Accuracy of Masimo Radical-7® Pulse CO-oximetry™ in Anaesthetized Dogs

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Abstract

Total haemoglobin concentration tHb, arterial haemoglobin saturation (SaO_2), and arterial oxygen content (CaO_2) are indicative of a patient's ability to transport oxygen (O_2) and can be used to guide clinical decisions. Laboratory-based methodologies such as the haematology analyser, laboratory CO-Oximetry, and point-of-care analysers have been used to assess these values, but despite being accurate all these instruments require a blood sample, allow only intermitted monitoring, are operator-dependent, and introduce a delay in obtaining results. Pulse CO-oximetry, by combining the principles of CO-Oximetry with pulse oximetry enables non-invasive measurements of the Hb (SpHb) and derived values. Whilst the Masimo pulse CO-oximeter has given the approval by the Food and Drug Administration (FDA) and European Medical Agency (EMA), there is still debate within the scientific literature regarding its accuracy. In a recent version of the pulse CO-oximeter software, a so-called *in-vivo* adjustment that allows initial calibration of the SpHb against a measured laboratory value has been introduced with the intent to increase accuracy (Miyashita et al. 2014; Frasca et al. 2015; De Rosa 2020).To date, in veterinary medicine only three studies have investigated the performances of pulse CO-oximetry, but none of them have investigated the accuracy of SpHb after *in-vivo* adjustment. With the hypotheses that *in-vivo* adjustment would increase the accuracy of subsequent SpHb measurements, the aim of this thesis was to assess the agreement of pulse CO-oximeter derived values of tHb [H], CaO_2 and SaO_2, using an optical fluorescence-based blood gas analyser and oximeter (VetStat®) as the reference method. This thesis hypothesises that the accuracy of SpHb and SpO_2 will be influenced by perfusion index (PI), mean arterial pressure (MAP) and tongue thickness. Furthermore, clinical significance and trending accuracy were tested with error grid and four quadrant plot analysis. A total of 39 data pairs of tHb were obtained before *in-vivo* adjustment in as many dogs. The mean [Hb]-SpHb difference was -2.7 g dL^{-1} with limit of acceptance (LoA) of -4.9 to -0.5 g dL^{-1}. After *in-vivo* adjustment from the same dogs, 104 data pairs were obtained; the mean [Hb]-SpHb difference, after *in-vivo* adjustment, was -0.2 g dL^{-1} with LoA of -1.1 to 0.6g dL^{-1}. The mean SaO_2-SpO_2 difference was 0.86% with LoA of -0.8 to 2.5% and between CaO_2 -SpOC was 0.66 ml $dL^{-1}$with LoA of -2.59 to 3.91 ml dL^{-1}. Zone A of the error grid encompassed approximately 98% of data pairs for SpHb. The concordance rate for consecutive changes in SpHb and [Hb] performed with four quadrant plot analysis was 92.6%. Before *in-vivo* adjustment, pulse CO-oximetry derived values overestimated the spectrophotometric-based blood gas analyser [Hb] and CaO_2 values. Following *in-vivo* adjustment, the accuracy, precision, and LoA markedly improved. The accuracy of SpHb and SpO_2 were not influenced by PI, MAP and tongue thickness and pulse CO-oximetry, after *in-vivo* adjustment, adequately tracked the changes of Hb within the time confirming a good trending accuracy. Furthermore, the Masimo's performance was evaluated in dogs referred to the University of Glasgow Small Animal Hospital for a variety of emergency surgeries and presented in hypovolemic states, and/or acute haemorrhagic states. The findings from our observational study shown an acceptable [Hb]-SpHb difference, and a consistent fall in SpHb values during bleeding episodes. This finding may support the use of pulse CO-oximetry devices as an intraoperative starting point for deciding when to perform an invasive tHb measurement. Nevertheless, in all patients receiving synthetic colloids and/or vasoactive drugs (noradrenaline infusion) the increase in [Hb]-SpHb difference suggests that values displayed by the Masimo Radical-7 under these circumstances should be considered carefully and always confirmed by an invasive blood sample. In conclusion, pulse co-oximetry and SpHb monitoring, after *in-vivo* adjustment, cannot completely replace invasive measurements, but show definite promise for use during surgical procedures.

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4Q	Four-Quadrant Concordance Plot
AAGBI	Association of Anaesthetists of Great Britain and Ireland
ABG	Arterial blood gas
B&A	Bland and Altman
CA	California
CaO_2	Arterial oxygen carrying capacity / arterial oxygen content
CC	Correlation Coefficient
CD	Coefficients of Determination
CO	Carbon monoxide
CO_2	Carbon dioxide
COHb	Carboxyhaemoglobin
dL	Decilitre
EGA	Error Grid Analysis
***F* HbO_2**	Fractional oxyhaemoglobin
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
Hb	Haemoglobin
HbO_2	Oxyhaemoglobin
HHB	Deoxyhaemoglobin
ICU	Intensive care unit
ICSH	International Committee for Standardization in Hematology
IR	Near infrared light
L	Litre/s
LFVR	Limited fluid volume resuscitation
LoA	Limit of acceptance
MetHb	Methaemoglobin
ml	Millilitre
O_2	Oxygen
PaO_2	Arterial partial pressure of oxygen
$PaCO_2$	Arterial partial pressure of carbon dioxide
PI	Perfusion index
PO_2	Partial pressure of oxygen
POC	Point of care
PRBCs	Packed Red Blood Cells
PVI	Plethysmography variability index
Q	Cardiac Output
RBC/RBCs	Red blood cell/s
R	Red light
r	Correlation Coefficient
r^2	Coefficient of determination
R	Relaxed haemoglobin structure
SaO_2	Haemoglobin arterial oxygen saturation
SD	Standard Deviation
$SpCaO_2$	Pulse CO-oximetery based CaO_2
SpHb	Pulse CO-oximetry base Hb concentration
SpO_2	Peripheral haemoglobin oxygen saturation
STP	Standard Temperature Pressure
T	Temperature
T	Tense haemoglobin structure
tHb	Total Haemoglobin Concentration
USA	United States of America
VO_2	Oxygen consumption
yrs	years

Declaration

I, Hamaseh Tayari, by submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Printed name: Dr Hamaseh Tayari

Signature: ……. Date: ……21/10/2021……

List of Publications

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Tayari H, Flaherty D, Yamaoka TT, Auckburally A. Assessment of pulse co-oximetry technology after in vivo adjustment in anaesthetized dogs. Vet Anaesth Analg. 2021

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BACKGROUND

1 INTRODUCTION

1.1.1 Haemoglobin and Oxygen Transport

Red blood cells, (RBCs) are components of the blood which functions to carry haemoglobin (Hb) around the body in sufficiently high concentration. They allow the effective transport of oxygen (O_2) from the lungs to the tissues, and the return of carbon dioxide (CO_2), produced during oxidative phosphorylation, back to the lungs (Gordon-Smith, 2013).

The Hb molecules contain a metallo cofactor, the haem group, which makes Hb capable of carrying in endothermic mammals and birds (haematocrit ~ 45%), about 9 mmol O_2 L^{-1} of blood (Hsia et al. 2013). The capability of RBCs to bind and to deliver the O_2 is due to a combination of the *cooperative binding* and *allosteric modulations* of Hb.

In nature, Hb molecules exist as an equilibrium between two different forms; the relaxed (*R*) form, where Hb has high O_2 affinity (oxyhaemoglobin, HbO_2) and the tense (*T*) form, with a low O_2 affinity (deoxyhaemoglobin, HHb).

At a high arterial oxygen partial pressure (PaO_2) context, such as at the respiratory surfaces, Hb becomes fully saturated with O_2, assuming its *R* form. Instead, as the blood enters into the microcirculation, the PaO_2 decreases, promoting the O_2 offloading and Hb shifting to the *T* form (Jensen 2009).

Each Hb molecule is a tetramer structure that contains 4 Hb chains and 4 atoms of iron capable of binding one molecule of O_2 each, for a total of 4 molecules of O_2 carried by each Hb molecule (***Figure 1-1***; ***1-2***).

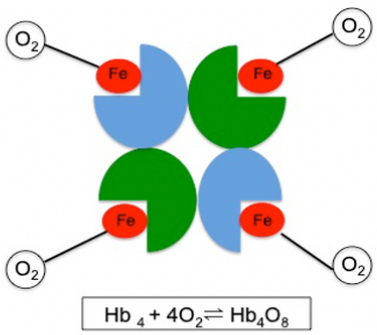

Figure 1-1 Structure of haemoglobin (Hb). In green 2 alpha chain, in blue to beta chains, each chain contains an haem-iron group (red spot)

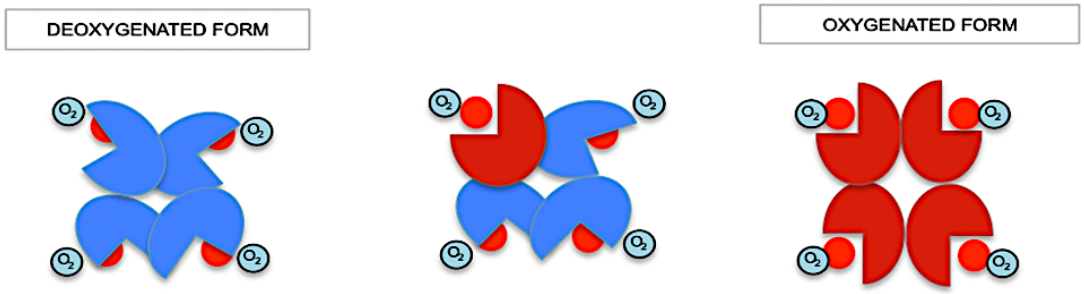

Figure 1-2 Functional States of Hb molecule. In blue deoxyhaemoglobin (HHb); in red oxyhaemoglobin (HbO_2). The red spots represent the Haem groups, not exposed in HHb while are exposed in HbO_2.

The ability of Hb to carry O_2 molecules is due to *cooperative binding*, a phenomenon displayed by receptors that have multiple binding sites and where the affinity of each of the binding sites for a ligand is increases (positive cooperativity) upon the binding of a ligand to a binding site. While the deoxyhaemoglobin has a relatively low affinity for O_2, once the first O_2 molecule is bound to a single haem, the O_2 affinity of Hb increases, allowing the second molecule to bind more easily, and then the third and fourth even more so.

The peculiarity that a binding of a particular ligand at one site affects the conformation of a second remote binding site for another ligand on the same protein is named *allosteric modulation*. As Hb is an *allosteric protein* it alters its affinity towards the first ligand, therefore the first O_2 molecule has to overcome strong electrostatic charges between the 4 Hb protein chains compared to the successive O_2 molecules that bind more easily. Due to the allosteric modulation and cooperative binding features the Hb molecule *cannot* be considered as made of four independently oxygen-binding subunits. The Hb molecule peculiarities allow it to deliver 1.7 times as much O_2 as it would if the sites were independent (Naik 2016). Regarding O_2 binding capacity, the amount of O_2 in millilitres carried by each gram of Hb, this is commonly referred to as *Hüfner's constant*, that, at STP – (standard temperature and pressure, 0° C and 760 mmHg), has a theoretical value of 1.39 ml O_2 for each gram of Hb (McDonell & Kerr 2017).

In reality, the Hb oxygen carrying capacity of blood is less than the 1.39 due to the presence of other forms of Hb in blood (e.g., carboxyhaemoglobin and methaemoglobin) with a different affinity for O_2. For this reason, oxygen carrying capacity is often theoretically reduced to 1.34 ml of O_2 per gram of Hb (Hüfner 1894) and a further reduction to 1.31 has

been proposed assuming a normal level of carboxyhaemoglobin and methaemoglobin in human blood (McLellan and Walsh 2004). In other species, the Hüfner's constant has been calculated as 1.33– and 1.35– ml of O_2 per g of Hb e.g., in dogs and mice (Shimizu S et al. 1986) and 1.38 ml g^{-1} in horses (Clerbeaux et al. 1986).

1.1.2 Oxygen Dissociation Curve

The oxygen dissociation curve (ODC) (***Figure 1-3***), describes the relation between the PaO_2 (x axis) and the haemoglobin oxygen saturation (SaO_2) (y axis). The shape of ODC is sigmoidal among the normal physiological range of PaO_2 (e.g., 40 to 100 mm Hg),typically the blood that leaves the lungs and enters into systemic arteries having a PaO_2 of about 95 mmHg, which from the OCD is equal to a SaO_2 of about 97% (Guyton and Hall 2016). Once the PaO_2 decreases to a level as low as 60 mmHg, arterial Hb remains 89% saturated with O_2 and conversely even if PaO_2 rises above 95mmHg, O_2 saturation can only increase to a maximum of 3 percent above normal. This is due to the oxygen that acts as a *buffer*, meaning that with a PaO_2 varies from 60 to 500 mmHg, SaO_2 in the peripheral tissue will not vary more than a few millilitres from normal (Guyton and Hall 2016).

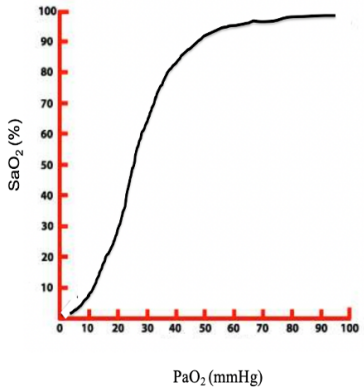

Figure 1-3 Oxygen Dissociation Curve

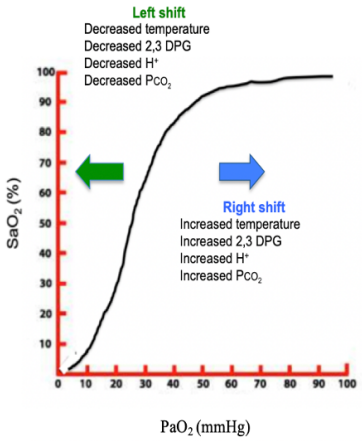

Figure 1-4 Factors Influencing the Oxygen Dissociation Curve

Another important value in the context of ODC is the P_{50}, that represents the PaO_2 value at which the Hb is 50% saturated with O_2 (typically about 26.6 mmHg in humans), and is conventionally used to define the Hb's affinity for O_2.

The value of P_{50} of each species is determined by natural selection according to animal size,

tissue metabolic requirements and ambient oxygen tension. As a rule of thumb, the smaller the animal, the lower its Hb's affinity for the O_2 would be (e.g. P_{50} for cats and dogs are 34 and 30 mmHg respectively; Cambier et al. 2004) and the ODC will shift to the right. A left shift of ODC would instead define a higher Hb's affinity for O_2, such as for the foetal human Hb, where the P_{50} value is about 19 mmHg (Thomas & Lumb 2012).

Several physiological factors may influence the ODC displacing the curve in one direction or the other (left or right shift) as shown in ***Figure 1-4***. While a right shift favours unloading the O_2, a left shift determines an increased Hb's affinity for O_2, that by favouring the O_2 binding makes its unloading more difficult. Increased CO_2 tension, decreases the pH (acidity), increases the 2,3-diphosphoglycerate (2,3-DPG) level, and increases the temperature, which are all factors that shift the curve to the right due to the *Bohr effect*. The *Bohr effect* facilitates the O_2 unloading, because once the CO_2 diffuses from the tissue into the blood where it reacts with water to form carbonic acid, the resulting decreases blood pH allowing the dissociation of O_2 away from the Hb towards the tissues (Bohr et al 1904). Henderson in 1920 was the first that showed the pH effect on Hb.

$H–Hb + O_2 \leftarrow \rightarrow Hb^- – O_2 + H^+$

This adaptation has been later shown to depend in part on the presence of 2,3-diphosphoglycerate (2,3-DPG) (Tomita & Riggs 1971). The 2,3-DPG which is a metabolic intermediate produced in tissues under heavy energy use (low ATP, high acid production). The oxygen binds to mammalian Hb with an exothermic reaction, as stated by Chatelier's principle, so increased temperatures are associated with reduced O_2 affinity, and vice versa (Weber & Campbell 2011), such that in humans extreme hypothermia is known to increases the affinity of Hb for O_2 more than 22-fold (Dash & Bassingthwaighte 2010). The decrease in Hb–O_2 affinity with increasing temperature, is advantageous in that it enhances O_2 unloading from blood that perfuses warm tissues, for example exercising muscles that have increased O_2 requirements (Barcroft & King 1909), but can become detrimental in regionally heterothermic animals, for example in cold-tolerant birds and mammals, where it may perturb the balance between O_2 unloading and O_2 requirement in organs with

substantially different temperatures (Weber & Campbell 2011).

