors determines the precise sample volume. The probe cleaner is an alkaline, and the E-Z cleaner an enzyme-based cleaning solution. They
are designed to clean the fluidic lines and bath (SHENZHEN MINDRAY BIO-MEDICAL ELECTRONICS CO. LTD., 2009).

The measurement methods used are the impedance method for determining WBC, RBC, and PLT data and the colorimetric method for determining HGB. The impedance method (see also Chapter 2.2.2.1) is based on the change in electrical resistance as a blood cell passes through the aperture with a diameter of 80 micrometers (μm) between two electrodes. This generates an electrical pulse, the amplitude of which is proportional to the particle’s volume. The number of pulses gives the number of blood cells of a certain volume that has passed through the electrodes. For the colorimetric method, lyse is added to release the hemoglobin from the RBC and to convert it to a complex that can be measured at a wavelength of 525 nanometers (nm). The extinction of a monochromatic light beam of 525 nm is recorded by a photo sensor and converted into hemoglobin concentration (SHENZHEN MINDRAY BIO-MEDICAL ELECTRONICS CO. LTD., 2009).

For the evaluation of the V-Sight for canine, feline, and equine blood, over 100 samples per species were analyzed by SCHWENDENWEIN (2010) in the Central Diagnostic Unit of the Veterinary University of Vienna. As reference, the Advia 120 (Siemens, Austria) and microscopic blood film evaluation were employed. Analyzed parameters include WBC with a three-part differential count, RBC parameters, platelets, as well as common pathologies. Precision was evaluated by calculating coefficients of variation. Method comparison was performed by correlation analysis, Passing-Bablok regression and analysis of difference plots (SCHWENDENWEIN, 2010). The results of the evaluation conducted by SCHWENDENWEIN (2010) are discussed in section 5.2.

3.3 Reference method: The Advia 2120i hematology analyzer

As reference method, the samples were analyzed in the Central Diagnostic Unit at the Veterinary University of Vienna with the hematology analyzer Advia 2120i
(Advia). Blood smears were prepared using the Bayer Hematek Stain Pak (Bayer AG, Leverkusen, Germany) and stored for further reference. In case of deviations of more than 25% from the reference range and defined abnormalities in the scattergrams, blood smear samples were examined microscopically by an experienced laboratory assistant to verify the result.

The Advia analyzers are based on flow cytometry and use light scatter, differential lysis and staining. They provide a complete blood cell count including a five-part WBC differential (MORITZ and BECKER, 2010). The predecessor model of the Advia 2120i, the Advia 120, was evaluated in detail by MORITZ (2002). It was approved to be suitable for veterinary routine diagnostics for several species including cattle (MORITZ, 2002). The Advia 2120 builds on the technology of the Advia 120, but uses a cyanide-free method for hemoglobin measurement, has a new user interface, and can analyze other fluids besides blood. HARRIS et al. (2005) evaluated the clinical performance of the Advia 2120 for human medicine and considered it equivalent to that of the Advia 120 (HARRIS et al., 2005; MORITZ and BECKER, 2010).

3.4 Statistical methods

All datasets (n = 97) were compiled from the V-Sight and Advia printouts by hand into the Microsoft Excel (Microsoft Excel® for Mac 2011, Version 14.2.2, Microsoft Co., Montrouge, France) program. Microsoft Excel was used to adapt the results from the V-Sight to the units used by the Advia using the conversion factors given in Table 4. Since the Advia measures different granulocyte populations, segmented cells, eosinophils and basophils were added to calculate an equivalent to the category ‘granulocytes’ used by the V-Sight.
Table 4: Advia - Units and conversion factors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit V-Sight</th>
<th>Unit Advia</th>
<th>Conversion factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes</td>
<td>$10^9/L$</td>
<td>$1/\mu L$</td>
<td>$/1,000$</td>
</tr>
<tr>
<td>Total lymphocytes</td>
<td>$10^9/L$</td>
<td>$1/\mu L$</td>
<td>$/1,000$</td>
</tr>
<tr>
<td>Total monocytes</td>
<td>$10^9/L$</td>
<td>$1/\mu L$</td>
<td>$/1,000$</td>
</tr>
<tr>
<td>Total granulocytes</td>
<td>$10^9/L$</td>
<td>$1/\mu L$</td>
<td>$/1,000$</td>
</tr>
<tr>
<td>Total erythrocytes</td>
<td>$10^{12}/L$</td>
<td>$10^9/\mu L$</td>
<td>1</td>
</tr>
<tr>
<td>Hemoglobin concentration</td>
<td>g/L</td>
<td>g/dL</td>
<td>$/10$</td>
</tr>
<tr>
<td>MCH concentration</td>
<td>g/L</td>
<td>g/dL</td>
<td>$/10$</td>
</tr>
<tr>
<td>Platelets</td>
<td>$10^9/L$</td>
<td>$10^3/\mu L$</td>
<td>1</td>
</tr>
</tbody>
</table>

The first three datasets ($n = 75$) were transferred from Microsoft Excel into the PASW (Predictive Analytics Software, Version 17.02, IBM, Armonk, New York, United States) program. Descriptive statistic parameters, such as minimum, maximum, range, mean, median, standard deviation (SD), variance, 95% confidence intervals around the mean, and kurtosis were calculated with PASW for each blood parameter and each method. PASW was also used to construct box plot diagrams and scatter plots to compare the results of the Advia and the V-Sight analyzer for each parameter. Further, a correlation analysis was run by PASW, calculating Pearson’s correlation coefficients between the two methods for each parameter.

In order to set the variation (i.e. range and standard deviation) into context, the V-Sight results were compared with Advia results and with the reference ranges (see Table 3). More specifically, four variation indices were calculated:

1) V-Sight range divided by Advia range (V/A range)
2) V-Sight standard deviation divided by Advia standard deviation (V/A SD)
3) V-Sight range divided by the range of the reference interval (V/RI range)
4) V-Sight 95% confidence interval (mean ± 1.96 standard deviations) divided by the range of the reference interval (V CI / RI range)

The purpose of the variation indices is to compare variation parameters, i.e. range and standard deviation, of the test results with the results of the reference method as well as the reference interval. While a certain variation might be acceptable if the
reference range is very wide, the same variation might cause problems with the interpretation of results if the reference range is narrow. With a variation within a healthy cow population that exceeds the reference range by a high factor, it might be possible that results outside of the reference range arise solely due to the high variation caused by insufficient precision.

Comparability to the reference method and precision, i.e. systematic and random error, were assessed by Bland-Altman analysis and Passing-Bablok regression (n = 75). Bland-Altman plots were constructed with the BiAS (Biometrische Analyse von Stichproben, Version 10.0, Epsilon-Verlag, Frankfurt, Germany) program. Passing and Bablok regression was performed with the MedCalc software (version 12.3.0, MedCalc software, Mariakerke, Belgium). As stated in Chapter 2.3.2, the definitions ‘agreement’ and ‘accuracy’ will be used interchangeably in this thesis, because the Advia is considered to deliver results that are sufficiently close to the ‘true value’.

For the determination of intra-assay precision, two randomly chosen samples were measured ten times each and coefficients of variation were calculated with Microsoft Excel. Inter-assay precision was not determined because repeated measurements must be taken over at least five days (see Chapter 2.3.3); however, whole blood samples cannot be stored more than 24 hours (SCHWENDENWEIN, 2009).

For the evaluation of carry-over effects, three consecutive measurements were performed of high and low results for WBC, RBC, and PLT selected from 22 samples, respectively. These parameters were chosen to represent the three gross fractions of blood. Since repeated measurements have to be made for every parameter, it was not possible due to time and economic reasons to assess carry-over for all parameters. Carry-over was calculated according to the method of BROUGHTON et al. (1969) as explained by SHINTON et al. (1982) (see Chapter 2.3.5). Linearity was not evaluated in this thesis.
For assessment of sensitivity and specificity, the Advia results are assumed to reflect the true situation. Negative test outcomes include all outcomes that fall into the reference interval given for the V-Sight as presented in Chapter 3.2. Positive test outcomes include all outcomes that do not fall into the reference range and are composed of results exceeding and falling short of the reference interval. Sensitivity, specificity, predictive values, and prevalence are calculated as described in Chapter 2.3.6 (n = 75).
4. Results

4.1 General assessment

For clinical purposes, the analyzer is operable with little training. Samples are aspirated directly from the test tube. Data can be entered conveniently via a keypad. Data output occurs via the screen and test results can be printed out on specially formatted paper rolls.

The V-Sight can analyze up to 25 samples per hour (MENARINI DIAGNOSTICS INTERNATIONAL, 2012), which results in a throughput time of approximately 2.4 minutes per sample. In the manual, maintenance procedures are suggested for certain periods. Reagents must be ordered as required. The number of analyses conductible with the remaining reagents is calculated by the software.

The evaluation of costs associated with the operation of the analyzer is not a part of this thesis.

4.2 Statistical and graphical overview of results

Results are based on the 75 bovine blood samples analyzed with the V-Sight and the Advia. Since HCT, MCH, and MCHC are calculated from RBC, HGB, and MPV and PCT is calculated from PLT and MPV, no graphs will be shown for HCT, MCH, MCHC, and PCT in the following chapters. However, all graphs in full size are included in the appendix. An overview over selected statistical parameters is given in Table 5 and Table 6.
Table 5: Range, minimum, and maximum for evaluated parameters of 75 samples by the Advia and the V-Sight

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Range Advia</th>
<th>Range V-Sight</th>
<th>Minimum Advia</th>
<th>Minimum V-Sight</th>
<th>Maximum Advia</th>
<th>Maximum V-Sight</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>/μL</td>
<td>3,360</td>
<td>48,800</td>
<td>2,480</td>
<td>2,000</td>
<td>9,840</td>
<td>50,800</td>
</tr>
<tr>
<td>Lymph#</td>
<td>/μL</td>
<td>3,483</td>
<td>42,200</td>
<td>1,216</td>
<td>500</td>
<td>4,699</td>
<td>42,700</td>
</tr>
<tr>
<td>Mon#</td>
<td>/μL</td>
<td>630</td>
<td>1,100</td>
<td>96</td>
<td>200</td>
<td>726</td>
<td>1,300</td>
</tr>
<tr>
<td>Gran#</td>
<td>/μL</td>
<td>5,199</td>
<td>7,100</td>
<td>746</td>
<td>1,000</td>
<td>5,945</td>
<td>8,100</td>
</tr>
<tr>
<td>RBC</td>
<td>10⁹/μL</td>
<td>2.8</td>
<td>2.1</td>
<td>4.6</td>
<td>4.0</td>
<td>7.4</td>
<td>6.1</td>
</tr>
<tr>
<td>HGB</td>
<td>g/dL</td>
<td>4.0</td>
<td>3.6</td>
<td>8.6</td>
<td>8.0</td>
<td>12.6</td>
<td>11.6</td>
</tr>
<tr>
<td>HCT</td>
<td>%</td>
<td>10.8</td>
<td>10.3</td>
<td>23.0</td>
<td>19.9</td>
<td>33.8</td>
<td>30.2</td>
</tr>
<tr>
<td>MCV</td>
<td>fL</td>
<td>14.3</td>
<td>16.1</td>
<td>41.0</td>
<td>41.5</td>
<td>55.3</td>
<td>57.6</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>5.5</td>
<td>6.1</td>
<td>14.6</td>
<td>16.4</td>
<td>20.1</td>
<td>22.5</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dL</td>
<td>4.3</td>
<td>5.0</td>
<td>34.7</td>
<td>37.2</td>
<td>39.0</td>
<td>42.2</td>
</tr>
<tr>
<td>PLT</td>
<td>10⁹/μL</td>
<td>508</td>
<td>642</td>
<td>67</td>
<td>68</td>
<td>575</td>
<td>710</td>
</tr>
<tr>
<td>MPV</td>
<td>fL</td>
<td>7.0</td>
<td>2.0</td>
<td>5.3</td>
<td>4.3</td>
<td>12.3</td>
<td>6.3</td>
</tr>
<tr>
<td>PCT</td>
<td>%</td>
<td>0.32</td>
<td>0.36</td>
<td>0.06</td>
<td>0.03</td>
<td>0.38</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Table 6: Mean, median, and standard deviation for evaluated parameters of 75 samples by the Advia and the V-Sight

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Mean Advia</th>
<th>Mean V-Sight</th>
<th>Median Advia</th>
<th>Median V-Sight</th>
<th>SD Advia</th>
<th>SD V-Sight</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>/μL</td>
<td>6,108</td>
<td>9,435</td>
<td>6,160</td>
<td>7,700</td>
<td>1,437</td>
<td>7,561</td>
</tr>
<tr>
<td>Lymph#</td>
<td>/μL</td>
<td>2,651</td>
<td>4,825</td>
<td>2,493</td>
<td>3,200</td>
<td>834</td>
<td>6,655</td>
</tr>
<tr>
<td>Mon#</td>
<td>/μL</td>
<td>312</td>
<td>607</td>
<td>303</td>
<td>600</td>
<td>119</td>
<td>223</td>
</tr>
<tr>
<td>Gran#</td>
<td>/μL</td>
<td>3,128</td>
<td>4,003</td>
<td>3,190</td>
<td>3,800</td>
<td>1,120</td>
<td>1,446</td>
</tr>
<tr>
<td>RBC</td>
<td>10⁹/μL</td>
<td>6.0</td>
<td>4.8</td>
<td>5.8</td>
<td>4.7</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>HGB</td>
<td>g/dL</td>
<td>10.5</td>
<td>9.6</td>
<td>10.5</td>
<td>9.5</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>HCT</td>
<td>%</td>
<td>28.3</td>
<td>24.2</td>
<td>28.1</td>
<td>24.1</td>
<td>2.4</td>
<td>2.1</td>
</tr>
<tr>
<td>MCV</td>
<td>fL</td>
<td>47.7</td>
<td>50.3</td>
<td>47.5</td>
<td>50.6</td>
<td>3.1</td>
<td>3.6</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>17.7</td>
<td>19.8</td>
<td>17.8</td>
<td>19.8</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dL</td>
<td>37.2</td>
<td>39.5</td>
<td>37.3</td>
<td>39.6</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>PLT</td>
<td>10⁹/μL</td>
<td>377</td>
<td>459</td>
<td>383</td>
<td>472</td>
<td>108</td>
<td>124</td>
</tr>
<tr>
<td>MPV</td>
<td>fL</td>
<td>7.2</td>
<td>5.1</td>
<td>7.0</td>
<td>5.2</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>PCT</td>
<td>%</td>
<td>0.26</td>
<td>0.24</td>
<td>0.27</td>
<td>0.24</td>
<td>0.66</td>
<td>0.63</td>
</tr>
</tbody>
</table>

For total WBC counts, the mean of the V-Sight results is 9,435 cells/μL and the median is 7,700 cells/μL. For the Advia, mean and median lie closer to each other, with 6,108 and 6,160 cells/μL, respectively. With regard to total WBC counts, range and standard deviation are considerably larger for the V-Sight (48,800 and 7,561 cells/μL) than for the Advia (7,360 and 1,437 cells/μL). This great variation is caused primarily by several very high measurements by the V-Sight, resulting in WBC counts over 30,000 cells/μL (see Figure 6 (a), and Figure 7 (a)).

Reasons for falsely high leukocyte counts include abnormally large or clumped platelets, nucleated red blood cells, insufficient lyses of erythrocytes or platelets, or
excessive Heinz bodies. Blood smears with abnormally high WBC counts were evaluated microscopically, but no evidence of any of these conditions was found.

These high WBC counts were not confirmed by two repeated measurements with the V-Sight for animals number 42 and 48 or by the results obtained from the Advia (see Table 7). Therefore, the four most extreme results were regarded as outliers and analysis was conducted including and excluding outliers, respectively.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Date</th>
<th>V-Sight result</th>
<th>Advia result</th>
<th>V-Sight repeat 1</th>
<th>V-Sight repeat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>22/08/12</td>
<td>31,800</td>
<td>5,930</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>22/08/12</td>
<td>34,300</td>
<td>3,880</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td>04/09/12</td>
<td>50,800</td>
<td>4,870</td>
<td>6,800</td>
<td>6,700</td>
</tr>
<tr>
<td>48</td>
<td>04/09/12</td>
<td>31,500</td>
<td>5,170</td>
<td>5,700</td>
<td>5,800</td>
</tr>
</tbody>
</table>

The adapted statistical parameters for WBC after excluding outliers are presented in Table 8.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Range</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>/μL</td>
<td>21,000</td>
<td>7,876</td>
<td>7,600</td>
<td>3,255</td>
</tr>
<tr>
<td>Lymph#</td>
<td>/μL</td>
<td>13,800</td>
<td>3,400</td>
<td>3,100</td>
<td>2,239</td>
</tr>
<tr>
<td>Mon#</td>
<td>/μL</td>
<td>1,000</td>
<td>587</td>
<td>500</td>
<td>206</td>
</tr>
<tr>
<td>Gran#</td>
<td>/μL</td>
<td>7,100</td>
<td>3,889</td>
<td>3,700</td>
<td>1,387</td>
</tr>
</tbody>
</table>

In order to set the variation into context, the V-Sight results are compared with the Advia results and the reference range. Four variation indices (see Chapter 3.4) are calculated and summarized in Table 9.
Table 9: Variation comparison indices of the V-Sight

<table>
<thead>
<tr>
<th>Parameter</th>
<th>V/A range</th>
<th>V/A SD</th>
<th>V/RI range</th>
<th>V CI / RI range</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC incl. outliers</td>
<td>6.63</td>
<td>5.26</td>
<td>4.44</td>
<td>2.69</td>
</tr>
<tr>
<td>WBC excl. outliers</td>
<td>2.85</td>
<td>2.27</td>
<td>1.91</td>
<td>1.16</td>
</tr>
<tr>
<td>Lymph#</td>
<td>12.12</td>
<td>7.98</td>
<td>5.63</td>
<td>3.48</td>
</tr>
<tr>
<td>Mon#</td>
<td>1.75</td>
<td>1.87</td>
<td>0.85</td>
<td>0.67</td>
</tr>
<tr>
<td>Gran#</td>
<td>1.37</td>
<td>1.29</td>
<td>1.04</td>
<td>0.83</td>
</tr>
<tr>
<td>RBC</td>
<td>0.75</td>
<td>0.83</td>
<td>0.41</td>
<td>0.38</td>
</tr>
<tr>
<td>HGB</td>
<td>0.90</td>
<td>0.89</td>
<td>0.73</td>
<td>0.64</td>
</tr>
<tr>
<td>HCT</td>
<td>0.95</td>
<td>0.88</td>
<td>0.57</td>
<td>0.46</td>
</tr>
<tr>
<td>MCV</td>
<td>1.13</td>
<td>1.16</td>
<td>1.07</td>
<td>0.94</td>
</tr>
<tr>
<td>MCH</td>
<td>1.11</td>
<td>1.08</td>
<td>1.02</td>
<td>0.85</td>
</tr>
<tr>
<td>MCHC</td>
<td>1.16</td>
<td>1.00</td>
<td>0.71</td>
<td>0.56</td>
</tr>
<tr>
<td>PLT</td>
<td>1.26</td>
<td>1.15</td>
<td>1.34</td>
<td>1.01</td>
</tr>
<tr>
<td>MPV</td>
<td>0.29</td>
<td>0.42</td>
<td>0.50</td>
<td>0.49</td>
</tr>
<tr>
<td>PCT</td>
<td>1.13</td>
<td>0.95</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n = 75, except WBC excl. outliers (n = 71)
n/a = not available (no reference interval for PCT given)
V/A range = V-Sight range / Advia range
V/A SD = V-Sight standard deviation / Advia standard deviation
V/RI range = V-Sight range / the range of the reference interval
V CI / RI range = V-Sight 95% confidence interval / range of the reference interval

In addition, the variation of the V-Sight results relative to the Advia results and the reference interval is presented graphically for selected parameters in Figure 5, Figure 12, Figure 13 and Figure 19. The left two vertical bars represent the Advia and the V-Sight range, respectively with the horizontal bars indicating the medians. The right vertical bars stand for a 95% confidence interval (equaling mean ± 1.96 standard deviations) around the means, which are represented by the horizontal bars. The dashed lines indicate the upper and lower limits of the reference interval.