1.1.3 Dysfunctional Haemoglobins

Not all the Hb are functional, meaning capable of transporting and unloading O_2 at the tissues; in fact, dysfunctional haemoglobins species such as sulfhaemoglobin, carboxyhaemoglobin (COHb) and methaemoglobin (MetHb), are Hb combined with other substance beside oxygen (Ralston et al. 1991). For instance, if Hb combines with carbon monoxide (CO), a colourless and odourless gas with a 200–times greater Hb affinity than O_2, a large proportion of the Hb binding sites will become occupied by CO forming COHb, even at low partial pressures.

$HbO_2 + CO \rightarrow COHb + O_2$

The formation of COHb not only displaces the O_2, but also causes a conformational change in Hb that results in greater affinity of the Hb for the CO then for O_2 and a shift of the ODC to the left, further compromising tissue oxygen delivery (***Figure 1-5***).

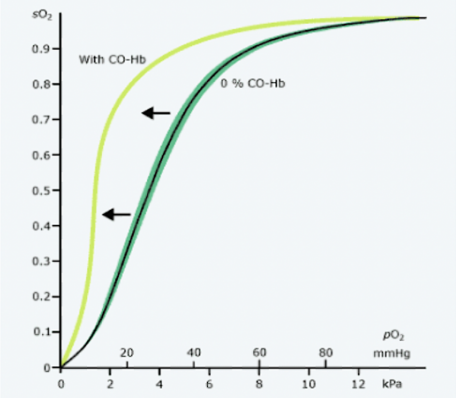

Figure 1-5 Carbon Monoxide shifts the oxygen dissociation curve to the left changing it in a more hyperbolic shape. Less oxygen is available for the tissue.

In humans the normal reference value for COHb ranges between 1% to 3% (Piantadossi 2002). Reports in the veterinary literature indicate a lack of consensus on the normal reference range in dogs but suggest that it is higher than in humans (between 5.6% - 6.4 %, median 6.1%) (Ashbaugh et al. 2012). Another dysfunctional haemoglobin species is the methaemoglobin; a Hb in the form of metalloprotein where the iron in the haem group is changed from the ferrous (Fe^{2+}) to the ferric state (Fe^{3+}) due to oxidative stress. The Fe^{3+} not only cannot bind O_2 and carry it to the tissues, but also prevents O_2 release from the other

Fe^{2+} on the same Hb molecule (Jaffe 1981). Under standard conditions, a small amount of methaemoglobin is always present [in the human blood < 1% of the total Hb (Jaffe 1981)] in the erythrocytes but is usually converted back to functional Hb by nicotinamide adenine dinucleotide (NADH)–dependent methaemoglobin reductase cytochrome b5 reductase (b5R), through the diaphorase pathway (***Figure 1-6***).

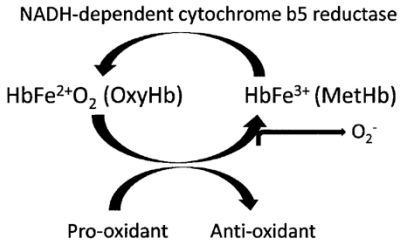

Figure 1-6 Formation of methaemoglobin

An increased level of MetHb relative to functional Hb, may cause tissue hypoxia and a left shift in the ODC as a result of increased affinity to bound O_2 in the remaining haem groups (Margulies & Manookian 2002). Diagnosis of methaemoglobinemia is extremely difficult; it is asymptomatic up to concentration of 10- 15%, with the colour of blood turning to chocolate brown when higher than 10%, exercising intolerance and cyanosis only with concentrations > 20%. Methaemoglobinemia occurs as a congenital or acquired condition; *congenita*l due to NADH-cytochrome b5 reductase deficiency (Atkins et al 1981; Harvey 2006; Shino et al. 2018), *acquired* as a result of exposure to toxic oxidizing agents such as benzocaine, prilocaine, sulphonamides, nitrates (Key et al. 1980).

1.1.4 Oxygen Content

The oxygen content (CaO_2) of blood is another important measurement, as neither SaO_2 nor PaO_2 provide information on the number of O_2 molecules within the blood. Of the main values used to assess blood O_2 levels, the *'how much'* is only provided by the CaO_2, which represents the total volume of O_2 in arterial blood and it is conventionally reported as millilitres of O_2 per decilitre (ml dL^{-1}) of blood. The CaO_2 value incorporates the O_2 bound to Hb and the O_2 dissolved in the plasma, where the amount of O_2 dissolved is proportional to the partial pressure exerted by oxygen on the plasma at a given temperature (Dunn et al.

2016) ***equation [1]***

$$Arterial\ oxygen\ conten = bound\ oxygen + dissolved\ oxygen$$

While CaO_2 is influenced by factors, such as PaO_2 and the adequacy of ventilation and gas exchange, its main determinant is the Hb concentration and affinity for the O_2, ***equation [2]***

$$CaO_2 = (\text{Hb} \bullet \text{Hüfner's constant}) \bullet \frac{SaO_2}{100} + (PaO_2 \bullet solubility\ coefficient\ of\ oxygen)$$

The Hb is the amount of haemoglobin in grams per decilitre (g dL^{-1}), the SaO_2 is the percentage arterial haemoglobin saturation, and the solubility coefficient of O_2 in blood is equal to 0.0031ml $mmHg^{-1}$ of O_2 dL^{-1} of blood and represents the solubility coefficient of oxygen at body temperature; the number of ml of O_2 dissolved per 100 ml of plasma per kilopascal (ml O_2 100 ml^{-1} plasma kPa^{-1}). PaO_2, partial pressure of oxygen in arterial blood, is expressed in kilopascals (kPa) (McLellan & Walsh 2004; West 2004).

The measurement of PaO_2 from a sample of arterial blood has been the traditional method for assessing oxygenation (Nishioka et al. 2017). Arterial blood gas analysis or arterial blood gas (ABG) is a widely available test used to directly measure PaO_2, $PaCO_2$, and pH, however some limitations include; as discrete measurements, the inability to give real-time measurement, possible pain and/or vascular trauma, difficulties in obtaining arterial blood samples from small sized patietns, sampling of venous as opposed to arterial blood, variable arterial oxygen tension associated with positioning for sample collection, operator dependency, and cost, can reduce or limit its use to only some contexts.

Dependent on the blood gas analyser used, the SaO_2 value can be measured or calculated from PaO_2 values (Nishioka et al. 2017), about which certain degree of inaccurancy has been shown when compared to the direct measurement of SaO_2 by laboratory CO-Oximetry (Johnson et al. 1993; Nierman & Schechter, 1994). The SaO_2, which is the percentage (%)

of tHb binding sites available and occupied by O_2 represents the measure of how much of the CaO_2 due to Hb is being utilised, as defined by the following ***equation [3]***

$$SaO_2 = \frac{[HbO_2]}{[HbO_2]+[HHb]} \times 100\%$$

It is important to note that the denominator of this equation is not the concentration of the total Hb, as MetHb and COHb are in fact not included. This means that pathologies such as carbon monoxide poisoning and methaemoglobinemia *(see previous sections)* may result in a reduction of the CaO_2 that is not reflected in a decrease of SaO_2%. In the same way, pathological states that determine reduction in Hb concentration (e.g. due to anaemia) decrease the CaO_2 values without eliciting changes in SaO_2.

In blood gas analysis, the overall percentage of Hb binding sites occupied by O_2, defined as SaO_2 % is often denoted as *functional SaO_2*. On the other hand, the term *fractional SaO_2*, often indicated as *fractional HbO_2* (abbreviated as *F* HbO_2) reflects the effects of COHb and MetHb giving a more accurate indication of the oxygen carrying capacity of the Hb (Chan et al. 2013), ***equation [4]***

$$F\,HbO_2 = \frac{[HbO_2]}{[HbO_2]+[HHb]+[COHb]+[MetHb]} \text{ x } 100\%$$

As previously mentioned, the SaO_2 % is generated with blood gas analysis, by one of the two following methods: from an indirect calculation from the measured PaO_2 or from a direct spectrophotometric measurement. The calculated methodology generates a SaO_2 from the pH and PaO_2 values and is based on their relationship as described by the ODC using a number of algorithms and variables including; PaO_2, pH, $PaCO_2$ and base excess (Breuer et al. 1989). Portable blood gas analysers such as i-STAT®, EPOC®, calculate SaO_2 from measured pH and $PaCO_2$ on the basis of standard human ODCs (pH 7.4, $PaCO_2$ 40 mmHg, and temperature 37°C) and assume a normal concentration of 2,3-DPG and dyshaemoglobins (COHb and MetHb). Unfortunately, as previously discussed, ODC is affected by a number of factors other than PaO_2 that make the calculated SaO_2 potentially

inaccurate.

Instead, the measured SaO_2 via spectrophotometric principles (shining light through the sample) is the method of choice for determining arterial oxygen saturation. Among the devices that measure the SaO_2, IDEXX VetStat® analyser enables total Hb and SaO_2 measuring through a red and infrared light coming from one light-emitting diode (LED) and two laser diodes, that are directed through an optically polished window to the blood in the cassette over the O_2 sensor. The light is partially absorbed and reflected by the erythrocytes to a photodiode. The intensity of reflected light varies in a well-defined way with the blood Hb and SO_2 used in their measurement. The output signal of the detectors is converted by the microprocessor to a number and displayed on the touch screen. Other values commonly used for the assessment of oxygen and acid-base status are calculated from these measured values (https://www.idexx.pl/files/vetstat-updated-operator-guide-en-gb.pdf). Clinicians should be aware of the methodology used to generate the SaO_2 value during blood gas analysis (***Table 1-1***), whether the methodology is based on calculation or direct measurements and for these reasons SaO_2 values should be interpreted with caution.

Calculated SaO_2 (blood gas analyser)	**Measured SaO_2** (CO-Oximeter)
I-STAT portable	
IL Gem Premier	
IL 1630, 1640, 1650, 1660, 1710, 1720, 1730, 1740	IL 682, 1715, 1725, 1735, 1745
Bayer 400	Bayer 405
Bayer 248, 278, 280, 288, 348, 840, 850, 860, 1200	Bayer 845, 855, 865, 1205
AVL Opti 1, 3, IDEXX VetStat	AVL Omni 3, 6
AVOXimeter 1000E	AVOXimeter 4000
Radiometer ABL 330	Radiometer ABL 520, 620, 625, 700 & 800 series
Radiometer 5, 50, 500, 505, 555, 600	Radiometer OSM, OSM3
AVL Compact 2, 3, AVL 995, AVL Omni 1, 2, 4, 5	

Table 1-1 Various blood gas machines (calculated SaO_2) and CO-Oximeters (measured SaO2). This is not an exhaustive list (Masimo web site).

1.2 METHODOLOGIES TO MEASURE HAEMOGLOBIN

Haemoglobin can be measured by several methodologies. The first clinical test of Hb measurement was developed more than a century ago, where after adding drops of distilled water to a measured volume of blood until its colour was matched that of an artificial colored standard (Gowers 1879). A later modification involved first saturating blood with coal gas (carbon monoxide) to convert haemoglobin to the more stable carboxyhaemoglobin and then to measure it.

Modern haemoglobinometry dates from the 1950's after the development of spectrophotometry and the haemiglobincyanide (cyanomethaemoglobin) method. Adaptation of this method and others for use in automated hematology analysers then followed. Over the past two decades advances have focused on the development of methods which allow point-of-care testing (POCT) of Hb.

1.2.1 Cyanomethaemoglobin assay

Nearly 50 years after it was first adopted as the reference method for measuring Hb by the International Committee for Standardization in Hematology (ICSH), the haemiglobincyanide (HiCN) assay remains the recommended method against which all new Hb methods were judged and standardised. The assay is performed by mixing the blood sample with a cyanide-containing reagent (*ferricyanide and potassium cyanide*) that converts the Hb molecule into cyanomethaemoglobin (ICSH 1995). The HiCN is a stable coloured product which in solution has an absorbance maximum at 540 nm. Absorbance of a diluted sample is compared with a standard HiCN solution whose equivalent Hb concentration is known. The major advantages of this type of Hb test is the availability of an internationally standard HiCN solution manufactured and assigned a concentration value

according to very precise criteria laid down and reviewed periodically by the ICSH (ICSH 1995) (Shah et al. 2011).

Advantages of HiCN	**Disadvantages** of HiCN
International standard – accurate	Manual method requires accurate pipetting and spectrophotometer
Easily adapted to automated hematology analyzers	Reagent (cyanide) hazardous
Well established and thoroughly investigated – ICSH recommended	Subject to interference from raised lipids, plasma proteins and leucocyte numbers
Inexpensive reagent	Does not distinguish those Hb derivatives which have no oxygen-carrying capacity (MetHb, COHb)

Table 1-2 Advantages and Disadvantages of HiCN methodology

The 'gold standard' HiCN technique, however it is both time and labor intensive as well as expensive, making it impractical for daily use in a clinical setting (Gehring et al. 2007) (***Table 1-2***).

1.2.2 Haematology analyser

The haematology analyser counts Hb proteins by detecting changes in conductance as cells suspended in a low concentration of electrolytes solution that pass through a small aperture. The volume of electrolyte displaced by the particle passing through the aperture causes a short-term change in the impedance across the aperture, which is measured as a voltage or current pulse. Thereafter, the characteristics of the pulse are used to calculate the number and volume of particles. Relative to the HiNDC this method has been reported to shown a bias ± SD of 0.3 ± 0.2 g dL^{-1} in Hb values (Pinkerton et al. 1970 ; Gehring et al. 2002).

1.2.3 Laboratory CO-Oximetry

Laboratory CO-Oximetry or haemoximetry, measure Hb and SaO_2 using a set of fixed wavelengths of light applying the principles of the Beer-Lambert law. This methodology of measurement is based on the fact that Hb and all its derivatives are coloured proteins which absorb light at specific wavelengths and thus have a characteristic absorbance spectrum (the range of Hb species absorb light 520-620 nm).

Once the operator has injected an arterial blood sample into the blood gas analyser CO-oximeter, the whole sample, or most likely a portion of it, is automatically pumped to a measuring cuvette, whereby either chemical or physical action, erythrocytes are lysed to release the Hb molecules. The sample is then spectroscopically scanned, then the absorbance measured to determine the Hb concentration and the concentration of each of the haemoglobin derivatives (HHb, O_2Hb, MetHb and COHb). The number of Hb derivatives detected by the CO-Oximeter is determined by the number of fixed wavelengths it is able to detect. Most clinically used CO-Oximeters detect over 100 wavelengths of light, providing the ability to discriminate between different Hb forms (***Table 1-3***).

There is no common agreement among researchers about the Hb results obtained by CO-oximetry. Several studies (Brunelle et al. 1996; Kuleš et al. 2011) have confirmed that Hb results obtained by CO-oximetry are not significantly different from those derived from reference laboratory methods, and that CO-oximetry is accepted to provide an acceptable means for urgent estimation of Hb in a critical care setting.

Gehring and colleagues (Gehring et al. 2007) measured the error among different CO-Oximeter devices, where the samples were tested on two identical devices from each manufacturer, and differences in [Hb] values up to 1.2 g dL^{-1} were detected between the pairs. When [Hb] values from six different CO-Oximeters were compared to that determined by a Coulter Counter, Patel et al. (Patel al. 2007) found biases ± standard deviation (SD) ranging from 0.0 ± 0.2 to 1.4 ± 0.4 g dL^{-1}.

Advantages of CO-oximetry
Speed of analysis
Easy of analysis
No capital or consumable cost beyond that required for blood gas analysis
Additional parameters (MetHb, COHb, O2Hb) measured
Not affected by high white-cell count

Table 1-3 Advantages of CO-oximetry

1.2.4 Conductometric point of care testing (POCT) devices (i-STAT®)

The conductometric methodology uses the conductivity properties to calculate haemoglobin concentration [Hb], haematocrit, blood gases and other parameters by measuring the current in a solution when a constant potential difference is applied and where the current (conductivity) is inversely related to the resistance (current = potential/resistance) (Ng et al 2014).

Systems using the conductivity method, such as the i-STAT® (Abbott Park, IL, USA) system, measures the electrical conductance of a whole blood sample where plasma conducts electrical current, and blood cells act as insulators. As an example, in a sample with a relatively high haematocrit, a larger proportion of volume is filled by the non-conductive RBCs, meaning that the overall conductance of the sample is relatively low.

In the i-STAT system corrections are applied for the temperature of the sample, the size of the fluid segment measured, and the relative conductivity of the plasma component (https://www.pointofcare.abbott/int/en/home). The i-STAT system provides a calculated [Hb] result which is determined as follows:

Hb (g dL^{-1}) = haematocrit (% PCV) x 0.34 or Hb (g dL^{-1}) = haematocrit (decimal fraction) x 34

Results of Hb levels from i-STAT, can be affected by elevated white blood cells values, high lipid levels, and low total protein levels, besides that i-STAT® is less accurate than a haematology analyser at low Hb level with sample discrepancies up to 2 g dL^{-1} (Hopfer et al. 2004). While the compactness of this method is desirable, a bias of 2 g dL^{-1} could affect patient management, and borderline measurements should be confirmed with another methodology (Berkow 2013).

1.2.5 Spectrophotometric point-of-care devices (HemoCue)

The HemoCue system provides an easy and convenient [Hb] estimation based on spectrophotometric readings from blood drawn into a single use cuvette by capillary action. In earlier generation devices (*HemoCue 201* and *HemoControl* [EKF Diagnostics, GmbH,

Barleben, Germany]), a blood drop was placed on a cuvette where sodium deoxycholate haemolysed erythrocytes to release the Hb. Sodium nitrite then converted the Hb to MetHb, which together with sodium azide gave azide-MetHb. The absorbance of which was measured photometrically at 565 and 880 nm wavelengths in order to compensate for turbidity in the sample (Chaudhary et al. 2017).

One of the advantages of the current available microcuvettes such as the HemoCue system, is that it can easily be used by non-laboratory-trained medical professionals at the site of care. It is fast, inexpensive and requires only small amounts of blood. It has been shown to have high accuracy and precision relative to haematology analysers in the hands of trained operators (Neville 1987; http://www.hemocue.com), but can be subject to large inter-operator variability (Neufeld et al. 2002). Reported sources of error with this system may come from incomplete filling, trapping of air bubbles and moisture, which can lead to errors of [Hb] of about 2 g dL^{-1} (Nguyen 2002). The mentioned limitations have however been mitigated in the newer modified devices (Hemocue 301 [HemoCue AB] and DiaSpect Haemoglobin T [DiaSpect Medical GmbH, Sailauf, Germany]) which use reagent-free cuvettes, not affected by temperatures (10°–40°C), humidity, and without special storage condition requirements (http://www.hemocue.com). The measurement of the absorbance of whole blood in this system is photometrically performed at the 506 nm isosbestic point, the wavelength at which the absorbance of the two main Hb derivatives oxy-haemoglobin and deoxyhaemoglobin are the same, and 880 nm which allows for compensation for turbidity (***Table 1-4***).

Advantages of HemoCue	**Disadvantages** of HemoCue
Small blood sample	Inter-operator variability
No needed laboratory-trained operator	Air buble and humidity may cause errors (old version)
Cost effective	
Quick results	

Table 1-4 Main advantages and disadvantages of HemoCue point of care device

1.2.6 Non invasive spectrophotometry

These methodologies use noninvasive, multiwavelength sensors to determine the [Hb] with sensors that automatically and continuously perform self-testing and calibration checks during measurement sessions (Chaudhary et al. 2017).

Presently, there are three technologies developed for human patients that use non invasive spectrophotometry for [Hb] measurement; the *occlusion spectroscope* (NBM 200; OrSense Co., Petah-Tikya, Israel); the *transcutaneous reflection spectroscopy* (HemoSpect; MBR Optical Systems GmbH & Co. Wuppertal, Germany); the *pulse co-oximeter* (Pronto-7; Radical-7 Masimo Co, Irvine, CA, USA).

The *occlusion spectroscope*, is a portable device that via a ring-shaped sensor fitted on the patient finger, this applies a pressure that temporarily stops the blood flow, creating an optical signal with a high signal-to-noise ratio. The optical elements in a multi-wavelength sensor (between 600 and 1500 nm) measure the light transmitted through the finger. Differential light absorption, before and after blood flow obstruction are then used to determine the [Hb] (Shah et al 2014).

The *transcutaneous reflection spectroscopy* has a button sensor adherent to the palm side of the finger of the nondominant hand. The sensor head, which is placed on the skin, projects a white light into the underlying tissue via a waveguide. Some of the projected light is absorbed by various tissue components, while some is reflected back to the device. The signal return to the unit is broken down into separate wavelengths, and an electronic evaluation unit connected to the system analyses the [Hb] (Chaudhary et al. 2017).

In the *pulse co-oximeter*, a sensor is placed over the individual's fingertip. The sensor acquires blood constituent data based on light absorption through this finger probe. Based on the light attenuation characteristics, the device calculates the [Hb]. Disadvantages of this method with regard to human medicine include the need for an adequate perfusion rate and the facts that dark skin color and metallic nail polishes may interfere with the results (Littlejohn & Applegate 2018).

1.3 PULSE OXIMETRY

Although arterial blood gas analysis is commonly considered the 'gold standard' for the assessment of oxygenation, oximetry may offer multiple advantages: as it provides continuous and transcutaneous estimations of the SaO_2, without the needed for repeated arterial punctures.

The accuracy of pulse oximeter readings of oxygen saturation (SpO_2) in estimating the SaO_2 among 23 studies available, reports that SpO_2 overestimated SaO_2 by 1.99% with a range of bias from -13.2 ± 8.0% to 12.0 ± 13.3% (Jensen et al. 1998).

In veterinary medicine, accuracy of oximetry in healthy and compromised horses during spontaneous and controlled ventilation suggested that SpO_2 has a general tendency to underestimate SaO_2 values (laboratory CO-Oximetry based) that were over 90% (Koening et al. 2003). Accuracy of pulse oximetry during arterial oxyhaemeglobin desaturation in a study involving dogs reported that the SpO_2 value closely reflected functional SaO_2 in the range of 22-100%, with a bias of +5.5% over this range (Sendak et al. 1988).

The agreement between SpO_2 and SaO_2 could be affected by confounding factors such as; complex physiological disturbances including altered blood flow, acid–base disturbances, abnormalities in temperature regulation, low perfusion status, sepsis, the presence of dysfunctional Hb, vital dyes, or due to motion (Jensen et al. 1998; Wilson et al. 2010; Hasanin et al. 2017). In regards to the last factor, a study performed in dogs, cats, and adult horses has also indicated considerable differences in accuracies between various type of monitors, sensors, and site of sensor placement, probably due to the different algorithms used (Matthews et al. 2003).

Nevertheless, pulse oximeter is routinely used to non-invasively measure SpO_2 as an estimation of SaO_2 and it is often considered as the additional vital sign (Branson & Mannheimer, 2004). Since, the use of pulse oximetry in human medicine has decreased the need for ABG by 37%, causing significant changes in the medical treatment of disorders in the emergency departments (Simon & Clark, 2002); nevertheless, several investigators demonstrated that episodic hypoxemia events are more common than previously suspected,

with an incidence ranging from 20–82% in the postoperative period (Roe & Jones, 1993; Bierman et al. 1992; Bowton et al. 1994).

For the safe use of pulse oximeters, it is critical to understand its multiple limitations and the two basic principles that govern its mechanism of function, in particular:

- How HbO_2 is distinguished from HHb.
- How the SpO_2 is calculated only from the arterial compartment of blood.

Pulse oximeters operate based on the principle of different absorption and light emission of the different Hb conformation states (Chan et al 2013); in fact by using an electronic processor and a pair of small LEDs a pulse oximeter emits red lights, with wavelength of 660 nm, and infrared lights with a wavelength of 940 nm.

Absorption of lights at these wavelengths differs significantly between blood loaded with oxygen and blood lacking oxygen; HbO_2 absorbs more infrared light and allows more red light to pass through while HHb allows more infrared light to pass through and absorbs more red light (Giguére et al. 2014).

The pulse oximeter's LEDs flash in a characteristic sequence: one on, then the other, then both off, to allow the photodetector to measure the background level of the ambient light in a triple sequence that happens 30 times per second. Furthermore, the microprocessor corrects for ambient light, and for the difference between arterial and venous saturation by deducting the minimum transmitted light during diastole from the maximum during systole (Al-Shaikh & Stacey 2013).

The peripheral oxygen saturation detected by the pulse oximeter is then displayed as the ratio between the concentrations of HbO_2 and other Hbs present in the blood, as defined by the following ***equation [6]***

$$SpO_2 = \frac{[HbO_2]}{[HbO_2] + [HHb]} \text{ x } 100\ (\%)$$

The ratio of absorbance at these two wavelengths is calibrated empirically against direct measurements of arterial blood oxygen saturation (SaO_2) in volunteers, and the resulting calibration algorithm is stored in a digital microprocessor within the pulse oximeter. During

subsequent use the calibration curve is used to generate the pulse oximeter's estimate of arterial saturation (Tremper & Barker, 1989) (***Table 1-5***).

SaO_2	660 nm (R)	940 nm (IR)	R/IR
0%			3.4
85%			1.0
100%			0.43

Table 1-5 Calibration curves. Red (R) and infrared (IR) scaled alternating current (AC) signals at arterial oxygen saturation (SaO2) of 0%, 85% and 100%.

All pulse oximeters are based on three main principles: *spectrophotometry*, *Beer's – Lambert's law*, and *optical plethysmography*.

1.3.1 Spectrophotometry

Every chemical compound absorbs, transmits, or reflects light over a certain range of wavelengths, which can be used to calculate the unknown amount of a known chemical substance. Spectrophotometry is a method used to measure how much a chemical substance absorbs light, by measuring the intensity of a light as it passes through a sample solution. Depending on the range of wavelength of the light sources used, spectrophotometers are classified into two types; *UV-visible spectrophotometer*, which uses light in the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum, and *IR-spectrophotometer*, that uses light in the infrared range (700 - 15000 nm) of the electromagnetic radiation spectrum.

Any spectrophotometer consists of two principal components; a *spectrometer* and a *photometer*. The first produces the desired range of light wavelengths by the transmitting straight beam of light (photons) into a collimator and through a monochromator (prism) that splits the light into several wavelengths (spectrum); then a wavelength selector (slit) will transmit only the desired wavelength. The photometer instead, measures the range of

wavelength of light that passes through a sample and sends a signal to a galvanometer or a digital display ***Figure 1-7***.

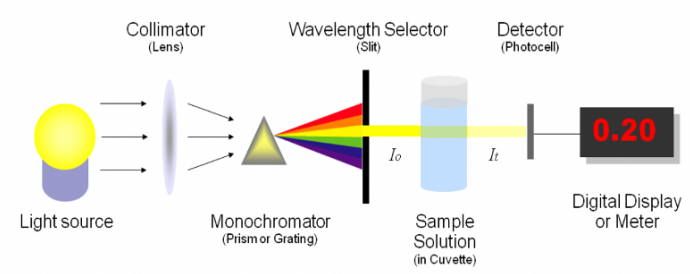

Figure 1-7 Basic structure of a spectrophotometer (illustrated by Heesung Shim)

The intensity of the light that passed through a sample is related to *transmittance* (the fraction of the light that passed through the sample) as illustrated by ***equation [7]***

$$Transmittance\ (T) = \frac{I_t}{I_o}$$

Where I_t is the light intensity after the beam of light passes through the sample and I_o is the light intensity before the beam passes through it. *Absorbance* on the other hand, is related to the transmittance as illustrated by ***equation [8]***

$$Absorbance(A) = -\log T - log\ (\frac{I_t}{I_o})$$

Well-oxygenated blood has a higher concentration of HbO_2 and appears bright red to the eye because it scatters more red light than HHb, while HHb absorbs more red light and appears less red. Unfortunately, because conventional pulse oximeters measure light absorbance at only two wavelengths, if any other substance is present in the sample this assumption is violated and the pulse oximeter cannot accurately estimate SpO_2.

1.3.2 Beer-Lambert's law in pulse oximetry

This law states that there is a linear relationship between the concentration and the absorbance of the solution, which enables the calculation of the solution concentration by measuring its absorbance.

- *Beer's law*: formulated by German mathematician and chemist August Beer in 1852, states that the intensity of transmitted light (I_t) decreases exponentially as the concentration of the medium increases.
- *Lambert's law*: formulated by German physicist Johann Heinrich Lambert (1728-1777), states that the intensity of transmitted light (I_t) decreases exponentially as the distance travelled through the substance increases (L).

Thus, the solute concentration can be calculated from the measurement of incident and transmitted light intensity at a known wavelength (Tremper & Barker, 1989).

1.3.3 Optical plethysmography

The ability of pulse oximeters to detect the SpO_2 'only' from arterial blood is based on the fact that the amount of red and infrared light absorbed fluctuates within the cardiac cycle.The volume of arterial blood increases during systole and decreases during the diastole (named here as pulsatile compartment, AC) while the blood volume in the capillaries, veins, skin and fat remains relatively constant (named here as no pulsatile compartment, DC) (Chan et al 2013) ***Figure 1-8***. This pulsatile expansion of the arteriolar bed produces an increase in path length that results in incresed absorbance, which is assumed as arterial blood by all pulse oximeters and the only pulsatile absorbance between the light source and the photodetector.

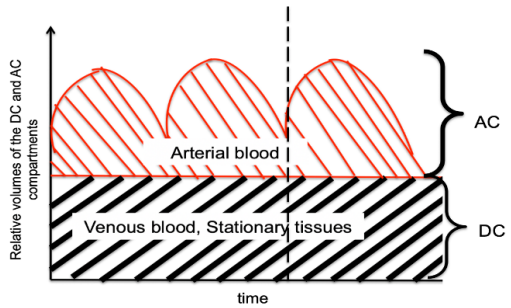

Fig 1-8 The onset of QRS complex coincides with the onset increase of arterial blood volume. (DC) non-pulsatile compartment (AC) pulsatile tissue compartment

In more detail, the electronic processor in the pulse oximeter is programmed to first determine the AC component of absorbance at each wavelength, 660 nm and 940 nm, then to divide it by the DC component to obtain a "pulse added" absorbance that is independent of incident light intensity. Lastly pulse oximeters calculate the ratio of these pulse added absorbances, which is empirically related to SaO_2 (Tremper & Barker, 1989) ***equation [9]***. *Ratio (r) =(AC660/DC660)/(AC940/DC940)*. The ***Figure 1-9***, illustrates an example of a pulse oximeter calibration curve (Pologe, 1987). This curve is determined on a theoretical basis,

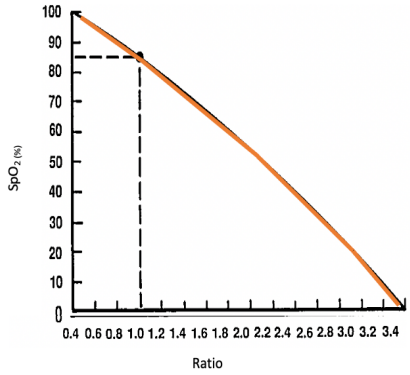

Figure 1-9 A standard curve of Red: Infrared Modulation Ratio in relation with SpO_2

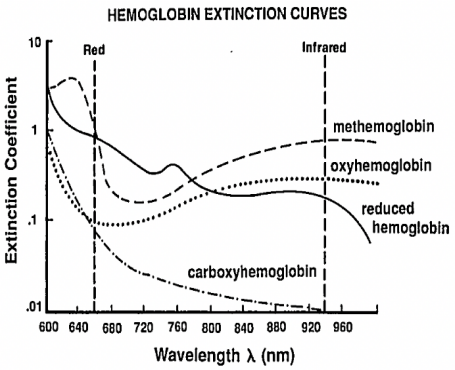

Fig 1-10 Extinction curve Transmitted light absorbance spectra of four Hb species (Tremper & Barker, 1987)

but for an accurate prediction of SpO_2 experimental data is required. The ratio of red to infrared absorbances vary from approximately 0.4 at 100% saturation to 3.4 at 0% saturation. Unfortunately, MetHb and COHb also absorb red and infrared light at the wavelength used by standard pulse oximeters (***Figure 1-10***) causing possible errors (Tremper & Barker, 1989). Now, how strongly a chemical species or substance absorbs light at a particular wavelength is termed the extinction coefficient (ε), which is an intrinsic property of a chemical species. With respect to ε in the IR range (940 nm) COHb absorbs very little light; whereas in the R range (660 nm) it absorbs as much light as HbO_2, therefore to the pulse oximeter COHb has a similar absorbance of HbO_2 at 660 nm. In a study conducted in dogs the effect of COHb on SpO_2 was found to be given by the following ***equation [10]*** (Barker & Tremper, 1987).

$$SpO_2 = \frac{HbO_2 + 0.9 \times COHb}{total\ Hb} \times 100\%$$

The effect of MetHb on pulse oximetry are also partially predictable from the extinction curves (***Figure 1-10***). MetHb has nearly the same absorbance as reduced Hb at 660 nm, while it has a greater absorbance at 940 nm. As MetHb levels increase, the SpO_2 tends to measure a value of 85% and eventually becomes almost independent of the actual SaO_2, it is erroneously low when SaO_2 is above 85% and erroneously high when SaO_2 is below 85% (Barker et al, 1989). This effect is due to an increased AC660 and AC940 on the *r equation [9]*.