Including outliers, the V-Sight range for WBC is 6.6 times as high as that of the Advia and 4.4 times a high as that of the reference interval (see Table 9 and Figure 5). Standard deviation of the V-Sight WBC results is 5.3 times as high as that of the Advia. The interval of mean ± 1.96 standard deviations is 2.7 times wider than the reference range. For lymphocytes, even higher comparison indices are calculated (see Table 9). For example, the V-Sight lymphocyte range is over 12 times as high as that of the Advia and over five times as high as the reference range.
Figure 5: Advia and V-Sight WBC (including outliers) range and median (left) and mean ± 1.96 SD (right) versus the reference interval (dashed lines) (n = 75)

After exclusion of outliers, range and standard deviation of the V-Sight WBC results decrease to 21,000 and 3,255 cell/µL, but are still greater by a factor of 2.9 and 2.3, respectively compared to the Advia results. The range of the V-Sight WBC is still almost twice as high (V/RI range = 1.9) and the 95% confidence interval 1.2 times as high as the reference range. Mean and median lie closer together (7,876 and 7,600 cells/µL) after excluding outliers. The same trend is observed for lymphocytes, for which the range decreases from 42,200 to 13,800 cells/µL and standard deviation from 6,655 to 2,239 cells/µL after excluding the four outliers. For monocytes, mean and standard deviation are 607 and 223 cells/µL and for granulocytes, they are 7,100 and 1,446 cells/µL, respectively. For monocytes and granulocytes, range, mean, median, and standard deviation do not change to the same extent after excluding outliers. This suggests that the outliers originate from the lymphocyte population (compare also Table 5, Table 6, and Table 8 and Figure 8 to Figure 11).

Scatter plots were constructed for each blood parameter with the Advia results on the x-axis and the V-Sight results on the y-axis. For optical reference, an equality line (dashed line) and a regression line (full line) were fitted into the diagrams.
From the scatter plots, a positive correlation between the two methods for WBC counts can be established because the regression line has a positive slope in all graphs for different WBC populations. For total WBC, monocytes, and granulocytes most of the points lie above the identity line (see Figure 6 (b) and Figure 11), which means that the V-Sight results on average are higher than the Advia results. This assumption is confirmed by the higher mean and median of the V-Sight WBC results compared to the Advia. Higher medians and 75% quartiles are also shown by the respective box plots (see Figure 7 (b) and Figure 10).

Figure 6: Scatter plots of WBC counts from the Advia and the V-Sight (a) including (n = 75) and (b) excluding (n = 71) outliers

Figure 7: Box plots of WBC counts from the Advia and the V-Sight (a) including (n = 75) and (b) excluding (n = 71) outliers
Figure 8: Scatter plots of lymphocyte counts from the Advia and the V-Sight (a) including (n = 75) and (b) excluding (n = 71) outliers

Figure 9: Box plots of lymphocyte counts from the Advia and the V-Sight (a) including (n = 75) and (b) excluding (n = 71) outliers

Figure 10: Scatter plots of (a) monocyte and (b) granulocyte counts from the Advia and the V-Sight (n = 75)
For RBC parameters, ranges and standard deviations between the two analyzers differ less than for WBC parameters. The V-Sight results for total RBC counts have a range of $2.1 \times 10^6$ cells/μL and a standard deviation of $0.5 \times 10^6$ cells/μL, which lie below those of the Advia (2.8 and $0.6 \times 10^6$ cells/μL, respectively). Mean and median of RBC counts of the V-Sight are $4.8$ and $4.7 \times 10^6$ cells/μL, respectively. For HGB, the V-Sight gives a range of 3.6, a mean of 9.6, and a standard deviation of 0.8 g/dL. For MCV, a range of 16.1, a mean of 50.3, and a standard deviation of 3.6 fL are calculated. The variation for RBC, HGB, and HCT results is smaller than that of the Advia or the reference range, with all variation comparison indices being smaller than 1.0. The comparisons of the variation indices for MCV, MCH, and MCHC are close to one and do not exceed 1.2 (see Table 9). A graphical comparison of ranges and medians as well as 95% confidence intervals around the mean for RBC and HGB is presented in Figure 12 and Figure 13. Compared to WBC (Figure 5), it can be seen that ranges and confidence intervals of the V-Sight are more similar to those of the Advia and smaller in relation to the reference interval for RBC and HGB.
Figure 12: Advia and V-Sight RBC range and median (left) and mean ± 1.96 SD (right) versus the lower limit of the reference interval (dashed lines) (n = 75)

Figure 13: Advia and V-Sight HGB range and median (left) and mean ± 1.96 standard deviations versus the reference interval (dashed lines) (n = 75)
In the scatter plots for RBC, HGB, and HCT, results cluster quite closely around the regression line (see Figure 14 (a), Figure 15 (a), and Figure 16 (a)). This indicates a higher correlation compared to WBC between the two methods. For RBC, HGB, and MPV, all results lie below the identity line. This means that the V-Sight results are lower on average compared to the Advia. This argumentation is confirmed by the box plots (see Figure 14 (b), Figure 15 (b), and Figure 16 (b)). For the MCV and the other two erythrocyte indices (MCH and MCHC) on the other hand, the results lie above the identity line, indicating that the V-Sight produces higher measurements on average (see Figure 16 (a)).

Figure 14: Scatter plot (a) and box plots (b) of RBC counts from the Advia and the V-Sight (n = 75)

Figure 15: Scatter plot (a) and box plots (b) of HGB results from the Advia and the V-Sight (n = 75)
For platelets, a range of 642, a mean of 459, and a standard deviation of $124 \times 10^3$ cells/$\mu$L are calculated for the V-Sight. The mean MPV is 5.1 fL and the standard deviation is 0.5 fL. For platelet counts, range and standard deviation as well as mean and median are slightly higher for the V-Sight than the Advia results and the majority of results lie above the identity line (see Table 5 and 6 and Figure 17). For MPV on the other hand, range and standard deviation as well as mean and median are lower for the V-Sight than the Advia results and all results lie below the identity line (see Table 5 and 6 and Figure 18).
Figure 18: Scatter plot (a) and box plots (b) of MPV results from the Advia and the V-Sight (n = 75)

The variation comparison indices for PLT are between 1.0 and 1.4 (see Table 9 and Figure 19), while those for MPV are not larger than 0.5 (see Table 9). This means that the variation of the V-Sight MPV results does not make up more than 50% of either the Advia variation or the reference range.

Figure 19: Advia and V-Sight PLT range and median (left) and mean ± 1.96 standard deviations versus the reference interval (dashed lines) (n = 75)
The correlation coefficient ($r$) was calculated for all parameters. The results are presented in Table 10.

Table 10: Correlation analysis for measurement results of the V-Sight and the Advia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Correlation coefficient ($r$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC incl. outliers</td>
<td>0.05</td>
</tr>
<tr>
<td>WBC excl. outliers</td>
<td>0.55</td>
</tr>
<tr>
<td>Lymph#</td>
<td>0.07</td>
</tr>
<tr>
<td>Mon#</td>
<td>0.28</td>
</tr>
<tr>
<td>Gran#</td>
<td>0.68</td>
</tr>
<tr>
<td>RBC</td>
<td>0.96</td>
</tr>
<tr>
<td>HGB</td>
<td>0.96</td>
</tr>
<tr>
<td>HCT</td>
<td>0.94</td>
</tr>
<tr>
<td>MCV</td>
<td>0.95</td>
</tr>
<tr>
<td>MCH</td>
<td>0.86</td>
</tr>
<tr>
<td>MCHC</td>
<td>0.18</td>
</tr>
<tr>
<td>PLT</td>
<td>0.82</td>
</tr>
<tr>
<td>MPV</td>
<td>0.24</td>
</tr>
<tr>
<td>PCT</td>
<td>0.63</td>
</tr>
</tbody>
</table>

$n = 75$, except WBC excl. outliers ($n = 71$)

For all parameters a positive correlation between the two methods was observed. However, for the WBC parameters the correlation coefficient was very low, with $r = 0.05$ for total WBC. This appears to be driven by an especially low correlation of lymphocyte counts ($r = 0.07$). After removing outliers, the correlation coefficient of WBC increases to 0.55. For the erythrocyte parameters RBC, HGB, HCT, and MCV, the correlation is higher with $r > 0.9$ each. MCH and especially MCHC exhibit a lower $r$. For platelet parameters, $r$ is 0.82 for PLT, 0.24 for MPV, and 0.63 for PCT.

### 4.3 Comparability and precision

#### 4.3.1 Bland-Altman analysis

The Bland-Altman analysis evaluates agreement between two measurement methods. Bias is estimated by the mean of differences ($d$) between two methods. Precision is indicated by the standard deviation ($s$ or SD) of differences. In total, 95% of differences are estimated to lie within the limits of agreement ($d \pm 1.96s$). For
further information, see Chapter 2.4.2. In Table 11, the key numerical indicators of the Bland-Altman analysis are summarized for the evaluated parameters.