Probably one of the most difficult engineering problems in pulse oximeter design is the identification of the pulsatile absorbance pattern of the arterial blood in the 'sea' of electromagnetic artifact (Tremper & Barker, 1989). Generally speaking, artifact has three major sources; *ambient light*, *low perfusion* (low AC/DC signal), and *motion* (large AC/DC signal), all of which result in poor signal-to-noise-ratio (Pologe, 1987).

A pulse oximeter probe has only 2 LEDs on the side of the detector, however the detector is exposed to three sources of light; red, infreared and ambient light. Due to the photodiodes used in the sensors as light detectors, they cannot discriminate one wavelength of light from the other, LEDs flash on and off in a particular sequence repeated 100 times per second (100 Hz) in order to try to eliminate the background light interference. First R light passes through the tissue and reaches the detector, but ambient light also reaches the detector, so the photodiodes record the R and ambient light. Next the pulse oximeter switches off the R LED and switches on the IR LED, now IR light and ambient light reach the photodiodes that record the IR and room light. Finally, the pulse oximeter switches off both LEDs and only

ambient light reaches the photodiodes that record the rooms light level, which it then subtracts from the reading of R and IR light levels.

In the event of small pulsatile absorbance (low AC-to-DC signal ratio) the pulse oximeter will amplify the signal and estimate the saturation from the *Ratio* of the amplified absorbance, unfortunately background noise is also amplified (static signal) and can be displayed as an artefactual SpO_2 value. To prevent these manufactures have incorporated minimum values for signal-to-noise ratio, below which the device will not display a SpO_2 value. Some pulse oximeters display a noise plethysmographic wave for visual identification of the noise. Regarding the motion (large AC/ DC signal) which is maybe the most difficult artefact to eliminate (Wukitsch et al. 1988), it is an artefact that rarely causes disturbance on the operating room, but rather in the recovery and ICU (Intensive Care Unit) it can make the pulse oximeter less reliable. Engineers have tried several approaches to address this problem such as *the signal averaging time*; if the device measures over a longer time period the effect of an intermitted artefact will be lessened, the downside is that will also slow the response time to an acute change in SaO_2 . To reduce the artefact due to motion, an algorithm present in most pulse oximeters identifies and *rejects spurious signals*. This shrewd mechanism assesses the AC-to -DC signal ratio, checking the validity of the saturation estimate by calculating its rate of change (Tremper & Barker, 1989). For example, if the saturation estimates change from 97% to 75% in one-tenth of second this sudden change may not be averaged into displayed SpO_2 (Wukitsch et al. 1988).

1.4 PULSE CO-OXIMETRY

Conventional pulse oximeters estimate SaO_2 and pulse rate by using a theoretical model which was developed over thirty years ago (Severinghaus & Honda, 1987), assuming absence of dyshaemoglobins forms and without accounting for motions or possible interference sources (Jubran 1993; Buckley et al. 1994).

Recently, manufacturers have developed other devices that by using more light wavelengths are able to measure other forms of Hb present in a blood sample (e.g., HbO_2 HHb, COHb and MetHb). The operation of pulse CO-oximetry (Radical-7® Pulse CO-Oximeter; Masimo Corp., Irvine, California, USA) is based on this relatively new technology, which furthermore has the ability to detect the true arterial signal by measuring and subtracting the noise signals during motion or low perfusion (Goldman et al. 2000).

The methodology of Hb measurement by pulse CO-oximetry is similar to that used for oxyhaemoglobin estimation by conventional pulse oximetry, except that instead of two wavelengths of light, multiple wavelengths are transmitted through the tissues to measure the light absorbance characteristics of the analyte [Hb]. The exact number of wavelengths and the specific wavelengths used however, are still proprietary and not available from the manufacturer. The performance of a conventional pulse oximeter, in a high motion environment or in patients with low perfusion is not always ideal and a high incidence of false alarms due to artefact has been reported (Moller et al. 1993). In this regard, medical equipment manufacturers have developed band-pass filtering in an attempt to address these confounding clinical problems. In fact, band-pass filters allow only a physiologic window of interest to pass while rejecting frequencies outside the desired frequency band.

Barker et al. (1997), by evaluating two conventional pulse oximeters accuracy during motion but a controlled oxygen desaturation protocol in human volunteers, revealed that the two pulse oximeters have displayed saturation values of about 23% and 13% lower than the stationary reference unit (Barker et al. 1997).

During motion, venous blood and other non-arterial absorptive substances generate a pulsatile optical signal attenuating the transmitted light. The result is a decrease in displayed SpO_2 which is not due to a reduced oxygen saturation, but rather to these 'non-arterial components in movements', with data strongly suggesting is the venous blood. During motion in fact, the low-pressure venous blood is susceptible to local effects of perturbation and creates a source of 'in-band' noise within the frequency bandwidth of interest. Furthermore, the venous blood is a strong absorber of light (Goldman et al. 2000; Swan 2007). In a pulse CO-oximeter the saturation values displayed account for not only the true arterial signal, by detracting those signals created by motion/noise, such that the detected IR and R signals coming from a venous (or non-arterial) motion/noise signal as shown by ***equation[11]***

$$IR = S + M$$

$$R = (r_a \times S) + (r_v \times M)$$

Where IR is normalised pulsatile IR signal, R is normalised red signal, S is IR signal vector from pulsatile arterial blood, M is IR motion signal vector generated by venous (or non-arterial) pulsation, r_a is optical density ratio corresponding to arterial saturation (this gives SpO_2), and r_v is optical density ratio corresponding to venous (or non-arterial) saturation (Goldman et al. 2000). Furthermore, adaptive filters (AF), an evolution of the band-pass filtering, are able to change their filtering characteristics based on the noise reference signal. This is obtained by subtracting the product of the arterial optical density ratio and the physiologic signal due to IR light from the physiologic signal due to R light. The resultant is a reference signal that contains only noise portions. Finally, a discrete saturation transform (DST) algorithm will allow one to separate and consequently calculate the optical density ratios that correspond to both the arterial oxygen saturation (r_a) and an estimate of the venous oxygen saturation (r_v) identifying the correct arterial saturation. As the pulse CO-oximeter calculates the arterial oxygen saturation without first extracting or determining discrete pulses it is able to display the values also when the pulse is poor

(Goldman et al. 2000; Swan 2007).

1.4.1 Pulse co-oximetry-based measurement of Hb

A pulse CO-oximeter based measurement of the O_2 carrying state of Hb as well as the dyshaemoglobins is obtained by measuring the absorption of light with multiple wavelengths (Masimo's rainbow technology®) through the blood. The use of the suffix 'CO' refers to the capacity of this device to measure also the carbon monoxide (Vos et al. 2012: Butwick et al. 2012; Patino et al. 2014).

Through the principles of s*pectrophotometry* and photo *plethysmography*, this so-called *rainbow technology* measures light absorption during the blood pulsatile cycle with the maximum radiant power of the strongest light rated at ≤ 25 mW. The detector receives the light, converts it into an electronic signal and sends it to the pulse CO-oximeter (Radical-7®) for calculation. Once the Radical-7 receives the signal from the sensor, it utilises proprietary algorithms to calculate the patient's functional oxygen saturation SpO_2 %, blood levels of carboxyhaemoglobin (SpCO%), methaemoglobin (SpMet %), total haemoglobin concentration (SpHb [g dL^{-1}]) and pulse rate (PR). Acquires absorbance signals from each wavelength allowing the non-invasive measurement of total Hb concentration (SpHb) and to distinguish between HbO_2, HHb, COHb, MetHb. In addition, other parameters such as perfusion index (PI), plethysmograph variability index (PVI), and oxygen content ($SpCaO_2$) are also calculated (Nicholas et al. 2015).

1.4.2 *In-vivo* Calibration

The measurement of blood components such as SaO_2, COHb, MetHb, and total Hb, vary depending on the method of measurement used. Several studies have compared laboratory methods with each other demonstrating that Hb measurements from the same blood sample can differ significantly due to inter-device variation. Rivas Chirino and colleagues (2006), measured Hb in human patient undergoing liver transplantation using Coulter and blood

gas analyser found a consistently higher Hb when measuring by the blood gas analyser (0.3–1.0 gdL^{-1}) (Rivas Chirino et al. 2006). The same inter-devices variation was found by Patel and colleagues testing eight different analysers (Patel et al. 2007). The Hb measurement can also vary within the same device. Gehring and colleagues documented variation of Hb level measured from the same blood sample analysed by two identical CO-oximeters, as large as 1.2 g dL^{-1} (Gehring et al. 2007). Laboratory measurement devices are often compared to a reference standard, but this standard is also subject to error (Bland & Altman, 1999). Therefore, inter- and intra-device variability differences can be a significant source of measurement variation, as well as inherent and expected variability within and between non-invasive and invasive measurement techniques.

Physiologic factors such as the blood source (venous or arterial), site and time of blood draws, blood draw technique, and patient body position are recognized in the clinical literature to add variability to Hb levels. The Radical 7® Pulse CO-oximeter (Radical-7® Pulse CO-Oximeter; Masimo Corp., Irvine, California, USA) has recently developed the ability to adjust the Hb value of displayed to a laboratory reference value. This should have the ability to mitigate some of the potential measurement variations and bring the SpHb value closer to laboratory instruments currently used for clinical decisions. Even though the ability to adjust the Hb value of device to a laboratory reference value is a retrospective application of a data set to a new algorithm. This coined '*in-vivo'* feature does not alter the way of measuring SpHb, but just adds or subtracts a constant to the reported values, based on a laboratory instrument. The measured reference [Hb] value is subtracted from the SpHb displayed on the monitor and this 'offset' value is than keyed into the monitor interface (this could be up to ± 3 g dL^{-1}).With this new feature, clinicians can adjust the non-invasive value at the beginning of a monitoring period to account for individual patient variation and the laboratory reference value (Isosu et al. 2013). With this adjustment the standard deviation and as such the limits of agreement (LoA) between the reference method and the pulse CO-oximeter should be reduced, for example with a significant increase of the

accuracy and reliability of SpHb within 2 g dL^{-1} in 98% of measures when compared with the HbLab obtained with a blood gas analyser (Model ABL700; Radiometer, Copenhagen, Denmark) (Miyashita et al 2014).

1.4.3 Perfusion Index

Clinicians often need to be aware of changes in peripheral perfusion and circulatory status, particularly in patients under general anaesthesia or who are in critical conditions in order to administer the best therapy for the case.

Tissue perfusion index (PI) varies with the quantity of RBCs in the skin microvasculature and it is a reliable indicator of changes in skin blood flow in humans and animals (Hales et al. 1989). The PI, which is derived from the photoplethysmographic signal on pulse oximetry (Lima & Bakker, 2013) is calculated as the ratio (%) between the pulsatile signal of light absorbed by the pulsating arterial inflow and the nonpulsatile signal (light absorbed by the skin, other tissues and venous or nonpulsatile blood) (De Felice et al. 2002). The PI may function as a marker for peripheral perfusion and resembles vasomotor tone, with low and high PI values indicating perfusion below and above average, respectively (van Gender et al. 2013; Lima et al. 2002). PI values in humans, may range from 0.02% (very weak pulse strength) to 20% (very strong pulse strength) (Mohamed et al. 2015), with a foot skin PI value ≤1.24% reported as accurate predictor of severe illness in neonates (De Felice et al. 2002), and a PI value ≤1.4% indicating hypoperfusion in adults (Lima et al.2002). PI has also shown to be useful as a more sensitive and earlier indicator of the sympatholytic effect after epidural block or sympathectomy compared with regional body temperature (Galvin et al. 2006; Ginosar et al. 2009). Microcirculatory disturbance and vascular hyporesponsiveness (i.e. in septic shock) lead to rapid changes on PI value, with poor perfusion usually associated with worse outcome (Lima et al 2002); which is why PI has become one of the resuscitation targets, helping clinicians to detect and to monitor microcirculatory disturbance (van Genderen et al. 2013). The impact of impaired perfusion

on the accuracy of non-invasive Hb measurement performed with pulse CO-oximeter has been evaluated in human patients during different volume and perfusion statues. Adel and colleagues (2018), concluded that a slightly better accuracy of SpHb when compared to LabHb was observed in fluid non-responsive patients compared to fluid responsive patients and in high-PI samples compared to low-PI samples (Adel et al. 2018; Isosu 2013). Conversely, in a volunteer study, Miller et al. had reported different results, where a higher PI (induced by digital nerve block) improved the accuracy of SpHb (Miller et al. 2014). The manufacturer of Masimo Radical-7^{TM} pulse CO-oximeter recommends caution interpreting SpHb results when PI is ≤1.4% (Bridges &Hatzfeld, 2016).

1.4.4 Conformity and Approval

Pulse oximeters are empirically calibrated on normal, healthy volunteers during desaturation studies which are then validated by the authorities [The European Medical Agency (EMA) in Europe; the Food and Drug Administration (FDA) in Canada and United States; the Medicines and Healthcare Products Regulatory Agency (MHRA) in United Kingdom)]. This procedure involves a *conformity assessment*; an audit of the manufacturer's quality system and a review of technical documentation from the manufacturer on the safety and performance of the device (https://www.ema.europa.eu/en/human-regulatory/overview/medical-devices), (Council Directive 93/42/EEC and subsequent modifications).

The study methodology for validating accuracy is outlined in the pulse oximetry International Standard, ISO 80601-2-61-2017 (https://www.iso.org/standard/67963.html). During these studies, warm healthy, young adult volunteers are slowly desaturated to as low as 75% SaO_2. Arterial samples are drawn during stable plateaus, and the SpO_2 pulse oximetry reading values are compared against functional SaO_2 from a laboratory CO-Oximeter. This comparison has routinely been reported in literature in terms of bias and precision. Bias is the mean difference between SaO_2 and SpO_2. Precision is defined as

standard deviation (SD) of the difference between SaO_2 and SpO_2 (Milner & Mathews, 2012; ISO 80601-2-61-2017; Singh et al. 2017). For the values of SpO_2, SpCO and SpMet, accuracy was determined by testing on healthy adult volunteers in the range of 60-100% for SpO_2, in the range of 0-40% for SpCO, and 0-15% for SpMet against a laboratory CO-Oximeter. SpO_2 and SpMet accuracy was determined on 16 neonates ranging in age from 7-135 days old and weighing between 0.5-4.25 kg from which seventy-nine (79) data samples were collected over a range of 70-100% SaO2 and 0.5-2.5% MetHb with a resultant accuracy of 2.9% for SpO_2 and 0.9% for SpMet (www.accessdata.fda.gov).

1.4.5 Pulse CO-oximetry in clinical setting

The ability to rapidly and accurately determine the Hb concentration is important to determine which patients, particularly those undergoing general anaesthesia and surgery, may require treatments such as blood transfusions (Villanueva et al. 2013).

The development of spectrophotometric methods for the non-invasive measurement of blood constituents such as Hb has been a highly desired yet largely unachieved goal of medical bioengineering (Berkow et al, 2011); although, with the introduction of pulse CO-oximetry (Masimo® Corp., Irvine, California, USA) the continuous non-invasive measurement of Hb, referred as SpHb and its components seems to be achieved (Linder & Exadaktylos, 2013).

In human medicine the performance of pulse CO-oximetry and in particularly the SpHb measurement has been assessed by comparing the results obtained from laboratory CO-Oximetry, haematology analyser, blood gas analysers, conductometric POC testing, and spectrophotometric POC devices with those obtained via pulse CO-oximetry.

Results from such studies report an absolute bias of about -0.2 to 0.8 ± 0.6 g dL^{-1} (Berkow et al. 2011;Causey et al. 2011, Lamhaut et. al 2011) and a precision of about 1.1 g dL^{-1} (SD 0.83). However, in comparison to haematology Hb analyser, the percentage of outliers resulted significantly higher with non-invasive than with capillary measurement (Lamhaut

et. Al 2011). Wittenmeier and colleagues 2018, have compared the simultaneous Hb values obtained by pulse CO-oximeter (SpHb; Radical-7 Pulse Co-Oximeter), a blood gas analyser (clinical standard, BGAHb; ABL 800 Flex), and a laboratory haematology analyser (used as reference method, labHb; Siemens ADVIA) in 60 healthy children (0.2–7.6 years of age). The bias/limits of agreement (LoA) between SpHb and labHb in that study resulted as −0.65/−3.4 to 2.1 g dL^{-1}. Interestingly, the bias/LoA between BGAHb and labHb were wider 1.14/−1.6 to 3.9 g dL^{-1} and it was concluded that both methods can show clinically relevant differences from the reference method, but that the non-invasive pulse CO-oximetry measurement of Hb agrees more with the reference method than the measurement of Hb obtained by using a blood gas analyser (Wittenmeier et al. 2018).

Conversely, Hiscock and colleagues reported that the SpHb values obtained from pulse CO-oximeters (Masimo® Rad-7 and Masimo® Pronto-7) and HemoCue photometers (201) diverged from the standard laboratory CO-Oximeter, by mean variances of -0.03 g dL^{-1} [95% confidence interval (CI) and LoA -3.0, 2.9 g dL^{-1}] and 0.08 g/dL (95% CI; and LoA -1.3, 1.4 g dL^{-1}) (Hiscock et al. 2015).

To date, the Radical-7™ Pulse CO-oximeter has been approved by the USA Food and Drug Administration, and by EMA for the non-invasive measurement of Hb in human, reporting a difference in Hb concentrations of ±1 g/dL^{-1} from the standard laboratory values in adult patients with Hb concentration ranging between 8 to 17 g dL^{-1} (Moore et al. 2013, von Schweinitz et al. 2015).

In human medicine, the use of pulse CO-oximetry technology and particularly the SpHb monitoring has been shown to reduce blood transfusion frequency in orthopaedic surgery (Ehrenfeld et al. 2014; Awada et al. 2015) and abdominal cancer surgery (Kamal et al. 2016) patients.

In fact, in a surgical setting in which blood loss may not be apparent or difficult to properly estimate, a continuous rather than intermittent Hb monitoring may provide an earlier warning, which is useful in the decision-making process. In this sense, SpHb represents a

good indicator of a need to measure invasively Hb if a decrease of more than − 0.5 g dL^{-1} is used as cut off (Applegate et al. 2020).

While the use of pulse CO-oximetry is being increasingly reported in human anaesthesia (Lamhaut et al. 2011; Skelton at al. 2013; Moore at al. 2013; Nicholas et al. 2015; De Rosa et al. 2020), only a limited number of studies have been published in veterinary species namely, dogs (Read et al. 2016), late gestation pregnant sheep (Quinn et al. 2013) and horses (Zoff et al. 2019).

Read and colleagues compared the SpHb values obtained from Radical-7™ against jugular venous blood samples analysed by a CO-oximeter laboratory haematology analyser (ADVIA 120; Siemens Healthcare GmbH, Germany) and reported that the Radical-7 Pulse CO-oximeter underestimated LabHb by 3.1 g dL^{-1} (bias) and had a wide LoA (-1.5 to 7.5 g dL^{-1}) concluding that pulse CO-oximeter cannot be safely used as the sole determinant to direct clinical decision-making for dogs (Read et al. 2016).

The study performed on ovine assessed the accuracy of the signal extraction technology (SET) of the Masimo Rad-7 pulse CO-oximeter for SpO_2 measurement against laboratory CO-Oximeter based SaO_2 values obtained from arterial blood gas analyses, investigating the failure rate of the pulse CO-oximeter, the accuracy and LoA, (Bland & Altman's analysis) and the effect of mean arterial blood pressure (MAP), perfusion index (PI) and haemoglobin (Hb) concentration on accuracy (regression analysis) (Quinn et al. 2013). The results of this study indicated that pulse CO-oximeter measurements tend to underestimate oxyhaemoglobin saturation compared to laboratory CO-Oximetry with a bias (mean difference) of 2% and precision (standard deviation of the differences) of 6%. Accuracy appeared to decrease when SpO_2 was <75%, however the authors believe that sample was too small for statistical comparisons. Among the other findings Quinn and colleagues reported that PI had minor influence on the accuracy of SpHb values obtained, however MAP was negatively correlated with SpO_2 bias. Quinn and colleagues concluded that Masimo SET pulse CO-oximeter provided reliable and continuous monitoring of arterial

oxyhaemoglobin saturation in anaesthetized pregnant sheep (Quinn et al. 2013).

In veterinary medicine, the most recent study is a retrospective one that involved equine species by comparing the performance of pulse Masimo Radical-7 CO-oximeter against laboratory CO-Oximeter (Zoff et al. 2019). Low bias and wide LoA were found between Masimo SpO_2 and SaO_2 (bias = -1.4%, LoA= -4.0 to 1.3%), and SpHb and Hb (bias = 0.6. g dL^{-1}, LoA -3.9 to 5.2 g dL^{-1}). When $SpCaO_2$ was compared with CaO_2, a bias of -0.2 ml dL^{-1} and a LoA –of 6.7 to 6.2 ml dL^{-1} were found. The authors of this study concluded that Masimo® pulse CO-oximeter was acceptable for SpHb measurement meeting the Clinical Laboratory Improvement Amendments for people (CLIA) limits. However, the wide LoA found in all compared measures suggest that pulse CO-oximeter cannot be recommended as a substitute of direct measurements.

The *in-vivo* method for adjusting SpHb, proposed recently by the manufacturer of pulse CO-oximeter Radical-7 (Masimo®), which allowed the clinicians to manually adjust the first displayed value of SpHb to match the corresponding Lab-Hb for continuous trending, has been evaluated only in three studies involving human patients (Isosu et a. 2013; Miyashita et al. 2014; Frasca et al 2015) which all concluded that *in vivo* adjustment may represents a significant advance in non-invasive monitoring of Hb as improved the accuracy and limit of agreement for SpHb. To date, no study has carried out the same evaluation in veterinary species.

1.5 METHOD-COMPARISON RESEARCH

In medicine new or revised versions of measurement techniques are developed on a regular basis, but one common issue with new instrumentation or clinical tests is their agreement with existing instruments or tests. In fact, before replacing an old device with a new one, for example a blood pressure device, it is essential to know whether the results of the two devices are similar. Method comparison research aims to evaluate the validity of a new monitor against an established reference technique by measuring its accuracy (the way in which an observed value of a quantity agrees with the true value) and precision (a measure of the extent to which repeated observations conform) (Watson & Petrie, 2010; McAlinden et al. 2011; Montenij et al. 2016). Specifically, in regards to the measurement of the 'true' Hb value, this should be obtained by using the reference technique of haemoglobin cyanide (HiCN) methodology, however it requires the waste disposal of large volumes of a reagent containing cyanide, it is bulky, time consuming and surely cannot be used in bedside monitoring. Therefore, less bulky and faster but reliable techniques for Hb determination have been developed by testing the agreement with a reference method. Subsequently the agreement between two measurement techniques, rather than validating the experimental technique against a perfect reference, have been carried out, letting only conclusions about interchangeability between the experimental and reference technique to be drawn (Giavarina, 2015; Montenij et al. 2016).

Saying that, if the disagreement between two devices is sufficiently small and the new device has advantages over the old one (for example because it is cheaper or less invasive), it is possible to replace the old method by the new one or use the two devices interchangeably.

Many methods have been developed to assess the agreement between two measurement methods, but the existing approaches can be classified into three main categories; (1) *hypothesis testing approach*, which tests the departure from the perfect agreement; (2) *index approach,* which includes the first commonly used correlation coefficient (CC), the

coefficient of determination (CD) and many more, (3) *interval approach* with the earliest approach in this category being the Bland-Altman (B&A) (Liao, 2015).

Outline guidelines on the design and analysis studies such the one reported in the present thesis, is based on Abu-Arafeh et al. 2016, and more recently on Riou 2018. Moreover, the American Society of Veterinary Clinical Pathologists (ASVCP) published guidelines for performing method comparison studies, recommend calculating the correlation coefficient (*r*) to help determine the appropriate statistical tests (Arnold et al. 2019).

1.5.1 Correlation Coefficient and Coefficient of Determination

The degree of association measured by a correlation coefficient is commonly abbreviated to *r*. It is sometimes called Pearson's correlation and measures linear associations between two variables on a scale that varies from + 1 through 0 to – 1. If the value of a variable increases and so does the value of the other variable, the correlation is recognised as positive and a full correlation between two variables would be expressed by + 1. On the opposite, if the value of a variable increases but the other variable value decreases, the correlation is recognised as negative and a full negative correlation would be expressed by -1. In case of a complete absence of correlation, the *r* value would be equal to 0 (***Figure 1-11***).

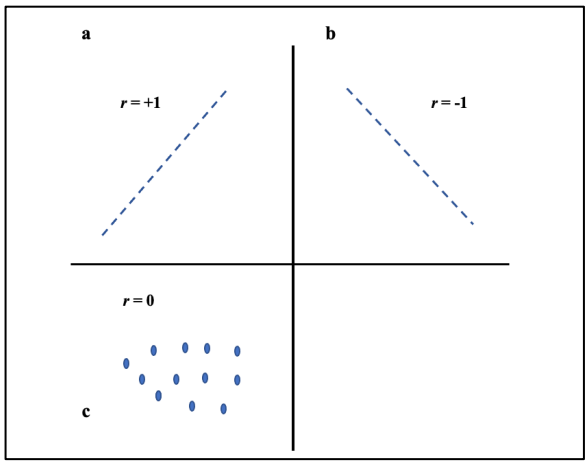

Figure 1-11 Scatter plot examples. (a)positive correlation, **(b)** negative correlation, **(c)** no correlation.

The correlation coefficient is sometimes criticized as having no obvious intrinsic interpretation, and researchers sometimes report the square of the correlation coefficient.

This r^2 is termed the "coefficient of determination". It can be interpreted as the proportion of variance in 1 variable that is accounted for by the other (Rodgers & Nicewander 1988). For example a r of 0.42 correspond to a r^2 of 0.18, meaning that in 18% of the variation can be explained by the relation between the two variables examined, however as more than 80% is not explained by this correlation there must be one or more other relevant factors that are related (Schober et al 2018). In interpreting the coefficient of determination, note that the squared correlation coefficient is always a positive number, so information on the direction of a relationship is lost.

Many studies use the product–moment correlation coefficient (r) between the results of two measurement methods as an indicator of agreement, with the null hypothesis that the measurements performed by using the two methods are not linearly related (Giavarina, 2015). Notably, by using correlation coefficients such investigations are often analysed inappropriately, as the (r) measures the strength of a relation between two variables, not the agreement between them. In fact, as the change in scale will not affect the correlation, it will certainly affect the agreement, and data which seems to be in poor agreement can produce quite a high correlation. Furthermore, the test of significance may show that the two methods are related, but it would be surprising if two methods designed to measure the same quantity were not related (Bland & Altman, 1986).

1.5.2 Bland-Altman

In 1983 Douglas G. Altman and Martin Bland (B&A) set out their view regarding the correct analysis and misconception regarding the Pearson correlation coefficient, they proposed an alternative analysis based on the quantification of the agreement between measurements, by studying the mean difference and limits of agreement (LoA) (Bland & Altman, 1983; 1986). In particular, Bland and Altman highlighted the need to assess two aspects of agreement: how well the methods agree on average and how well the measurements agree for individuals. As an example in regards to the first assumption, if

one of the methods reads lower than the other for half of the subjects but higher for the other half of subjects, the net average discrepancy (the difference between measures on the same subject) is close to 0, despite the discrepancy for individuals being high. The average agreement, or bias, can be estimated by the mean of the differences for individuals, with a *t* test conducted against the null hypothesis of no bias. Estimates of bias then can be reported with 95% confidence intervals (CIs), computed as the mean difference ± 1.96 × standard error of the differences. The LoA summarises the agreement for individuals, which involves the analysis of the variability of the differences. In case of a reasonably normal distribution of the differences (assessed by a histogram), and provided that the level of discrepancy does not depend on the level of the characteristic being measured, then a 95% LoA can be computed as the mean of the differences ± 1.96 × standard deviation (SD) of the differences (Bunce 2009).

In regards to the second assumption, as the B&A plot is a simple scatterplot of the difference between the measurements against their averages (Odor et al., 2017), this should be looked at to detect whether there seems to be any relationship between discrepancy and the level of measurement [e.g. increasing discrepancy between reference and tested method with increased Hb level (***Figure 1-12 a*** simulated data) or increasing variability of differences between instruments with increased Hb level (***Figure 1-12 b*** simulated data)].

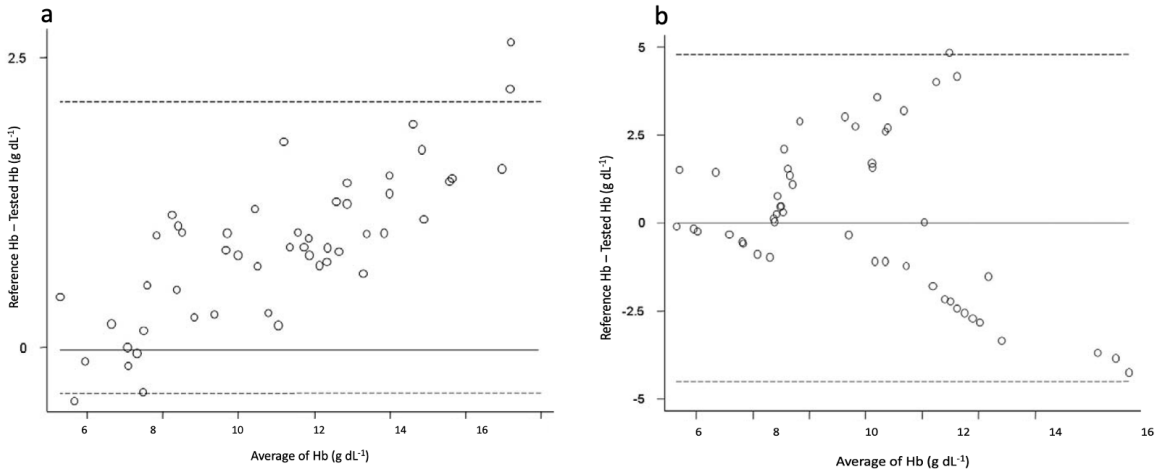

Figure 1-12a-b Bland-Altman Scatter plots simulating (a) an increase of discrepancy with the increase of haemoglobin concentration, (b) an increase of variability with the increase of haemoglobin concentration.

The B&A scatterplots on the x axis represent the average of a pair of measurements (method A + method B/2), and the y axis shows the difference between the two paired measurements (method A – method B); a line of perfect agreement (0 bias), and two external dashed lines indicating the limit of agreements (LoA) (within which approximately 95% of all population differences would lie) (Bland & Altman, 1999).

The 95% LoA quantifies the range of values that can be expected to cover agreement for most of the subjects and guides the clinician as to whether the two methods agree sufficiently for use in clinical assessment. How small a LoA should be to conclude that two methods agree sufficiently is a clinical and not a statistical decision, that should ideally be made in advance of the analysis (Bunce 2009).

Subsequently, B&A (Bland & Altman, 1999) have provided a modification for analysing repeated measures under stable or changing conditions, where repeated data were collected over a period of time (e.g., consecutive Hb concentrations during surgery). This tool allows traditional bias and LoA, but also within-subject and between-subject variability. When repeated measurements (replicantes) are made for each subject, it is inefficient to estimate average bias and LoA using only the first measurement, rather than for all measurements.

The Guidelines for Reporting Reliability and Agreement Studies (GRRAS) by Kottner et al. (2011) comprise of a comprehensive checklist of fifteen items that support the transparent reporting of agreement and reliability studies (Kottner et al. 2011). More recently, these aspects have been commented upon in more general terms (Gerke et al. 2018).

1.5.3 Assessment of Repeatability

The repeatability (i.e., the single operator or intralaboratory precision) and reproducibility (the interlaboratory precision), of a measurement are important attributes that should be quantified to enalbe the user to understand the variability of test results. In order to investigate the repeatability of measurements, a repeatability study should consider at least

two measurements per subject under identical conditions (same measurement method or same observer or rate). The ASTM E691 Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a test method in terms of repreatibility and reproducibility specifies analysis of variance (ANOVA) for repeated measures to quantify the single-operator or multilaboratory errors (ASTM E691-15, 2015). Once repeatability is quantified, and the possibility of bias between measurements excluded, the agreement between measurements made on the same subject would depend only on the within-subject standard deviation, which measures the size of measurement errors (Bartlett & Frost 2008).

Once repeatibility is assessed, a *systematic effect*, which implies that there is a tendency for the differences in the paired results to go in one direction (e.g., to be positive if the variable of interest is numerical) or a *random effect*, which implies that sometimes the differences go in one direction and sometimes they in the opposite direction, but they tend to balance out on avarage. To answer this question, we need to calculate the differences between each of the *n* pairs of measurements. Generally, a paired *t-test* to test the null hypothesis that the true means the difference is zero. If the mean of these differences is zero, then it may be concluded that there is no systematic difference between the pairs of results (i.e., on average, the results are reproducible or repeatable, as relevant) (Siegel & Castellan, 1988).

1.5.4 Trending Analysis

An increased number of studies focus on the ability to track changes in Hb, in addition to determining its absolute value (Chang et al. 2019; De Rosa et al. 2020).