Table 11: Analysis of differences between the V-Sight and the Advia according to Bland-Altman

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bias (d)</th>
<th>Standard deviation (s)</th>
<th>Lower limit of agreement (95%)</th>
<th>Upper limit of agreement (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC incl. outliers</td>
<td>-3.327</td>
<td>7.624</td>
<td>-18.270</td>
<td>11.617</td>
</tr>
<tr>
<td>WBC excl. outliers</td>
<td>-1.704</td>
<td>2.747</td>
<td>-7.087</td>
<td>3.680</td>
</tr>
<tr>
<td>Lymph#</td>
<td>-2.174</td>
<td>6.651</td>
<td>-15.211</td>
<td>10.861</td>
</tr>
<tr>
<td>Mon#</td>
<td>-295</td>
<td>222</td>
<td>-729</td>
<td>140</td>
</tr>
<tr>
<td>Gran#</td>
<td>-875</td>
<td>1.120</td>
<td>-2.981</td>
<td>1.231</td>
</tr>
<tr>
<td>RBC</td>
<td>1.11</td>
<td>0.22</td>
<td>0.68</td>
<td>1.53</td>
</tr>
<tr>
<td>HGB</td>
<td>0.94</td>
<td>0.26</td>
<td>0.44</td>
<td>1.44</td>
</tr>
<tr>
<td>HCT</td>
<td>4.1</td>
<td>0.9</td>
<td>2.3</td>
<td>5.8</td>
</tr>
<tr>
<td>MCV</td>
<td>-2.6</td>
<td>1.3</td>
<td>-4.8</td>
<td>0.3</td>
</tr>
<tr>
<td>MCH</td>
<td>-2.04</td>
<td>0.67</td>
<td>-3.36</td>
<td>-0.73</td>
</tr>
<tr>
<td>MCHC</td>
<td>-2.3</td>
<td>1.3</td>
<td>-4.8</td>
<td>0.2</td>
</tr>
<tr>
<td>PLT</td>
<td>-82</td>
<td>71</td>
<td>-221</td>
<td>56</td>
</tr>
<tr>
<td>MPV</td>
<td>2.0</td>
<td>1.2</td>
<td>-0.3</td>
<td>4.3</td>
</tr>
<tr>
<td>PCT</td>
<td>0.03</td>
<td>0.06</td>
<td>-0.08</td>
<td>0.14</td>
</tr>
</tbody>
</table>

n = 75, except WBC excl. outliers (n = 71)

In the Bland-Altman plots (see Appendix for all diagrams in full size), the mean of the result obtained from the Advia analyzer and the result from the V-Sight is plotted on the x-axis. The y-axis shows the difference between the two methods (Advia result minus V-Sight result). The diagram includes a horizontal line representing the mean difference and two dotted lines representing a 95% confidence interval around the mean difference. The forth, non-horizontal line is a regression line.

For all WBC parameters, negative biases are observed (see Table 11). For total WBC counts including outliers, a bias of -3.327 cells/µL, a standard deviation of differences of 7,624 cells/µL, and limits of agreement from -18,270 to 11,617 cells/µL are calculated. After excluding the four outliers referred to in Chapter 4.2, the bias is reduced to -1.704 cells/µL, the standard deviation to 2,747 cells/µL, and the limits of agreement to -7,087 to 3,680 cells/µL. In the Bland-Altman plot for WBC (see Figure 20 (a) and (b)), the majority of the results are clustered between the zero difference and the mean difference line for mean differences smaller than 1,000 cells/µL on the
x-axis. It can also be observed that the negative bias between the two methods is increasing with higher measurement results. Four results lie below the limits of agreement. This is still the case after removing the four most significant outliers.

Figure 20: Bland-Altman plots of WBC counts (a) including (n = 75) and (b) excluding (n = 71) outliers

The Bland-Altman plot of lymphocytes resembles the pattern observed for total WBC to a large extent (see Figure 21 (a)). For monocyte and granulocyte counts, the bias is -295 and -875 cells/μL and the standard deviation of differences is 222 and 1,120 cells/μL, respectively. The limits of agreement are -729 to 140 cells/μμL for monocytes and -2,981 to 1,231 cells/μL for granulocytes. In the Bland-Altman plots for monocytes and granulocytes (see Figure 21 (b) and (c)), the results are more dispersed than for total WBC and lymphocytes. For granulocytes, five results lie considerably below the limits of agreement.
Figure 21: Bland-Altman plots of (a) lymphocyte, (b) monocyte, and (c) granulocyte counts (n = 75)

A graphical comparison of Bland-Altman analysis results for WBC parameters is provided in Figure 22.
A positive bias is recorded for RBC, HGB, and HCT, and a negative bias for the erythrocyte indices MCV, MCH, and MCHC (see Table 11). For RBC, the bias is $1.11 \times 10^6/\mu L$, the standard deviation of differences is $0.22 \times 10^6/\mu L$, and the limits of agreement are $0.68$ to $1.53 \times 10^6/\mu L$. The positive bias between the two methods is rising with a higher mean of measurement results. For HGB, HCT, and MCV, the bias is $0.94$ g/dL, 4.1%, and -2.6 fL and the standard deviation of differences is $0.26$ g/dL, 0.9%, and 1.3 fL, respectively. The limits of agreement are 0.44 to 1.44 g/dL for HGB, 2.3 to 5.8% for HCT, and -4.8 to -0.3 fL for MCV. Results for MCH and MCHC are presented in Table 11. All results for RBC parameters are relatively wide dispersed (see Figure 23), especially those for HGB and MCV. A few results lie outside of the limits of agreement.
Figure 23: Bland-Altman plots of (a) RBC counts, (b) HGB, (c) HCT, and (d) MCV (n = 75)

A comparison of Bland-Altman biases and limits of agreement for different RBC parameters is presented in Figure 24.
Figure 24: Bland-Altman bias (dark lines) and limits of agreement (grey bars) for RBC parameters (n = 75)

For PLT, the bias is $-82 \times 10^3/\mu\text{L}$, the standard deviation of differences is $71 \times 10^3/\mu\text{L}$, and the limits of agreement are $-221$ to $56 \times 10^3/\mu\text{L}$. For MPV, a bias of 2.0 fL, a standard deviation of 1.2 fL, and limits of agreement of -0.3 to 4.3 fL were recorded.

For platelets, results are more dispersed for lower means, while for MPV the bias increases with higher means (see Figure 25).

Figure 25: Bland-Altman plots of (a) PLT counts and (b) MPV (n = 75)

Bland-Altman results for platelet parameters are compared in Figure 26.
4.3.2 Passing-Bablok regression

Passing-Bablok regression tests the relationship between two measurement methods and provides confidence intervals for the intercept $a$ and the slope $b$ of the regression line ($y = a + bx$). The intercept $a$ reflects systematic, and $b$ proportional differences. The hypotheses that $a = 0$ and $b = 1$ are tested and accepted if the 95% confidence intervals contain the values 0 and 1, respectively (see also Chapter 2.4.3).

Passing-Bablok regression was conducted for all parameters. The results are summarized in Table 12. Results for which the confidence interval for intercept $a$ includes 0 or the confidence interval for slope $b$ includes 1 are highlighted in bold. In the regression plots, results obtained from the V-Sight are plotted against the x-axis and results from the Advia against the y-axis. The outcome does not depend upon the assignment of the methods to x and y. The solid line is the regression line. The dashed lines represent the confidence intervals, and the dotted line the identity line ($x = y$) (see also Chapter 2.4.3). With the exception of MPV, the Cusum test for linearity does not indicate any significant deviation from linearity for all parameters.
Table 12: Passing-Bablok regression analysis of the V-Sight and the Advia results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intercept $(a)$</th>
<th>Slope $(b)$</th>
<th>Confidence interval for intercept $a^*$</th>
<th>Confidence interval for slope $b^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC incl. outliers</td>
<td>1,623</td>
<td>0.63</td>
<td>983 to 2,675</td>
<td>0.49 to 0.73</td>
</tr>
<tr>
<td>WBC excl. outliers</td>
<td>1,168</td>
<td>0.71</td>
<td>635 to 1,900</td>
<td>0.60 to 0.78</td>
</tr>
<tr>
<td>Lymph#</td>
<td>1,076</td>
<td>0.51</td>
<td>523 to 1,528</td>
<td>0.33 to 0.66</td>
</tr>
<tr>
<td>Mon#</td>
<td>71</td>
<td>0.43</td>
<td>-6 to 162</td>
<td>0.27 to 0.60</td>
</tr>
<tr>
<td>Gran#</td>
<td>-16</td>
<td>0.87</td>
<td>-285 to 347</td>
<td>0.77 to 0.95</td>
</tr>
<tr>
<td>RBC</td>
<td>-0.53</td>
<td>1.33</td>
<td>-1.00 to -0.09</td>
<td>1.24 to 1.43</td>
</tr>
<tr>
<td>HGB</td>
<td>-0.98</td>
<td>1.20</td>
<td>-1.77 to -0.16</td>
<td>1.11 to 1.29</td>
</tr>
<tr>
<td>HCT</td>
<td>-0.84</td>
<td>1.20</td>
<td>-2.90 to 1.27</td>
<td>1.12 to 1.29</td>
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<tr>
<td>MCV</td>
<td>3.49</td>
<td>0.89</td>
<td>0.65 to 6.55</td>
<td>0.82 to 0.94</td>
</tr>
<tr>
<td>MCH</td>
<td>-0.72</td>
<td>0.94</td>
<td>-3.23 to 1.78</td>
<td>0.81 to 1.07</td>
</tr>
<tr>
<td>MCHC</td>
<td>2.66</td>
<td>0.88</td>
<td>-17.02 to 14.14</td>
<td>0.58 to 1.38</td>
</tr>
<tr>
<td>PLT</td>
<td>-7</td>
<td>0.85</td>
<td>-62 to 41</td>
<td>0.75 to 0.97</td>
</tr>
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<td>MPV</td>
<td>-10</td>
<td>3.38</td>
<td>-21 to -5</td>
<td>2.31 to 5.50</td>
</tr>
<tr>
<td>PCT</td>
<td>-0.0015</td>
<td>1.15</td>
<td>-0.10 to 0.05</td>
<td>0.92 to 1.54</td>
</tr>
</tbody>
</table>

n = 75, except WBC excl. outliers (n = 71)

* Results, for which the confidence interval for intercept $a$ includes 0 or the confidence interval for slope $b$ includes 1 are highlighted in bold.

The regression equation for WBC including outliers is $y = 1,623 + 0.63x$ and that for WBC excluding outliers is $y = 1,168 + 0.71x$. The respective regression and residual plots can be seen in Figure 27. For WBC including outliers, the 95% confidence interval for $a$ is 983 to 2,675 and the interval for $b$ is 0.49 to 0.73. After excluding outliers, the confidence intervals for WBC are 635 to 1,900 for $a$ and 0.60 to 0.78 for $b$. For WBC including and excluding outliers as well as for lymphocytes, the confidence intervals for $a$ do not include 0 and the confidence intervals for $b$ do not include 1. Thus, the hypotheses that $a = 0$ and $b = 1$ are rejected. This means that significant proportional and systematic difference is present between both methods for these parameters and that the results cannot be regarded as identical.

The regression and residual plots for monocytes and granulocytes are presented in Figure 29 and Figure 30. For monocytes and granulocytes, both confidence intervals for $a$ contain the value 0. The confidence intervals for $b$ do not contain 1. Therefore, for both parameters the hypothesis that $a = 0$ is accepted whereas the hypothesis that $b = 1$ is rejected. This means that no systematic, but proportional difference is detected for monocytes and granulocytes.
Figure 27: Passing-Bablok regression plot and residuals of WBC counts: (a) regression plot and (b) residuals including outliers \(n = 75\) and (c) regression plot and (d) residuals excluding outliers \(n = 71\)

Figure 28: Passing-Bablok (a) regression plot and (b) residuals of lymphocyte counts \((n = 75)\)
The regression equation for RBC is $y = -0.53 + 1.33x$, the equation for HGB is $y = -0.98 + 1.20x$ and that for MCV is $y = 3.49 + 0.89x$ (see Table 12 and Figure 31, Figure 33, and Figure 34). The hypotheses that $a = 0$ and $b = 1$ are rejected for RBC, HGB, and MCV. For HCT, the hypothesis that $a = 0$ is accepted while the hypothesis that $b = 1$ is rejected. Thus, no systematic but only proportional difference is proven for HCT. For MCH and MCHC, the confidence intervals for $a$ contain 0 and those for $b$ contain 1 (see Table 12). It can thus be concluded that both parameters are free...
from proportional and systematic difference and that the V-Sight and the Advia deliver identical results for MCH and MCHC.

Figure 31: Passing-Bablok (a) regression plot and (b) residuals of RBC counts (n = 75)

Figure 32: Passing-Bablok (a) regression plot and (b) residuals of HGB (n = 75)
The regression equation for PLT is $y = -7 + 0.85x$ (see Table 12 and Figure 34). The confidence interval for $a$ is -62 to 41 and contains 0, and the interval for $b$ is 0.75 to 0.97 and does not include 1. Therefore, PLT is assumed to be free from systematic, but not from proportional differences. The equation for MPV is $y = -10 + 3.38x$. Proportional as well as systematic differences for MPV exist between the two methods. Furthermore, the Cusum test predicts a deviation from linearity for MPV and the residuals exhibit a linear relationship (see Figure 35 (b)). For PCT, the regression equation is $y = -0.0015 + 1.15x$. The hypotheses that $a = 0$ and $b = 1$ are accepted, meaning that the results obtained by both methods should be identical.
In conclusion, Passing-Bablok regression observes that the V-Sight measurements can be regarded as identical to those of the Advia for MCH, MCHC, and PCT. No systematic, but proportional differences exist for monocyte and granulocyte counts, HCT, and PLT. For all other parameters, the two methods cannot be regarded as identical.

4.4 Intra-assay precision

In order to estimate intra-assay precision, two randomly chosen samples were measured ten times each. During the first measurement set, two measurements with exceptionally low values for all parameters were observed, which were regarded as outliers. Two additional measurements of the same sample were conducted to replace the outliers. Mean, standard deviation, and coefficient of variation (CV) in % of the two data sets are summarized in Table 13.
Table 13: Intra-assay precision of the V-Sight*

| Parameter | Sample 1** | | | Sample 2 | | |
|-----------|------------|-------------|-------------|-------------|-------------|
|           | Mean       | SD          | CV          | Mean        | SD          | CV          |
| WBC       | 10,340     | 1,540       | 14.9        | 6,650       | 392         | 5.9         |
| Lymph#    | 3,140      | 1,956       | 62.3        | 2,620       | 469         | 17.9        |
| Mon#      | 910        | 152         | 16.7        | 550         | 53          | 9.6         |
| Gran#     | 6,290      | 300         | 4.8         | 3,480       | 148         | 4.2         |
| RBC       | 5.2        | 0.5         | 9.6         | 4.8         | 0.3         | 6.4         |
| HGB       | 9.9        | 0.9         | 9.2         | 8.4         | 0.1         | 1.8         |
| HCT       | 25         | 3           | 10.4        | 21          | 0.3         | 1.2         |
| MCV       | 48         | 0.5         | 1.1         | 45          | 0.2         | 0.5         |
| MCH       | 18.9       | 0.2         | 1.2         | 17.9        | 0.3         | 1.4         |
| MCHC      | 40         | 0.8         | 2.0         | 40          | 0.7         | 1.6         |
| PLT       | 172        | 27.4        | 16.0        | 585         | 19.0        | 3.2         |
| MPV       | 5.2        | 0.1         | 2.2         | 5.1         | 0.1         | 1.3         |
| PCT       | 0.09       | 0.02        | 16.9        | 0.30        | 0.01        | 3.7         |

* 10 repeated measurements of two randomly chosen samples
** Two outlier measurement cycles were replaced by new measurements

Sample 1 has higher means for all parameters except MCHC, PLT, and PCT and higher standard deviations for all parameters except MCH and MPV than sample 2. The CV is also higher for all parameters except MCH and MPV for sample 1 compared to sample 2.

If a CV of smaller than 5% is considered acceptable (see Chapter 2.3.3), then the CV for WBC, lymphocyte, monocyte, and RBC counts is not acceptable for both samples. The CV for granulocyte counts, MCV, MCH, MCHC, and MPV is smaller than 5% for both samples and is therefore acceptable for sample 1 and for sample 2. With regard to HGB, HCT, PLT, and PCT the results for the CV for both samples lead to different conclusions. Whereas the CV for HGB, HCT, PLT, and PCT is larger than 5% and thus not acceptable for sample 1, it is smaller than 5% and thus acceptable for the same parameters of sample 2.

4.5 Carry-over

Carry-over assesses whether a test result is influenced by previous results (see Chapter 2.3.5). It was conducted by testing a sample with a high concentration of a
WBC, RBC, and PLT three times (H₁, H₂, H₃), followed immediately by running a sample with a low concentration of the same parameter thrice (L₁, L₂, L₃). Carry-over was calculated according to the formula given in Chapter 2.3.5. The results are summarized in Table 14.

Table 14: Carry-over of the V-Sight

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WBC (/μL)</th>
<th>RBC (10⁶/μL)</th>
<th>PLT (10³/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₁</td>
<td>7,100</td>
<td>5.82</td>
<td>673</td>
</tr>
<tr>
<td>H₂</td>
<td>7,300</td>
<td>5.82</td>
<td>715</td>
</tr>
<tr>
<td>H₃</td>
<td>7,300</td>
<td>5.74</td>
<td>690</td>
</tr>
<tr>
<td>L₁</td>
<td>4,300</td>
<td>3.15</td>
<td>227</td>
</tr>
<tr>
<td>L₂</td>
<td>4,700</td>
<td>3.14</td>
<td>242</td>
</tr>
<tr>
<td>L₃</td>
<td>4,600</td>
<td>3.11</td>
<td>230</td>
</tr>
</tbody>
</table>

Carry-over (%) = -11.11, 1.52, -0.91 for WBC, RBC, and PLT, respectively.

4.6 Sensitivity and specificity

For assessment of sensitivity and specificity, outcomes were divided into four groups for each parameter (see Table 15): True-positive (TP), false-positive (FP), false-negative (FN), and true-negative (TN). True-positive outcomes include all outcomes that do not fall into the reference range when measured with either analyzer. False-positive outcomes are outcomes, for which the V-Sight gives a result that does not lie within the reference range, but which is within the reference range when determined with the Advia. False-negative outcomes are defined as within the reference interval by the V-Sight but not by the Advia. True-negative outcomes are outcomes that fall for both analyzers into the reference range. An overview of all outcomes is presented in Table 15. Sensitivity, specificity, and related parameters cannot be calculated for PCT, because no reference range is given.
Table 15: Number of true-positive (TP), false-positive (FP), false-negative (FN), and true-negative (TN) outcomes of the V-Sight (n = 75)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>true-positive (TP)</th>
<th>false-positive (FP)</th>
<th>false-negative (FN)</th>
<th>true-negative (TN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>10</td>
<td>6</td>
<td>5</td>
<td>54</td>
</tr>
<tr>
<td>Lymph#</td>
<td>2</td>
<td>12</td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td>Mon#</td>
<td>2</td>
<td>0</td>
<td>34</td>
<td>39</td>
</tr>
<tr>
<td>Gran#</td>
<td>8</td>
<td>0</td>
<td>12</td>
<td>55</td>
</tr>
<tr>
<td>RBC</td>
<td>4</td>
<td>44</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>HGB</td>
<td>3</td>
<td>11</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>HCT</td>
<td>34</td>
<td>36</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>MCV</td>
<td>3</td>
<td>16</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>MHC</td>
<td>13</td>
<td>41</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>MCHC</td>
<td>47</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PLT</td>
<td>2</td>
<td>9</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>MPV</td>
<td>2</td>
<td>22</td>
<td>4</td>
<td>47</td>
</tr>
</tbody>
</table>

Overall, a high number of false-positive outcomes are noted (see Table 15). With the exception of monocytes and granulocytes, false-positive outcomes are present for all parameters. Especially for RBC, HCT, MHC, MCHC, and MPV, the number of false-positives is very high. For monocytes and granulocytes on the other hand, several false-negative outcomes are found. For WBC, lymphocytes, and MPV, false-positive and false-negative outcomes are recorded. In addition, for WBC and MPV, two contradictory outcomes are present: For WBC, the Advia indicates two results as above the reference range whereas the respective V-Sight results fall short of the reference range. For MPV, the situation is reversed in two cases.

From TP, FP, FN, and TN, sensitivity, specificity, accuracy, and the Youden index as well as positive predictive value, negative predictive value, and prevalence are calculated as described in Chapter 2.3.6. The results are presented in Table 16 and Table 17. To facilitate interpretation of the results, they will be grouped into high (>90%), intermediate (50% to 90%) and low (<50%).
Sensitivity is high for all RBC counts and indices as well as platelets (see Table 16). Intermediate sensitivity is recorded for WBC and lymphocytes, and low sensitivity for monocytes, granulocytes, and MPV. However, with regard to the high sensitivity results it has to be considered that a high number of false-positives are recorded for RBC, HCT, MHC, MCHC, and MPV. Specificity is high for monocytes and granulocytes, and intermediate for WBC, lymphocytes, HGB, MCV, PLT, and MPV. Low specificity is calculated for RBC, HCT, MHC, and MCHC. Because of the high number of false-negative outcomes for monocytes and granulocytes, the high specificity results for these parameters should be interpreted with care.