Although the Bland-Altman analysis can provide insights within a trending analysis, the two most frequently used graphical statistical methods for trending analysis are; the 4-quadrant (4Q) concordance and the polar plot methodology. The 4Q method plots the change in experimental Hb (e.g.; ΔSpHb) against the change in reference Hb (ΔHb ref). The percentage

of data points in which ΔSpHb and ΔHb ref change in the same direction is called 4Q concordance.

Four-Quadrant Concordance Analysis

The 4Q plot was first used for the description of trending capabilities in studies comparing cardiac output measurement technologies by Perrino and colleagues (Perrino et al.1994; Perrino et al. 1998). This statistical tool illustrates the trending ability of two devices (reference and studied) on measuring a quantity, allowing for the fast visual assessment of accuracy besides information about the magnitude and direction of changes detected by both technologies. ***Figure 1-13*** shows an example of a 4Q plot with 9 artificial data points.

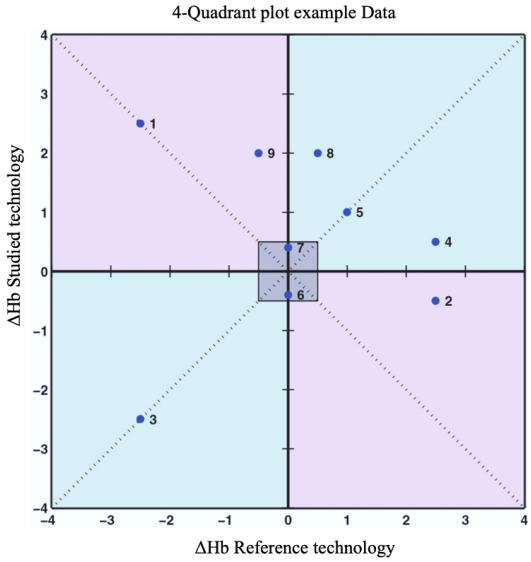

Figure 1-13 Four quadrant plot reporting 9 artificial data for explanation purpose

Once data are plotted, the points will be distributed in one of the four quadrants. When both the studied technology and the reference technology indicate an increase or decrease in [Hb], the respective data points will appear in the upper right and lower left respectively, quadrant of the 4-quadrant plot. For example point 8 in *figure 1-13*, indicates a reference method detected a [Hb] change by 0.5 g dL^{-1}, whereas the studied technology showed a change of about 2 g dL^{-1}. Despite the two technologies both indicating a positive change in [Hb], the numerical values are far from being equal. Points with equal numerical values will be located on the 45° diagonal within the quadrant (the dotted line in the blue quadrants). When

measurements of ΔHb ref methodology and ΔHb-studied methodology disagree in regards to the direction of change, the respective data points will appear in the upper left or lower right quadrant of the plot (purple areas). The higher the number of data points in the blue quadrants compared with those in the purple quadrants, the higher the concordance between the measurement devices is. In order to quantify the level of concordance between the two methodologies, the proportion of data points in the quadrants representing direction of change agreement (blue quadrants) in all data points need to be calculated. Very small changes in [Hb] readings can be attributed to noise, and are not supposed to contribute sufficiently to, or even disturb trending analysis; to mitigate the inconvenience the concept of *exclusion zone* was introduced. In the exclusion zone, which is represented by the center of the 4Q plot, as it is not clear whether the points measured are related to real changes in the measurements of Hb or are mainly driven by noise, data are excluded from the analysis of the trending ability. In *figure 1-13* the exclusion zone was set as 0.5 g dL^{-1} and marked as a grey area. The limitations of the 4Q concordance analysis are related to the lack of cutoff values defining good, acceptable, and poor agreement. Moreover, as the results of the 4Q plot depend on the time interval between consecutive measurements, the plot can be influenced by choosing different time intervals for the analysis.

1.5.5 Clinical Significance Analysis

Error Grid Analysis

In 1987 Clarke and colleagues (Clarke et al, 1987) developed the error grid analysis (EGA), originaly to assess the performance of blood glucose values obtained from a blood glucose meter in comparison to reference values obtained from 'gold standard'. As opposed to the traditional statistical method comparison approaches such as; Deming regression, Passing-Bablok regression or Ordinary Least Squares regression. Subsequently the 'Clarke Error Grid' or EGA, was also used for analysing non-glucose analytes comparison. The Error Grid Analysis consists of a predefined grid of several regions that indicate whether the

measured test *vs* reference data paired observations are within clinically acceptable boundaries, with five typically different zones called zone A to E (***Figure 1-14***). In particular, zone A should contain values that are within 20% of reference values and are considered "clinically accurate/acceptable". Zone B will contain values that are greater than 20% of the reference value but would "not lead to inappropriate treatment"; zone C contains values that would "lead to unnecessary treatment"; zone D contains values that would indicate a "potentially dangerous failure" to detect low or high analyte value (e.g.; Hb); last, zone E will contain values that would confuse treatment of low or high (e.g. Hb) and vice-versa.

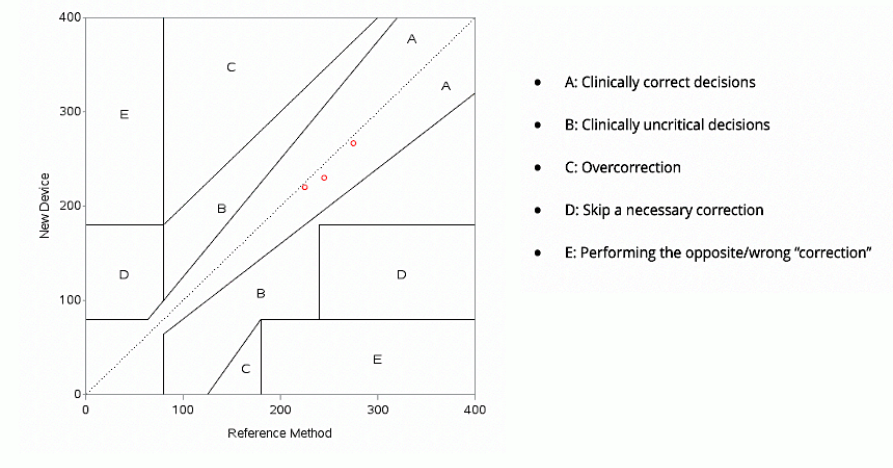

Figure 1-14 Clarke Error Grid for venous *vs* Fingerstick Glucose (mg dL^{-1})

In 2011 this statistical tool was applied by Morey and colleagues as a method to assess the clinical significance of the accuracy of Hb measurement, where by placing the reference and tested measurements in x and y axes respectively, they divide the grid in 3 zones: A, B, and C, which is based on the American Society of Anesthesiologists guidelines and clinical relevance for blood transfusion (ASA guidelines 2006; Morey et al. 2011). With this setting, zone A begins with an isthmus of a 10% error on either side of a perfectly accurate measurement between the Hb values of 6 and 10 g dL^{-1}. Furthermore, as below 6 g dL^{-1}, transfusion will likely occur as well as Hb measurements >10 g dL^{-1} where the likely result in no transfusion, the accuracy at these levels is considered less important. Zone C, signifies where errors may be critical. If the 'true' Hb is <6 g dL^{-1}, but the device reports a value >10

g dL^{-1}, PRBCs may be withheld, resulting in possible harm to the patient. Conversely, if the reference Hb is >10 g/ dL^{-1}, but the device reads <6 g dL^{-1}, an unnecessary transfusion would occur. Zone B is between zone A and zone C, where errors might result in harm depending on the circumstances, but not as serious as a zone C error.

1.5.6 Cohen's Kappa

To further assess the agreement between pulse CO-oximetry and IDEXX VetStat, Cohen's kappa coefficient (κ) statistics was calculated. The Cohen's kappa is a statistical coefficient that measures the agreement between two raters, who each classify N items into mutually exclusive categories negating the agreement by chance. Although not perfect, this value is superior to percent agreement because it accounts for random agreement (Morey et al 2011).

2 OBSERVATIONAL PROSPECTIVE STUDY (Agreement)

2.1 Thesis aim and hypothesis

Perioperative bleeding remains a major complication during and after surgery, resulting in increased morbidity and mortality (Ghadimi et al. 2016). In dogs as in humans the intraoperative estimation of total blood loss is anything but easy (Clark et al. 2010; Nuttall et al. 2000) and beside a patient's vital parameters, the measurement of Hb, SaO_2, and CaO_2 are important determinants for transfusion decision making (Colquhoun et al 2012; Frasca et al 2015).

Although the 'gold standard' of Hb determination remains the cyanomethaemoglobin assay (HiCN), the complexity of this methodology does not allow its routine use in a hospital setting as it is time intensive and labour intensive, and expensive (Berkow et sl. 2013).

While the laboratory-based methodologies such as the haematology analyser, the laboratory CO-Oximetry, the conductometric point of care, and the spectrophotometric point of care are effective for assessing [Hb], SaO_2, and CaO_2, all require a blood sample which needs to be analysed, introducing a delay in result obtainment and therefore are ineffective for tracking on going changes (Joseph et al 2016).

In this scenario a non-invasive methodology to analyse the haematological parameters offers the advantages of continuous monitoring, allowing clinicians to react to ongoing changes that may reduce over or under transfusion events (Berkow 2013; Iamaizumi et al 2016).

Recent technological advances in the field of multiwavelength pulse oximetry (Masimo Rainbow SET® Radical-7 Pulse CO-oximeter, Masimo Corp., Irvine, CA, USA) enables non-invasive and real-time measurements of Hb, SaO_2, and CaO_2 as derived pulse CO-oximeter Hb concentration (SpHb), SaO_2 (SpO_2), and calculated pulse CO-oximeter derived oxygen content ($SpCaO_2$), and perfusion index (PI) values. The spectrophotometry-based technology of the probe allows continuous haemoglobin determination; as light passes

through the tongue, it is received by the photodetector and generates electrical signals that are processed by a Masimo algorithm. This provides an estimation of [Hb] based on its absorbance characteristics (De Rosa et al. 2020).

Pulse CO-oximetry technology has been tested in various clinical scenarios, with results from human and veterinary studies regarding its accuracy (Macknet et al. 2010; Miller et al. 2011; Nguyen et al. 2011; Read et al. 2016; Zoff et al. 2019) to date have been inconsistent, and two meta-analyses concluded with an alert about clinical decision making based on these device (Kim et al. 2014; Hiscock et al. 2015).

As a strategy to improve the accuracy of SpHb, a software feature called *in-vivo'* adjustment has recently been introduced in the new version of pulse CO-oximeter, which uses a haemoglobin value provided by invasive methods (Miyashita et al 2014; Frasca et al 2015). Based on a search of scientific literature published to date about *in-vivo* adjustment applied to SpHb values, no study in veterinary medicine has been carried out. Consequently, the aim of this study was to evaluate if *in-vivo* adjustment using the Hb value provided by blood gas analyser measurement from arterial blood sampled at the first SpHb measurement, would increase the accuracy of the monitor of the subsequent haemoglobin values obtained by Masimo Radical-7 pulse CO-oximeter, when compared with the standard value provided by the laboratory blood gas analyser.

2.2 Ethical Approval

This study received University ethical approval (REF13a/16). No further medical or pharmacological treatment, except for the routine clinical treatments, was carried out at any point and in any of the animals enrolled. All animals enrolled in the study underwent general anaesthesia and their lungs were mechanically ventilated during the surgery.

During hemilaminectomy among other complications, major blood loss is common (Nowicki 2014) and as the neurological outcome is also linked to normotension (Vale et al. 1997; Guha et al. 1989), the assessment of arterial blood pressure is mandatory. Considering

that the non-invasive blood pressure (NIBP) technique is inherently inaccurate (MacFarlane et al. 2010), in all the animals enrolled in this study a peripheral arterial catheter was placed for measuring invasively the arterial blood pressure as per routine anaesthetic management. In fact, the recognized 'gold-standard' for blood pressure measurement in small animal clinical anaesthesia is the invasive measurement via cannulation of a peripheral artery (Bodey & Michell 1996; Valerio et al. 2006).

Considering that all patients' lungs were mechanically ventilated during the surgery, arterial blood gas sampling was performed under the Veterinary Surgeons Act 1966, as per routine monitoring. All arterial catheters were removed in all dogs at the end of the surgery.

2.3 Informed Consent

Informed owner consent, to allow anonymous use of patient clinical data, was obtained upon admission to the hospital and recorded via a signed form.

2.4 Materials & Methods

2.4.1 Hypothesis

The null hypothesis of this study was that '*in-vivo'* adjustment using the first invasive haemoglobin value provided by a blood analyser could increase the accuracy of subsequent SpHb, $SpCaO_2$ measurements performed by Masimo rainbow-SET® Radical-7 pulse CO-oximetry™, when compared to the laboratory blood gas analysis (LabHb and $LabCaO_2$) in dogs undergoing general anaesthesia for spinal surgery. The primary endpoint of the study was to compare the accuracy in measuring the Hb concentration using a non-invasive method (Masimo rainbow SET® Radical 7 Pulse CO-oximetry™) to the values provided by the invasive method (IDEXX VetStat® analyser). As a second endpoint, the influence of PI, mean arterial pressure (MAP) and tongue thickness on the accuracy of SpHb and SpO_2 readings after *in vivo* adjustment was evaluated. The third endpoint was to assess the

trending ability of pulse CO-oximetry on measuring haemoglobin by comparing it to the trend provided by IDEXX VetStat® analyser.

2.4.2 Experimental Design

The study was designed as a prospective single-site field study performed in the UK at the University of Glasgow, Small Animal Hospital between March 2017 and March 2019 where a method comparison study was conducted.

2.4.3 Sample Size Calculation

The population size to compare SpHb and [Hb], considering the power of the test set at 0.8 (1- β error), with a significant level of 0.05, α (error of 5%) and a correlation coefficient of r =0.5 was about 30 patients according to a previous study in human patients (Riess & Pagel; 2016). The sample size for repeated measurement for a population with a mean of Hb of 12 dL^{-1} with an estimated SD of 3.5 g dL^{-1} with a significant level of 0.05 α (error of 5%) and to detect a mean difference (bias) of ±1 g dL^{-1} was about 96 sample. The animals enrolled and the samples collected were rounded to 39 dogs, and 39 time-matched blood samples before *in-vivo* adjustment and 104 time-matched blood samples after *in-vivo* respectively, in order to compensate for possible lost and to reduce the number of blood sampling for each dog. Due to the adjustment of the first measured SpHb to first measured VetStat [Hb] value, the data pairs from the first time point were not included in the linear regression and Bland-Altman analysis for repeated measurements for [Hb] and SpHb.

$$N = \frac{\sigma^2 (z_{1-\beta} + z_{1-\alpha/2})^2}{(\mu_0 - \mu_1)^2}$$

$$N = \frac{(3.9^2 (0.84 + 1.96)^2}{(12 - 10)^2}$$

$$N = 30$$

μ_0 = population mean
μ_1 = mean of study population
N = sample size of study population
σ = variance of study population
α = probability of type I error (usually 0.05)
β = probability of type II error (usually 0.2)
z = critical Z value for a given α or β

$$N = \frac{\sigma^2 (z_{1-\beta} + z_{1-\alpha/2})^2}{(\mu_0 - \mu_1)^2}$$

$$N = \frac{(3.5^2 (0.84 + 1.96)^2}{(12 - 13)^2}$$

$$N = 96$$

μ_0 = population mean
μ_1 = mean of study population
N = sample size of study population
σ = variance of study population
α = probability of type I error (usually 0.05)
β = probability of type II error (usually 0.2)
z = critical Z value for a given α or β

Sample size calculation for assessing the agreement between two methods of haemoglobin measurement: *Laboratory IDEXX VetStat [Hb]* vs *Masimo Radical-7 pulse CO-oximetry [SpHb]*. The Equations here reported were used to calculated the numbers of blood sample pairs needed to assess the accuracy of the *SpHb* by Bland-Altman analysis *before* ***(a.)*** *and after* ***(b.)*** *in vivo-adjustment.* Further information are reported in the text.

2.4.4 Inclusion & Exclusion Criteria

Based on physical examinations preanaesthetic haematology and biochemistry analysis, only dogs classified as the American Society of Anesthesiologists (ASA) physical status II and with a body condition score (BCS) ranging between 4 and 6 out of 9 were included (Laflamme, 1997). Animals with preoperative haemoglobin levels outside the Masimo's validated range for humans [(Hb) 8 – 17 g dL^{-1}], or outside the paediatric rainbow® reusable pulse CO-oximeter sensor range (10 – 50 kg) were excluded. A total of 39 dogs of various breeds, undergoing spinal surgery for decompressive hemilaminectomy were included in this study. Animals that had diagnostic procedures and surgery carried out within the same day or same general anaesthetic event were also excluded. If the same animal had hemilaminectomy performed twice, only at the time of the first event was considered for the present study.

2.5 Description of the method

Food, but not water, was withheld for approximately 8 hours prior to general anaesthesia. Dogs were premedicated with a variety of drugs based on clinical requirements and anaesthetist preference. Once sedation was achieved, an intravenous (IV) cannula (Biovalve safe, Vygon, UK), was aseptically placed into a peripheral vein and general anaesthesia was induced with either propofol (PropoFlo Plus, Zoetis, UK) or alfaxalone (Alfaxan, Jurox, UK) titrated to effect. Orotracheal intubation was performed to maintain anaesthesia with either isoflurane (IsoFlo, Zoetis, UK) or sevoflurane (SevoFlo, Zoetis, UK) vaporised in either oxygen or in a mixture of medical air and oxygen. The fractional inspired oxygen (FIO_2) was maintained between 0.7 - 0.98 and was delivered via a rebreathing system (Datex Ohmeda, GE Healthcare, Chalfont St Giles, UK). Volume controlled, mechanical ventilation was instituted in all dogs (Aestiva/5 Datex Ohmeda, GE Healthcare, Chalfont St. Giles, UK) with inspiratory: expiratory ratios between 1:2.5 and 1:3, with tidal volumes and respiratory rates

(f_R) adjusted based on the animal's requirements to maintain an end-tidal carbon dioxide concentration ($PE'CO_2$) between 35 to 45 mmHg and peak inspiratory pressures kept below 20 cmH_2O. Hartmann's solution (Vetivex II Solution, Dechra, UK) was administered at 5 ml kg^{-1} $hour^{-1}$ throughout anaesthesia. Hypotension (mean arterial blood pressure less than 60 mmHg) was managed with a fluid bolus (5 to 10 ml kg^{-1} over 10 minutes) or drugs (antimuscarinic or dopamine) or both where appropriate. As part of standard care procedures for continuous invasive arterial blood pressure (IBP) monitoring and intermittent arterial blood samples, 20- or 22-gauge cannula (Biovalve Safe, Vygon, UK), depending on the size of the dog, was aseptically placed in a dorsal pedal artery. All dogs were administered fentanyl (Fentadon 50 µg ml^{-1}, Dechra, UK) and ketamine (Anesketin 100 mg ml^{-1}, Dechra, UK) as variable rate IV infusions throughout surgery. During anaesthesia, heart rate (HR*),* f_R , IBP, $PE'CO_2$, FIO_2, end-tidal isoflurane ($FE'Iso$) or end-tidal sevoflurane ($FE'Sevo$) were continuously monitored using a multiparameter monitor (S5 Compact Anaesthesia Monitor; Datex Ohmeda, Clafont St. Giles, UK) and recorded every 5 minutes. For IBP, the transducer was zeroed to atmospheric pressure and positioned at the level of the right atrium (point of shoulder). By using a clip-type pulse co-oximeter probe (paediatric rainbow® reusable Sensor DCI-P SC 200, Masimo Corporation, CA, USA) positioned on the apex of the tongue laterally to the median groove, SpHb, SpO_2, SpOC, and PI values were displayed on a pulse CO-oximeter monitor (Masimo Radical-7, Masimo Corporation, CA, USA). All data generated were automatically and continuously recorded using the Masimo collect program (RDS Docking Station. Model/Cat # Radical-7) throughout the duration of the procedure. A calliper was used to measure the maximal vertical dimension and thickness of each dog's tongue at the level of the median groove, prior to placement of the probe, and this value was recorded in centimetres (cm). The probe sensor was left in place for 5 minutes until the device obtained a stable measurement. If no reading was obtained at the location after three attempts, it was recorded as an undetectable reading. Once the reading had stabilised, the SpHb value recorded as *before in-vivo* adjustment was the one displayed by the pulse CO-

oximeter 15 minutes from the first reading; at this point, an arterial blood was simultaneously sampled via the dorsal pedal arterial catheter. Thereafter, every 30 minutes until the end of the surgery, arterial blood was sampled, and pulse CO-oximetry detected values were recorded simultaneously. For each arterial blood gas (ABG) measurement, to prevent hemodilution or contamination of the blood sample, the first 2 ml of arterial blood was withdrawn from the attached extension tubing and three-way tap connected to the arterial catheter, using a 2.5 ml syringe and discarded; a further 1 ml was collected anaerobically using arterial blood gas lyophilized calcium-balanced heparin syringes (BD Preset, Becton-Dickinson, UK). The maximum volume of arterial blood collected from each dog was 0.5% of total circulating blood volume (estimated as 90 ml kg^{-1}) (Haneda & Horiuchi, 1986). All ABG samples were analysed within 1 - 2 minutes from collection using a fluorescence-based blood gas analyser and oximeter (IDEXX VetStat, Electrolyte and Blood Gas Analyser, Laboratories, Inc., ME, USA) using respiratory blood gas cassettes (Electrolyte 8 Plus, IDEXX Laboratories, Westbrook, USA) that were kept at a room temperature between 4°C and 30°C. The VetStat uses red and infrared light from one light-emitting diode (LED) and two laser diodes. This light is directed through an optically polished window to the blood in the cassette. The light is partially absorbed and reflected by the erythrocytes to a photodiode. The intensity of reflected light varies in a well-defined way, with the blood Hb and SaO_2 used in their measurement. Haemoglobin saturation (SaO_2) and [Hb] were measured. Temperature correction was not applied, and samples were analysed at 37° C. The VetStat blood gas analyser was installed to the manufacturer's specifications and serviced as recommended with daily and monthly quality control checks, using quality control products supplied by the manufacturer (IDEXX Laboratories, 2019, IDEXX Laboratories, 2010). Results were shared anonymously with IDEXX's SmartServiceTM allowing for automatic software updates and remote calibration of the analysers. The before *in-vivo* adjustment [Hb] values, obtained by the VetStat were used to calibrate the pulse co-oximeter using the *in-vivo* feature of this device. The measured reference [Hb] value from the VetStat was

subtracted from the SpHb displayed on the monitor and this 'offset' value was keyed into the monitor interface (this could be up to ± 3 g dL^{-1}). Arterial oxygen content was calculated manually, using the arterial partial pressure of oxygen (PaO_2), SaO_2, and [Hb] values obtained from ABG and by using the following equation:

$$CaO_2 \text{ ml } dL^{-1} = ([Hb] \text{ g } dL^{-1} \times SaO_2/100 \times \text{Hüfner's constant}) + (0.0031 \text{ ml } dL^{-1} \text{ mmHg}^{-1} \times \text{arterial partial pressure of oxygen } (PaO_2) \text{ mmHg})$$

The Hüfner's constant of 1.3 ml g Hb^{-1} was applied in the Masimo Radical-7 algorithm (Masimo Corporation 2014) as used for calculation purposes. The same investigator maintained intraoperative anaesthesia in all dogs, recorded all the relevant variables, and collected the arterial blood to be analysed. A different investigator analysed all the arterial samples according to the manufacturer instructions

2.6 Statistical Analysis

All data was transferred from paper records onto a digital spreadsheet (Microsoft Excel, USA).

2.6.1 Statistical Software

Most statistical analyses were performed using MedCalc (MedCalc, v17.1 64-bit, Ostend, Belgium) except for Error-Grid analysis and Four-quadrant plot that were performed using an open-source program R 3.6.2 statistical software (GNU General Public License) https://www.r-project.org/.

2.6.2 Normality Testing

A Shapiro-Wilk test was performed to confirm normality of the continuous variables.

2.6.3 Bland Altman Analysis

The agreement between methods [Hb] provided by IDEXX VetStat® Blood Gas Analyser, and SpHb provided by the tested method, Masimo rainbow SET® Radical 7 Pulse CO-oximetry™, was assessed by calculating the bias and displayed using Bland-Altman plot (1986; 2012) for each variable (Hb; SpO_2; CaO_2) of paired data before *in-vivo* adjustment. In this context, bias (error), SD (precision) and LoA (mean difference ± two standard deviations of the differences) between the two methods were calculated. Our predefined Δ was ±1.0 g dl^{-1} (O'Reilly 2011; Johnson et al. 2020).

2.6.4 Bland Altman Analysis for repeated measures

For paired data after *in-vivo* adjustment, Bland-Altman analysis for repeated measures per subject was performed (Bland & Altman, 2007; 2012). The bias which value could be either negative (the pulse CO-oximeter overestimated the value compared to the reference method) or positive (the pulse CO-oximeter underestimated the value compared to the reference method), precision, and LoA between the two methods were calculated. Our predefined Δ was ±1.0 g dl^{-1} (O'Reilly 2011; Johnson et al. 2020).

2.6.5 Regression Analysis

The effect of mean arterial blood pressure (MAP), PI and tongue thickness on accuracy (the difference between [Hb] and SpHb and difference between SaO_2 and SpO_2), was assessed by regression analysis.

2.6.6 Clinical Significance Analysis – Error Grid Analysis

Clinical significance analysis was evaluated by plotting paired Hb values using the graphical technique described by Clarke et al. and adapted by Morey et al. for Hb (Clarke et al. 1987; Morey et al. 2011). The reference method is plotted on the abscissa versus the SpHb measures on the ordinate. Zones are defined that demarcate acceptable and unacceptable errors. Of particular interest is the Hb concentration range of 6–10 g dL^{-1}, which involves critical decisions concerning blood transfusions and within which range only a 10% error is generally regarded as permissible (ASA guidelines 2006; Morey et al. 2011).

2.6.7 Trending Capability

Trending capability of the pulse CO-oximeter to follow [Hb] measured by the reference method (IDEXX VetStat® Blood Gas Analyser) was assessed in addition to accuracy analysis. A modified four-quadrant plot method was used to test the magnitude and directionality of the change in [Hb] values. A central exclusion zone for values ± 0.5 g dL^{-1} of change in [Hb] was applied to rule out pairs of data with minimal difference. For the four-quadrant plot, the change in [Hb] measured by the reference method was plotted against the change in [Hb] measured by the test method in a regression style. The concordance rate of the four-quadrant plot simply describes the number of points which lie within the 2 quadrants of agreement (lower left and upper right).

2.6.8 Cohen's Kappa

The Cohen κ statistic for agreements to transfuse beyond chance, between pulse CO-oximetry device (SpHb) and VetStat [Hb], was calculated as recommended by Morey et al. (Morey et al. 2011). Results were collected into five categories based on if they fell below, within or above the reference interval. The agreement was considered *poor* if $\kappa < 0.2$, *fair* if $\kappa = 0.21$ to 0.40, *moderate* if $\kappa = 0.41$ to 0.60, *substantial* if $\kappa = 0.61$ to 0.80 and *almost perfect or perfect* if $\kappa > 0.81$ (Landis and Koch, 1977).

2.7 Number of Samples per dog

Based on a prior study performed in human patients (Applegate et al. 2012), which reported a median of 4 blood samples to allow an average of 3 trend calculation per patient using a standard deviation of 1.5, a median of 4 blood samples per dog was performed (of which, one was before the *in-vivo* calibration).

2.8 Sample Test Ranges

The range over which samples were tested on IDEXX VetStat® analyser are listed below in ***Table 2-1***.

Parameter	Unit	Dynamic Range
pH	pH unit	6.6–7.8
PCO_2	mmHg	10–200
PO_2	mmHg	10–700
tHb	g dL^{-1}	5-25

Table 2-1 Range over which samples were tested on IDEXX VetStat™

2.9 Results

Fifty-two animals were eligible for the study; of these 13 were excluded (2 due to the incompleteness of the anaesthetic record, 2 had PI values missed, in 1 dog the thickness of the tongue was not recorded, and in 8 dogs arterial catheterisation was not successfully achieved). The remaining 39 animals were included in the study and their data therefore analysed. A flow chart according to consolidated standards of reporting trials (CONSORT) is presented in ***Figure 2-1***

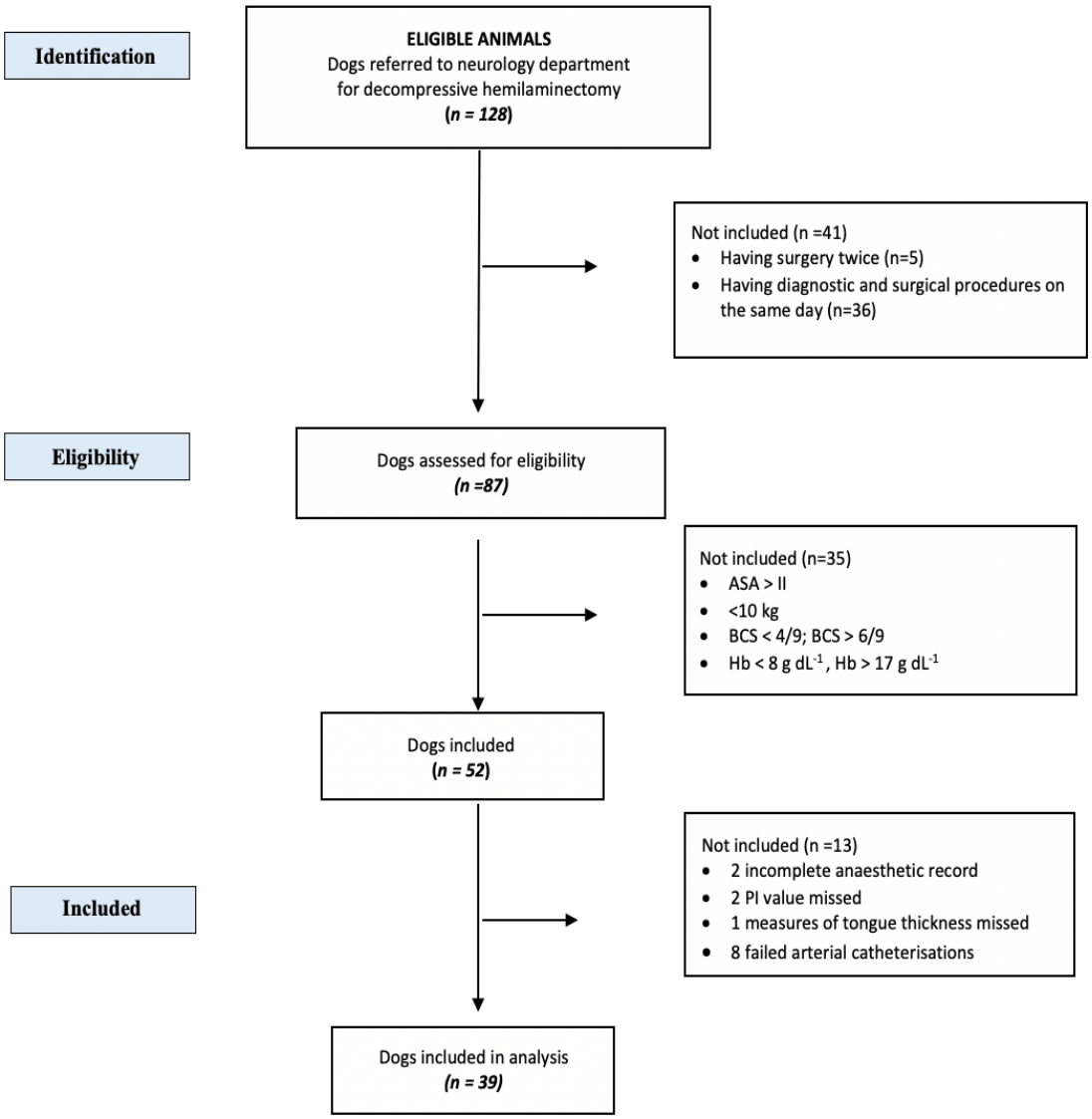

Figure 2.1 CONSORT chart for animal recruitment

The means and standard deviations and range for weight and age of dogs were 20.8 ± 10.4 kg (10.2 to 55.5 kg) and 84 ± 33 months (6 to 156 months) respectively. Median BCS was 5 (range 4 to 6 out of 9) (***Table 2-2***).

(*n*= 39)	mean* ± SD	range
Age (month)	84 ± 33	6 – 156
Weight (kg)	20.8 ± 10.4	10.2– 49.5
PaO_2 (mmHg)	460 ± 96	238 – 626
$PaCO_2$ (mmHg)	37 ± 8	36 –44
BCS (over 9)	5	4 – 6
ASA	2	1 – 2
Gender		
male	3	
female	4	
male neutered	13	
female neutered	19	
Anaesthesia time (minutes)	198 ± 89	79 – 380
Number of samples (median)	4 ± 1.5	3 – 8
Tongue thickness (cm)	0.7± 0.2	0.3 – 1

Table 2-2 Demographic and clinical data of 39 dogs. Data are described as mean and standard deviation (SD), range, number of animals or proportion (%). [Hb] haemoglobin concentration measured by the reference method (VetStat); (SpHb) haemoglobin concentration measured by pulse CO-oximetry; (BCS) body condition score (over 9); (ASA) American Society of Anesthesiologists. * Mean if not indicated differently.