Accuracy calculated from the four-field matrix does not exceed 90% for any analyte (see Table 16). Intermediate accuracy is noted for WBC, lymphocytes, monocytes, granulocytes, HGB, HCT, MCV, MCHC, PLT, and MPV. The parameters RBC and MHC exhibit low accuracy. The Youden index is intermediate for WBC, HGB, MCV, and PLT and low for all other parameters (see Table 16).

The positive predictive value is high for monocytes and granulocytes, intermediate for WBC and MCHC, and low for all other parameters (see Table 17). No low negative predictive values are recorded. Intermediate negative predictive values are present for monocytes and granulocytes. The negative predictive value for MCHC cannot be calculated because it would require division by zero, which is not allowed. All other
parameters exhibit a high negative predictive value. Prevalence is not high for any parameter. Intermediate prevalence is noted for monocytes and MCHC, whereas for all other parameters low prevalence is observed.

**Table 17: Predictive values and prevalence of the V-Sight (n = 75)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>63</td>
<td>92</td>
<td>20</td>
</tr>
<tr>
<td>Lymph#</td>
<td>14</td>
<td>97</td>
<td>5</td>
</tr>
<tr>
<td>Mon#</td>
<td>100</td>
<td>53</td>
<td>50</td>
</tr>
<tr>
<td>Gran#</td>
<td>100</td>
<td>82</td>
<td>27</td>
</tr>
<tr>
<td>RBC</td>
<td>8</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>HGB</td>
<td>21</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>HCT</td>
<td>49</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td>MCV</td>
<td>16</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>MHC</td>
<td>24</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>MCHC</td>
<td>63</td>
<td>n/a</td>
<td>63</td>
</tr>
<tr>
<td>PLT</td>
<td>18</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>MPV</td>
<td>8</td>
<td>92</td>
<td>8</td>
</tr>
</tbody>
</table>
5. Discussion

5.1 Discussion of the V-Sight results for bovine blood

5.1.1 White blood cell counts

The total WBC and lymphocyte measurement results of the V-Sight are characterized by a high variation. Variation, i.e. range and standard deviation should be interpreted relative to the reference. A certain variation might be acceptable with a wide reference range, but lead to interpretation problems with a narrow reference range. For example, a high variation within a healthy cow population might be caused by insufficient precision. This should be kept in mind during interpretation.

For example, the range of the V-Sight results for WBC is 6.6 times higher than that of the Advia and 4.4 times as high as that of the reference interval. Standard deviation of WBC measurements is 5.3 times higher than that of the Advia. This great variation is partly caused by several extremely high measurements, which are regarded as outliers. These outliers originate exclusively from the lymphocyte subpopulation.

However, the reference method did exhibit neither leukocytosis nor lymphocytosis in these cases. Even after removing the four most severe outliers, variation remains high. WBC range excluding outliers remains 2.9 times as high as that of the Advia and 1.9 times as high as that of the reference interval, while standard deviation is higher by a factor of 2.3 versus that of the Advia. An interval of mean ± 1.96 standard deviations versus the reference interval is also higher by the factor 2.7 for WBC including outliers. This high variation should be kept in mind because it influences further analysis. The chance that a WBC measurement by the V-Sight is not within the reference range is increased solely by high variation. This might result in false-positive results. The mean and median of the V-Sight total WBC and lymphocyte results lie above those of the Advia. Almost no correlation is present for WBC including outliers ($r = 0.05$) and lymphocytes ($r = 0.07$). The correlation coefficient is
still low after removal of WBC outliers \((r = 0.55)\). This indicates an unexpectedly weak correlation between the V-Sight and the Advia results.

Bland-Altman analysis calculates a negative bias for WBC (-3,327 cells/µL including outliers and -1,704 cells/µL excluding outliers) and lymphocytes (-2,174 cells/µL). The standard deviation is high and the limits of agreement are very wide and thus only of very limited use for clinical purposes. This conclusion is confirmed by the results of the Passing-Bablok regression, which detects significant proportional and systematic difference for total WBC including and excluding outliers as well as lymphocytes with regard to the reference method. Intra-assay precision, as estimated by the coefficient of variation between two sample sets, is not acceptable for WBC \((CV \text{ of } 14.9\% \text{ in sample 1 and } 5.9\% \text{ in sample 2})\) or lymphocytes \((CV \text{ of } 62.3\% \text{ in sample 1 and } 17.9\% \text{ in sample 2})\). Sensitivity, specificity, and accuracy are intermediate (between 50% and 90%) for WBC and lymphocytes, whereas specificity is higher than sensitivity. The Youden index is 57% for WBC and 33% for lymphocytes.

Overall, it can be concluded that the V-Sight does not deliver identical results to the reference method for total WBC and lymphocytes. Accuracy and precision are poor. The clinical usefulness of results for WBC is severely impaired by great variation, poor precision, and false-positive outliers.

Range and standard deviation of monocytes and granulocytes as well as mean and median of granulocytes are moderately higher for the V-Sight than for the Advia. Mean and median of monocytes are approximately twice as high for the V-Sight. Compared to the range of the reference interval, the range of V-Sight monocyte results is smaller (factor 0.85), while the range of granulocyte results is approximately equal (factor 1.04). The correlation coefficient is 0.28 for monocytes and 0.68 for granulocytes, indicating a weak, but positive association between the two methods. Bland-Altman analysis results in negative biases for both parameters, whereas the results are widely dispersed. Passing-Bablok regression suggests no systematic, but
proportional differences for monocytes and granulocytes. Intra-assay precision is acceptable for granulocytes ($CV < 5\%$ in both samples), but not for monocytes ($CV$ of 16.7$\%$ in sample 1 and 9.6$\%$ in sample 2). Sensitivity is low for monocytes and granulocytes. Specificity is high (100$\%$) for both parameters, however, a high number of false-negative outcomes were observed. Overall, results are more accurate for monocytes and granulocytes than for other WBC parameters. However, proportional differences are present with regard to the reference method. Precision is acceptable for granulocyte, but not for monocyte results. Sensitivity is low and specificity high for both parameters.

The interpretation of carry-over makes sense only for a positive result, because only a high result might influence a low result and not vice versa. For the V-Sight, the negative carry-over for WBC is assumed to be due to the high overall standard deviation for these parameters and does not provide any useful information.

### 5.1.2 Red blood cell counts and indices

The V-Sight provides slightly lower means and medians for RBC, HGB, and HCT than the Advia. Ranges and standard deviations of the V-Sight for these parameters are also lower than those of the Advia. The range of the V-Sight results is smaller than that of the reference interval for all parameters (factor 0.41 for RBC, 0.73 for HGB, and 0.57 for HCT). The 95$\%$ confidence interval of the V-Sight results is also smaller than the reference range. Both the Advia and the V-Sight results for RBC are concentrated at or below the lower end of the reference interval. The correlation between both analyzers is high ($> 0.9$), which is also observed in the scatter plots.

Bland-Altman analysis results in positive biases for RBC ($1.11 \times 10^6$ cells/$\mu$L), HGB (0.94 g/dL), and HCT (4.1$\%$) and standard deviations of differences of $0.22 \times 10^6$ cells/$\mu$L for RBC, 0.26 g/dL for HGB, and 0.9$\%$ for HCT.
Passing-Bablok regression detects significant systematic and proportional differences between the V-Sight and the Advia results for RBC and HGB and proportional differences for HCT. The intra-assay precision $CV$ with 9.6% and 6.4%, respectively is over 5% for RBC in both samples and thus not acceptable. The intra-assay precision results for HGB and HCT are inconclusive, with an acceptable $CV$ in one sample and a non-acceptable $CV$ in the other sample. Carry-over for RBC is 1.52%, which is higher than the cutoff stated in the V-Sight specifications ($\leq 0.5\%$). Sensitivity of all three parameters is high. However, a high number of false-positive outcomes are noted, especially for RBC and HCT. All false-positive outcomes are results that fall short of the reference interval. Specificity is intermediate (85%) for HGB and low ($>50\%$) for RBC and HCT.

The overall conclusion is that agreement and precision for RBC, HGB, and HCT are clearly better than for WBC parameters, but not sufficient to suggest agreement between the V-Sight and the Advia. The results can thus not be considered to exhibit a high accuracy. The Passing-Bablok regression concludes that results for both methods are not identical for RBC and HGB and that proportional differences exist for HCT. Intra-assay precision does not result in an unambiguous acceptable result. However, considering the positive bias, systematic adjustments might be made to achieve a better comparability with the Advia measurements. With regard to the large number of RBC and HCT results that fall at or below the lower end of the reference range and suggest anemia, the conduction of further studies is suggested to evaluate if the reference interval for RBC is still valid. Interestingly, a considerable number of the Advia HCT results also fall short of the reference range, raising the question if the reference interval should be lowered. If supported by additional studies, an adjustment of the reference interval should be considered to reflect up-to-date parameters of the modern Austrian cow population.

For the erythrocyte indices MCV, MCH, and MCHC, range and standard deviation are slightly higher for the V-Sight results, whereas the mean and median are lower than that of the Advia. The variation comparison indices for MCV, MCH, and MCHC
are close to one and do not exceed 1.2. The correlation coefficient is high for MCV and MCH, but low for MCHC. Bland-Altman analysis suggests a negative bias for all parameters and a standard deviation of differences of 1.3 fL for MPV, 0.67 pg for MCH, and 1.3 g/dL for MCHC.

For MCH and MCHC, the regression does detect neither systematic nor proportional differences between the V-Sight and the Advia for both parameters. For MCV on the other hand, systematic and proportional differences are found. The intra-assay precision is acceptable (≤5%) for all erythrocyte indices. As for the other RBC parameters, a high number of false-positive results occur when measuring MCV, MCH, and MCHC with the V-Sight. All false-positive results exceed the reference range. Accordingly, sensitivity is high for all three parameters and specificity intermediate (MCV) to low (MCH, MCHC).

In conclusion, the results of this study suggest that the V-Sight results for MCH and MCHC are characterized by a high accuracy as well as a high precision. Therefore, they can be considered as comparable to those of the Advia. The V-Sight results for MCV are precise, but not comparable to the reference method. With regard to the false-positive results it should be considered that a substantial number of Advia results for MCH and even more so for MCHC exceed the reference interval as well. An increased MCHC and MCH might be caused by hemolysis, which can be induced by pre-analytic conditions. Alternatively, it might suggest that the upper limit of the reference interval is outdated and calls for further research.

5.1.3 Platelet counts and indices

Platelets mean and median of the V-Sight are higher, but MPV and PCT mean and median are lower than that of the Advia. Range and standard deviation of the V-Sight are higher for PLT and PCT, but lower for MPV compared to the Advia. The variation comparison indices are between 1.0 and 1.4 for PLT and less than 0.5 for MPV. The
correlation coefficients for platelet parameters are intermediate for PLT ($r = 0.82$) and PCT ($r = 0.63$) and low for MPV ($r = 0.24$).

Bland-Altman analysis calculated a negative bias (-82 x 10$^3$ cells/$\mu$L) and a standard deviation of differences of 71 for PLT. For MPV and PCT, positive biases are determined. Passing-Bablok regression indicates proportional, but no systematic differences between the V-Sight and the Advia results for PLT. For MPV, a significant deviation from linearity is detected. The regression, which states that significant systematic and proportional difference exists for MPV, must thus be interpreted carefully. PCT is free from both types of differences.

Intra-assay precision is acceptable for MPV ($CV < 5\%$ in both samples) and inconclusive for PLT ($CV$ of 16.0$\%$ in sample 1 and 3.2$\%$ in sample 2) and PCT ($CV$ of 16.9$\%$ in sample 1 and 3.7$\%$ in sample 2). Carry-over is negative for PLT and does therefore not allow interpretation. Sensitivity is high for PLT and low for MPV. Specificity is intermediate for both parameters. For MPV, a high number of false-positive outcomes and also some false-negative outcomes were detected.

In total, PLT is characterized by proportional difference with reference to the Advia and measurements can therefore not be regarded as identical. With regard to precision, the results are inconclusive and might warrant further studies. MPV exhibits a low comparability to the reference method and thus poor accuracy. The non-linear relationship with regard to the reference method could be analyzed in more detail in future studies. Precision is acceptable for MPV. PCT results are comparable to the reference method, implying a high accuracy, but precision is inconclusive.

5.2 Discussion of the V-Sight results for canine, feline, and equine blood

The evaluation of the V-Sight for canine, feline, and equine blood by SCHWENDENWEIN (2010) found precision to be excellent for most canine
parameters. For equine blood, the CV is < 5% for RBC parameters, total WBC, and granulocytes and ≥ 5% for lymphocytes, monocytes, and platelets. For feline blood, a CV of < 5% is calculated for RBC parameters, while WBC and PLT parameters exhibit CVs of ≥ 5%. The CV for platelets in cats is 36%. In total, a CV of < 5% is listed for 9 out of 11 parameters for dogs, 4 out of 11 parameters for cats, and for 6 out of 10 parameters for horses (SCHWENDENWEIN, 2010).

Overall method comparison performance is found to be very good (correlation ≥ 0.95) to good (correlation 0.85 to 0.94) for total WBC, granulocytes, HGB, and HCT. Fair performance (correlation 0.75 to 0.84) is recorded for PLT in all examined species, for MCV in dogs and horses, for RBC in horses, and for lymphocytes in cats. Agreement is poor (correlation < 0.75) for MCHC and RDW (with the exception of dogs) as well as monocytes. Compared to the Advia, good or very good method comparison performance is indicated for 8 out of 11 parameters for canines, 6 out of 11 parameters for felines, and for 6 out of 11 parameters for equines (SCHWENDENWEIN, 2010).

The poor performance with regard to platelet counts is attributed to the small volume difference between PLT and RBC in cats and horses as well as to platelet clumping. MPV performance is thought to be influenced by different cell swelling in different diluents. Poor agreement of monocyte counts is considered to be caused by difficulties to differentiate between monocytes and lymphocytes. Overall, it is recommended to confirm severely abnormal results by microscopic evaluation, however, it is stressed that this is suggested for any hematology analyzer (SCHWENDENWEIN, 2010).

5.3 Overall conclusion

The purpose of this study is to evaluate the suitability of the V-Sight for the analysis of bovine blood for clinical use. From usability's point of view, the A. Menarini V-Sight
hematology analyzer is a practical in-house device, which offers easy handling, quick throughput, and limited size. No safety hazards were recorded during normal use.

The V-Sight provides accurate and precise results for only two out of 13 parameters: MCH and MCHC. PCT results are comparable to the reference method according to Passing-Bablok regression, implying a high accuracy, but precision is inconclusive. Significant proportional difference, but no systematic difference with regard to the reference method was detected for monocytes, granulocytes, HCT, and PLT. For all other parameters, significant proportional and systemic difference is discovered with the Passing-Bablok regression. Therefore, the V-Sight results cannot be regarded as identical with the reference method. This means that the V-Sight does not provide sufficiently accurate results for the important clinical parameters WBC, lymphocytes, RBC, HGB, MCV, and MPV.

A correlation coefficient of $r > 0.9$ was achieved only for 4 out of 13 parameters: RBC, HGB, HCT, and MCV. All other parameters exhibit a surprisingly low correlation, considering that the same sample was analyzed. While a high correlation does not necessarily equal good agreement between two measurement methods, a low correlation coefficient does hint towards poor agreement. In contrast, SCHWENDENWEIN (2010) detected correlation of 0.9 or higher between the V-Sight and the reference method for 8 out of 11 parameters for canine and for 6 out of 11 parameters for feline and equine blood. Fair or poor correlation was found mainly for PLT, MCV, MCHC, RDW as well as monocytes. This suggests that the correlation between the V-Sight and the Advia results for WBC parameters is higher for canine, feline, and equine than for bovine blood. High correlation for all evaluated species is found for RBC (with the exception of horses), HGB, and HCT. MCV correlation is good for bovine and feline blood, but not for canine or equine blood samples. For PLT, fair or poor correlation was calculated for all species.

Intra-assay precision for bovine blood is acceptable ($CV < 5\%$) for 5 out of 13 evaluated parameters (38.4%), namely for granulocytes, MCV, MCH, MCHC, and
MPV. For all other parameters, intra-assay precision is inconclusive or poor. In comparison, SCHWENDENWEIN (2010) calculated a CV of < 5% for 9 out of 11 parameters (81.8%) for canine blood, 6 out of 10 parameters (60%) for equine blood, and 4 out of 11 parameters (36.4%) for feline blood. However, it should be considered that not exactly the same set of parameters was analyzed. Furthermore, the quality of parameters with an acceptable precision might be interesting. Acceptable precision was found for important parameters like total WBC and RBC parameters in dogs and horses, as well as RBC parameters in cats. In contrast, total WBC and RBC counts exhibit poor precision (CV ≥ 5%) in cattle. This suggests that the V-Sight’s precision for key parameters is low for cattle compared to blood from other species.

Carry-over is higher than stated in the V-Sight specifications for RBC. It is negative and thus not meaningful for WBC and PLT, possibly due to high variation of measurement results.

Sensitivity is high (> 90%) for all RBC parameters as well as platelets. Intermediate sensitivity (50% to 90%) is found for WBC and lymphocytes, and low sensitivity (< 50%) for monocytes, granulocytes, and MPV. A high number of false-positive outcomes are recorded for RBC, HCT, MHC, MCHC, and MPV. Specificity is > 90% for monocytes and granulocytes, and intermediate for WBC, lymphocytes, HGB, MCV, PLT, and MPV. Specificity of < 50% is determined for RBC, HCT, MHC, and MCHC. A high number of false-negative outcomes are recorded for monocytes and granulocytes, thus the high specificity results for these parameters should be interpreted with care. Accuracy calculated from the four-field matrix is < 90% for any parameter. Intermediate accuracy is calculated for WBC, lymphocytes, monocytes, granulocytes, HGB, HCT, MCV, MCHC, PLT, and MPV. The parameters RBC and MHC exhibit low accuracy. The Youden index is < 90% for WBC, HGB, MCV, and PLT and < 50% for all other parameters.
Special attention must be directed towards the V-Sight results for total WBC and lymphocyte counts of bovines. Several measurements resulted in abnormally high results, which were neither confirmed by repeated measurements with the V-Sight nor by measurement with the Advia. In the microscopic evaluation of blood smears, no morphologic condition was found that might cause falsely high leukocyte counts. Potential further causes include insufficient lysis of erythrocytes or thrombocytes as discussed by METZGER (2006) or WEBB and LATIMER (2011) or inadequate counting and calculation algorithms in the V-Sight software. Insufficient lysis can be tested by setting up a dilution experiment, which might be done in additional studies.

Falsely flagged leukocytosis might contribute to incorrect diagnosis of a number of conditions associated with elevated leukocytes as described by KRAFT (2005), GRÜNDER (2006), and WEBB and LATIMER (2011). Among them are infectious diseases, other inflammatory conditions (e.g. due to puerperal disease, acute bacterial mastitis, or foreign body peritonitis), intoxication, endocrine conditions, central nervous disorders, anaphylactic shock, leukemia, and leukocyte adhesion deficiencies. To avoid detrimental effects, it is advised to diagnose a disease not based on pathological blood parameters only. Specifically in the case of abnormally high WBC and lymphocytes, repeated measurements should be undertaken when using the V-Sight. Furthermore, a microscopic blood film evaluation should be carried out, as recommended for example by BUTTARELLO and PLEBANI (2008) or MORITZ and BECKER (2010).

Linearity and inter-assay precision as well as an analysis of costs were not part of this study. Intra-assay precision and carry-over were partly inconclusive. These topics might be subject to further research.

For some hematological parameters, the question may be raised if the reference intervals are still valid for modern Austrian dairy cows. This concerns especially the parameters RBC, HCT, MHC, and MCHC. As GEORGE et al. (2010) demonstrated, several hematological parameters have changed significantly within the past fifty
years in North American dairy cows. It might be possible that bovine hematology parameters in Austrian Simmental, Brown Swiss, and Holstein Friesian cows have undergone similar or different changes and that the reference intervals used in local hematology labs do not reflect the current population. Studies to establish up-to-date reference intervals and to compare them with the intervals used currently in Austrian veterinary hematology laboratories might provide an answer to this issue.

A potential weakness of this study is the composition of the sample set. The sample is comprised of dairy cows, the majority of which did not exhibit any signs of clinical disease during the sampling period. Therefore, this assessment of the V-Sight hematology analyzer is based mainly on physiological samples. A more diverse sample set including calves, heifers, steers, and diseased animals might result in a wider range of hematological parameters. Especially the inclusion of samples from diseased animals might help to study the effect of pathological change and thus provide a more general assessment, as suggested by SHINTON et al. (1982) and LUNDORFF JENSEN and KJELGAARD-HANSEN (2011).

A further potential shortcoming includes the choice of the reference method, the Advia 2120i hematology analyzer. Some authors argue that a true gold standard does not exist for hematology (see Chapter 2.3.2). However, the Advia has been evaluated extensively and is currently used as the main diagnostic tool in hematology in the Central Diagnostic Unit of the Veterinary University of Vienna. It is thus considered as a reliable tool for hematologic analysis. Potential insecurities were decreased by microscopic blood smear evaluation in case of major deviations from the reference range. However, it should be kept in mind that failures by the Advia to correctly measure blood parameters cannot be excluded for sure.

Finally, the clinician must decide for himself or herself if the V-Sight hematology analyzer is a suitable tool for his or her practice. He or she should keep in mind that according to this evaluation the V-Sight provides unambiguous accurate and precise results only for MCH and MCHC. For total WBC and lymphocytes, abnormally high
results should be verified by repeated or alternative measurements. With the current settings of the analyzer it is not recommended to use the V-Sight either for research purposes or to base clinical decisions on a sole measurement by the V-Sight.
6. Zusammenfassung


Die Ergebnisse der Parameter für weiße Blutzellen sind durch eine hohe Spannweite und Standardabweichung gekennzeichnet. Auch ohne Berücksichtigung der vier

Für Parameter des roten Blutbilds mit Ausnahme von MCHC besteht eine hohe Korrelation zwischen dem V-Sight und der Referenzmethode. Die Bland-Altman-Analyse ergibt eine positive Verzerrung für RBC (1,11 x 10⁶ Zellen/µL), HGB (0,94 g/dL) und HCT (4,1%) und eine negative Verzerrung für die Erythrozytenindizes. Mittels Passing-Bablok-Regression wurden signifikante systematische und proportionale Unterschiede für RBC und HGB sowie proportionale Unterschiede für HCT ermittelt. Für MCH und MCHC wurden weder systematische noch proportionale Differenzen im Vergleich mit der Referenzmethode festgestellt. Die Intra-Assay-Präzision ist für die Erythrozytenindizes, nicht aber für RBC akzeptabel. Insgesamt liefern die Test- und die Referenzmethode keine identischen Ergebnisse für RBC und HGB und proportionale Unterschiede für HCT. Lediglich die Ergebnisse für MCH und MCHC sind sowohl präzise als auch vergleichbar mit der Referenzmethode.

Die Bland-Altman-Analyse für Thrombozyten-Parameter ergibt eine negative Verzerrung für PLT (-82 x 10³ Zellen/L) und positive Verzerrungen für MPV und PCT. Die Passing-Bablok-Regression ermittelt proportionale Unterschiede für PLT sowie proportionale und systematische Differenzen für MPV. Für PCT stimmen die Ergebnisse von Test- und Referenzmethode überein.
7. Summary

The aim of this thesis is to evaluate the suitability of the A. Menarini V-Sight hematology analyzer (V-Sight) for the analysis of bovine blood. In total, 97 blood samples were obtained from 75 dairy cows located at the Teaching and Research Farm Kremesberg of the Veterinary University of Vienna, Austria. Accuracy was tested by comparison with a reference device (Siemens Advia 2120i) employing Bland-Altman analysis, Passing-Bablok regression, and correlation analysis. Coefficients of variation (CV) were used to estimate intra-assay precision. Further, carry-over, sensitivity, and specificity were used to estimate extrinsic parameters. Analyzed parameters include counts of white blood cells (WBC), lymphocytes, monocytes, granulocytes, red blood cells (RBC), and platelets (PLT), as well as hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), PLT, mean platelet volume (MPV), and thrombocrit (PCT).

The V-Sight provides accurate and precise results for only two out of 13 parameters: MCH and MCHC. PCT results are comparable to the reference method, but precision is inconclusive. Significant proportional difference, but no systematic difference with regard to the reference method was detected for monocytes, granulocytes, HCT, and PLT. For all other parameters, significant proportional and systemic difference was discovered with the Passing-Bablok regression. Therefore, these V-Sight results cannot be regarded as identical with the reference method. Sensitivity is high for all RBC counts and indices as well as PLT and low for monocytes, granulocytes, and MPV. Specificity is high for monocytes and granulocytes, and low for RBC, HCT, MHC, and MCHC. A high number of false-positive outcomes were recorded for RBC parameters and a high number of false-negative outcomes for monocytes and granulocytes. In the following, the method comparison and precision results are presented for WBC, RBC, and PLT parameters in more detail.

The total WBC measurement results of the V-Sight are characterized by a high range and standard deviation. Even after removing the four most severe outliers, variation
remains high. Correlation coefficients between the V-Sight and the reference method for WBC are low. Bland-Altman analysis indicates negative biases for total WBC (-3,327 cells/μL) as well as for each subpopulation. Passing-Bablok regression detects significant proportional and systematic differences compared to the reference method for total WBC and lymphocytes. For monocytes and granulocytes only proportional differences were found. Intra-assay precision is acceptable for granulocytes (CV < 5%), but not for WBC, lymphocytes, or monocytes (CV ≥ 5%). Overall, the V-Sight results for WBC parameters are not comparable with the reference method. Accuracy and precision are especially poor for total WBC and lymphocytes. The clinical usefulness of WBC results is impaired by high variation, poor precision, and false-positive outliers.

For all RBC parameters except MCHC correlation between both analyzers is high (r > 0.9). Bland-Altman analysis results in positive biases for RBC (1.11 x 10^6 cells/μL), HGB (0.94 g/dL), and HCT (4.1%) and a negative bias for the erythrocyte indices. Passing-Bablok regression detects significant systematic and proportional differences for RBC and HGB and proportional differences only for HCT. For MCH and MCHC, the regression detects neither systematic nor proportional differences. The intra-assay precision is acceptable for all erythrocyte indices, inconclusive for HGB and HCT, and not acceptable for RBC. In total, the results for both methods are not identical for RBC and HGB. Proportional differences exist for HCT. MCH and MCHC results are precise and comparable to the reference method.

For platelet parameters, Bland-Altman analysis calculates a negative bias for PLT (-82 x 10^3 cells/L) and positive biases for MPV and PCT. Passing-Bablok regression detects proportional, but no systematic differences for PLT. MPV exhibits both proportional and systematic differences, whereas PCT is free from both types of differences. Intra-assay precision is < 5% for MPV and inconclusive for PLT and PCT.
Disclaimer

The A. Menarini V-Sight hematology analyzer was placed at the disposal of the Section for Herd Health Management, Clinic for Ruminants, Vetmeduni Vienna by the A. Menarini Pharma GmbH, Vienna, Austria, for test use and evaluation. Reagents and printing paper rolls were partly provided free of charge. Besides this, the Section for Herd Health Management has not received any kind of monetary or other compensation for the purpose of evaluating the V-Sight hematology analyzer for bovine blood. The author has conducted this study independently.
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MINERVINO, A.H.H., BARRETO, R.A., FERREIRA, R.N.F., RODRIGUES, F.A.M.L.,


Appendix

1. Box plots

WBC including outliers

WBC excluding outliers
Lymphocytes including outliers

Lymphocytes excluding outliers
2. Scatter plots

WBC including outliers

WBC excluding outliers
Lymphocytes including outliers

Lymphocytes excluding outliers
3. Bland-Altman plots

**WBC including outliers**

**WBC excluding outliers**
4. Passing-Bablok regression and residual plots

WBC including outliers
WBC excluding outliers
5. Variation comparison plots

### WBC including outliers: means +/- two standard deviations vs. reference interval

<table>
<thead>
<tr>
<th>cells/μL</th>
<th>Advia range and median</th>
<th>V-Sight range and median</th>
<th>Advia mean ± 1.96 SD</th>
<th>V-Sight mean ± 1.96 SD</th>
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### WBC excluding outliers: means ± 2 standard deviations vs. reference interval

<table>
<thead>
<tr>
<th>cells/μL</th>
<th>Advia range and median</th>
<th>V-Sight range and median</th>
<th>Advia mean ± 1.96 SD</th>
<th>V-Sight mean ± 1.96 SD</th>
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</table>
Lymphocytes: means ± 2 standard deviations vs. reference interval

Monocytes: means ± 2 standard deviations vs. reference interval
Granulocytes: means ± 2 standard deviations vs. reference interval

```
cells/μL
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
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<thead>
<tr>
<th>Advia range and median</th>
<th>V-Sight range and median</th>
<th>Advia mean ± 1.96 SD</th>
<th>V-Sight mean ± 1.96 SD</th>
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</table>

RBC: means ± 2 standard deviations vs. reference interval

```
10^6/μL
11
10
9
8
7
6
5
4
3
```

| Advia range and median | V-Sight range and median | Advia mean ± 1.96 SD | V-Sight mean ± 1.96 SD |
HGB: means ± 2 standard deviations vs. reference interval

![Graph showing HGB values with reference intervals and comparison between Advia and V-Sight](image)

Advia range and V-Sight range and median

Advia mean ± 1.96 SD

V-Sight mean ± 1.96 SD

HCT: means ± 2 standard deviations vs. reference interval

![Graph showing HCT values with reference intervals and comparison between Advia and V-Sight](image)

Advia range and V-Sight range and median

Advia mean ± 1.96 SD

V-Sight mean ± 1.96 SD
**MCV: means ± 2 standard deviations vs. reference interval**

- **fL**
  - Advia range and median
  - V-Sight range and median
  - Advia mean ± 1.96 SD
  - V-Sight mean ± 1.96 SD

**MCH: means ± 2 standard deviations vs. reference interval**

- **pg**
  - Advia range and median
  - V-Sight range and median
  - Advia mean ± 1.96 SD
  - V-Sight mean ± 1.96 SD
MCHC: means ± 2 standard deviations vs. reference interval

<table>
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<th>g/dL</th>
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<th>V-Sight range and median</th>
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PLT: means ± 2 standard deviations vs. reference interval

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<th>V-Sight range and median</th>
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MPV: means ± 2 standard deviations vs. reference interval

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