Breeds distribution of the 39 dogs included in the study are reported below (***Table 2-3***).

(Total *n* = 39)	*n*
Basset Hound	1
Beagle	3
Cavalier King Charles Spaniel	2
Cocker Spaniel	3
Collie	1
Crossbreed	7
Dachshund	1
Doberman	1
English Bulldog	2
French Bulldog	6
German Shepherd	2
Golden Retriever	1
Great Dane	1
Greyhound	1
Labrador	4
Lhasa Apso	1
Pointer	1
Welsh Corgi	1

Table 2-3 Breed distribution among the 39 dogs included in the data analysis

A total of 143 time-matched blood samples and pulse CO-oximetry values were obtained, of which 39 pairs of data for [Hb] measurements were obtained before *in-vivo* adjustment and 104 pairs of data for [Hb], SaO_2, and CaO_2 measurements were obtained after *in-vivo* adjustment. The average number of measurements (pulse CO-oximetry measurements simultaneously to arterial samples) performed per dog was four (range 3-8). In all dogs, pulse CO-oximetry provided detectable SpHb, SpO_2 and SpOC readings after 2 (1-3) attempts from the first attempt of probe positioning on the tongue, with a prevalence in delayed reading in dogs administered dexmedetomidine and methadone as premedication (7 out of 10 dogs premedicated only with dexmedetomidine and methadone *vs* 3/23 dogs premedicated with acepromazine and methadone, and 0/6 dogs premedicated with dexmedetomidine acepromazine and methadone).

The recorded PaO_2 was 460 ± 96 (238 – 626) mmHg and 480 ± 84 (227 – 657) mmHg before and after *in-vivo* adjustment respectively. The recorded $PaCO_2$ was between 36–44 mmHg for all samples. A total of 23 dogs were sedated with a combination of acepromazine (ACP Injection 2 mg ml^{-1}, Elanco, UK) with a dose range of 0.005 - 0.02 mg kg^{-1} and methadone (Comfortan Solution for Injection,10 mg ml^{-1}, Dechra, UK), range 0.3 – 0.4 mg kg^{-1} administered either IV or intramuscularly (IM). In 10 dogs, sedation consisted of a combination of dexmedetomidine (Dexdomitor 0.5 mg ml^{-1}, Vetoquinol, UK), with a range 0.5 - 5 µg kg^{-1} and methadone, range 0.3 – 0.4 mg kg^{-1}, and 6 dogs were sedated with a combination of acepromazine, dexmedetomidine, and methadone (same dose ranges). General anaesthesia was induced with IV propofol in 25/39 dogs (dose range 1 – 3 mg kg^{-1}) and with IV alfaxalone in 14/39 dogs (dose range 1 – 2.5 mg kg^{-1}) and maintained with either isoflurane in 30/39 dogs or sevoflurane in 9/39 dogs.

2.9.1 Before in-vivo Adjustment

Thirty-nine samples were taken from 39 dogs (1 from each) of which 39 data pairs were analysed. The mean (± SD) values for [Hb] and SpHb were 10.6 ± 1.8 g dL^{-1} and 13.4 ± 1.7 g dL^{-1}, respectively; normality distribution, assessed with Shapiro-Wilk is reported in (***Figure 2-2***).

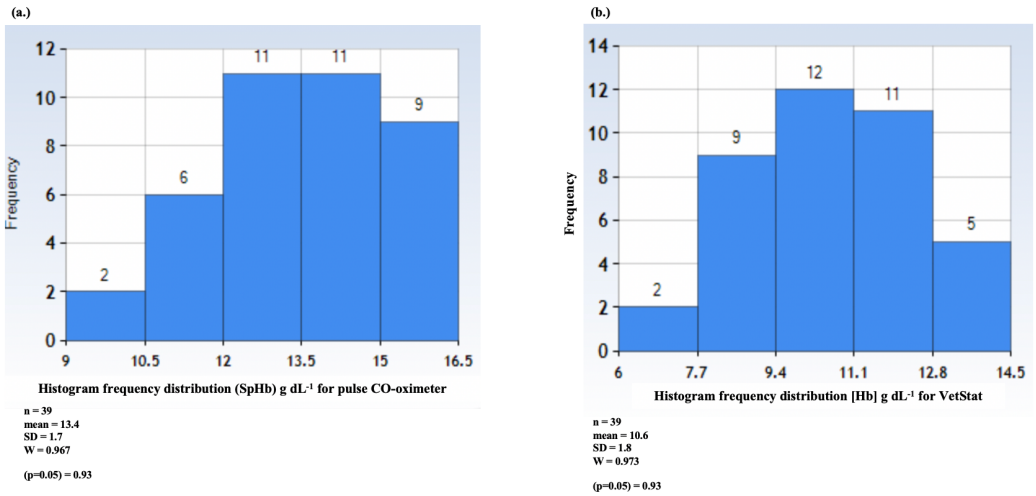

Figure 2-2 Histogram frequency distribution before *in-vivo* adjustment of haemoglobin measured **(a.)** by pulse CO-oximetry, **(b.)** by VetStat. Normal distribution confirmed with W close to 1, and large *p*. Standard deviation (SD); number of measurements (*n*) of haemoglobin (Hb) as g dL^{-1} performed using each methodology; SpHb haemoglobin-based pulse CO-oximetery; [Hb] haemoglobin-based VetStat.

With the quantile-quantile plot (QQ-plot) a graphical presentation of the distribution for Hb concentration measured by the two methods has been assessed, which resulted normally distributed (**Figure 2-3; 2-4**).

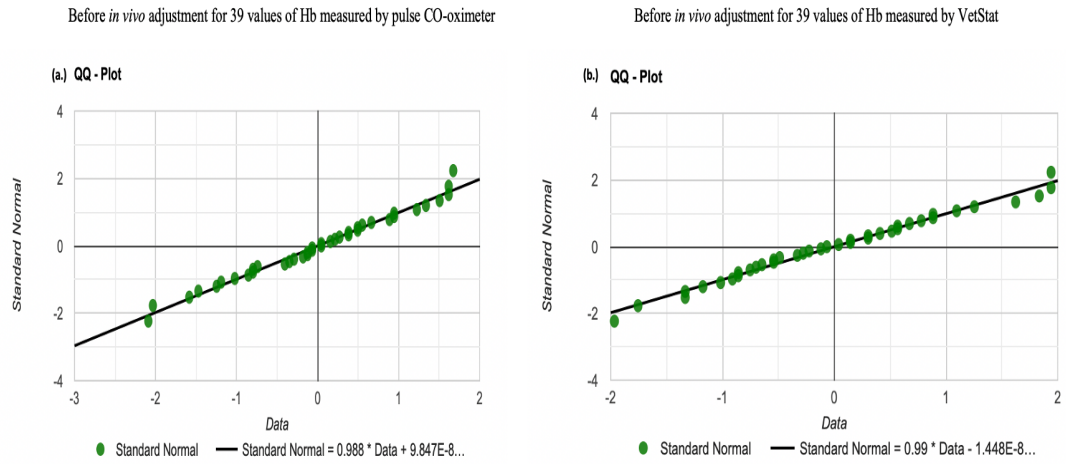

Figure 2-3 Quantile-quantile plot (QQ-plot) for haemoglobin (Hb) measured by **(a.)** pulse CO-oximeter, **(b.)** VetStat laboratory blood gas analyser.

Since there was no evidence of a systematic effect, the intraclass correlation coefficient (ICC) was estimated by creating a sample of 78 pairs of observations by adding to the original sample of 39 pairs and a set of 39 pairs of observations in which the value in each pair from the original sample are interchanged (Petrie & Watson, 2013).

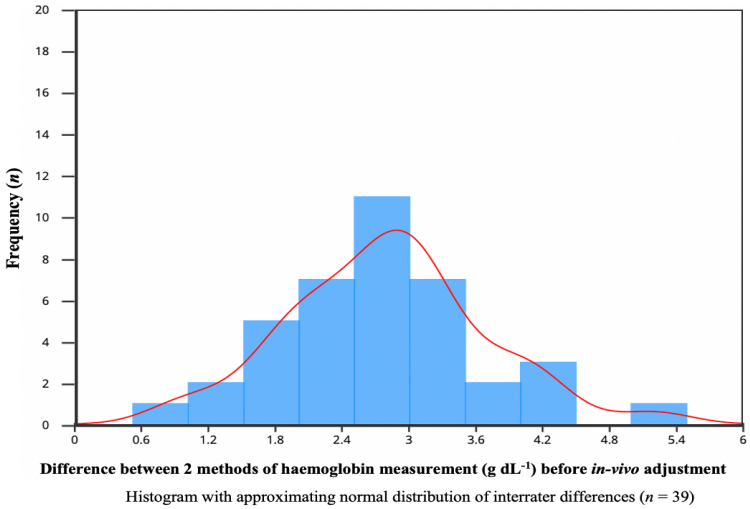

Figure 2.4 Distribution plot of differences between measurements by VetStat and pulse CO-oximeter. The red line represents Normal distribution.

The estimated Pearson correlation coefficient calculated using all 78 pairs of observations created was 0.8, i.e., from this intracalss correlation coefficient, 88.4% of the variability in the observations is due to the difference between the pairs, and 11.6% is due to difference within a pair. The coefficient of determination (r^2 values) was 0.78. Bias, LoA and 95% confidence intervals (CI) are reported in ***Table 2-3***. The Bland and Altman plot showed that the values obtained by using the pulse CO-oximeter overestimated [Hb] compared with the value obtained by the VetStat and the bias was consistent across the range of measured values (***Figure 2-5***). The difference between [Hb] and SpHb was < 1 g dL^{-1} in 1/39 (2.6%)

pairs, 1 - 2 g dL^{-1} in 7/39 (17.9%) pairs, 2 - 3 g dL^{-1} 18/39 (46.1%) pairs, > 3 g dL^{-1} 13/39 (33.3%) pairs. Distribution plots of differences between measurements by VetStat and pulse CO-oximeter before *in-vivo* adjustment are reported in ***Figure 2-5***.

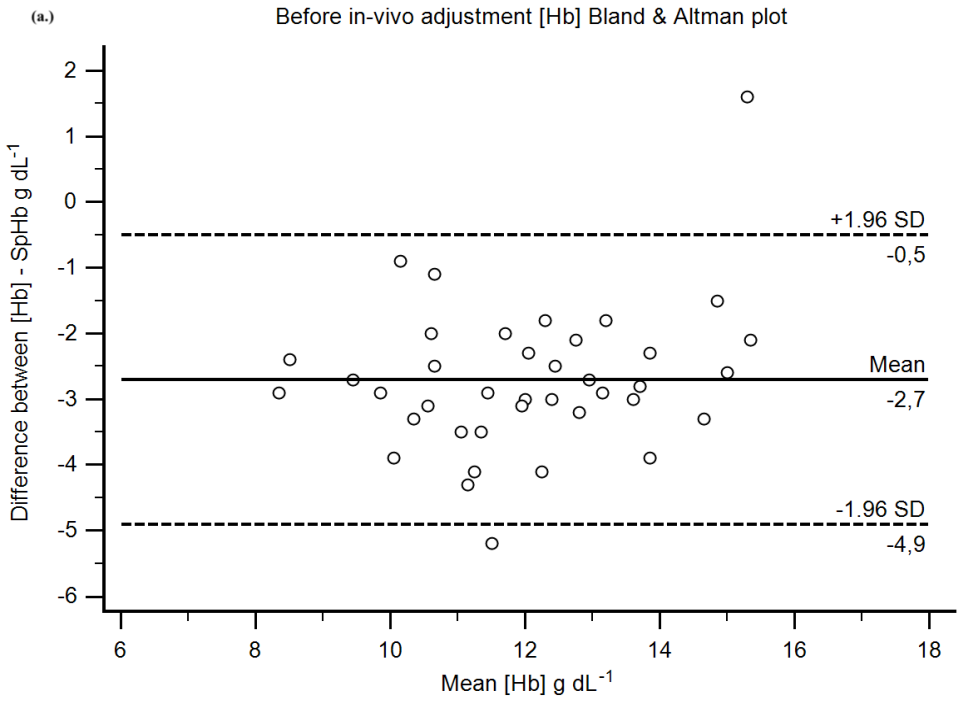

Figure 2-5 Bland-Altman plot to assess agreement between Vetstat haemoglobin concentration ([Hb]) and pulse CO-oximeter derived haemoglobin (SpHb) before *in-vivo adjustment* (39 data pairs). The mean difference between the two methods (bias) is shown as a solid black line; the 95% limits of agreement are shown as dashed lines. Negative bias indicates that the pulse co-oximeter overreads compared to the reference method.

The mean ± SD values for CaO_2 and SpOC were 15.3 ± 2.4 ml dL^{-1} and 17.7 ± 2.6 ml dL^{-1}, respectively. There was a tendency for SpOC to be overestimated and SpO_2 to be underestimated compared to the reference method.

2.9.2 After in-vivo Adjustment

One hundred and four samples were taken from 39 dogs (average 3 samples from 39 dogs) of which all 104 data pairs were analysed. The mean (± SD) values for [Hb] and SpHb, were 10.3 ± 1.8 g dL^{-1} and 10.6 ± 1.5 g dL^{-1}, respectively. Bias, LoA and 95% confidence intervals (CI) are reported in ***Table 2-4***. The Bland-Altman plot showed that the pulse CO-oximeter overestimated [Hb] compared with the VetStat and the bias was consistent across the range of measured values (***Figure 2-6***). Once the threshold for accuracy (± 1 g dL^{-1}) is applied to our data, difference between [Hb] and SpHb was < 1 g dL^{-1} in 100/104 (96.1%) pairs, and 1 - 2 g dL^{-1} in 4/104 (3.84%) pairs. The correlation coefficient (*r*) between simultaneous [Hb] and SpHb measurement pairs was depicted in a scatter plot, resulting as 0.97. To assess SpHb accuracy in time (*n*=104), consecutive r^2 values were calculated for all measured intervals and resulted as 0.94.

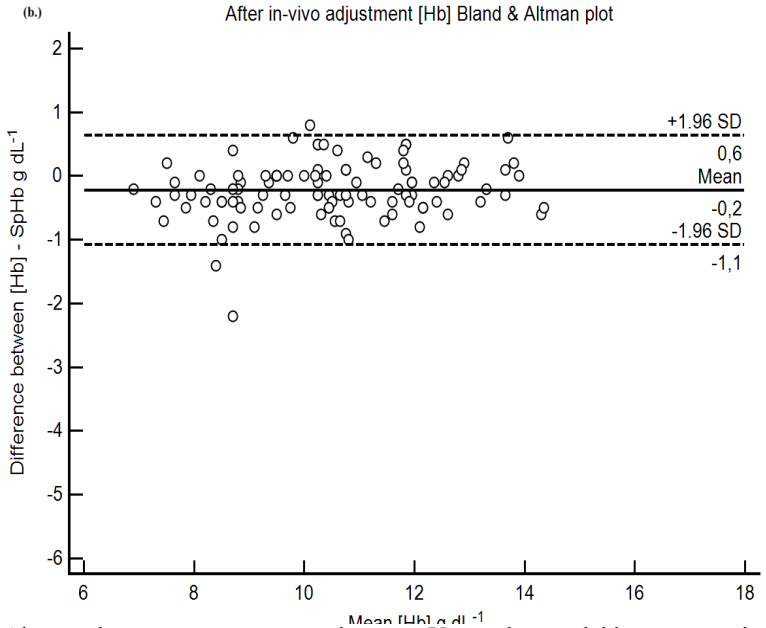

Figure 2-6 Bland-Altman plot to assess agreement between Vetstat haemoglobin concentration ([Hb]) and pulse CO-oximeter derived haemoglobin (SpHb) after *in-vivo* (104 data pairs) adjustment. The mean difference between the two methods (bias) is shown as a solid black line; the 95% limits of agreement are shown as dashed lines. Negative bias indicates that the pulse co-oximeter overreads compared to the reference method.

There was a tendency for SpOC and SpO_2 to be underestimated compared to the reference method following *in vivo* adjustment (***Figure 2-7***). Regarding arterial oxygen saturation, the mean ± SD values for SaO_2 and SpO_2, were 100 ± 0% and 98.6±1.05% respectively. SaO_2 had a bias of 0.9% and LoA of -0.81 to 2.6% *after in-vivo* (***Figure 2-8***).

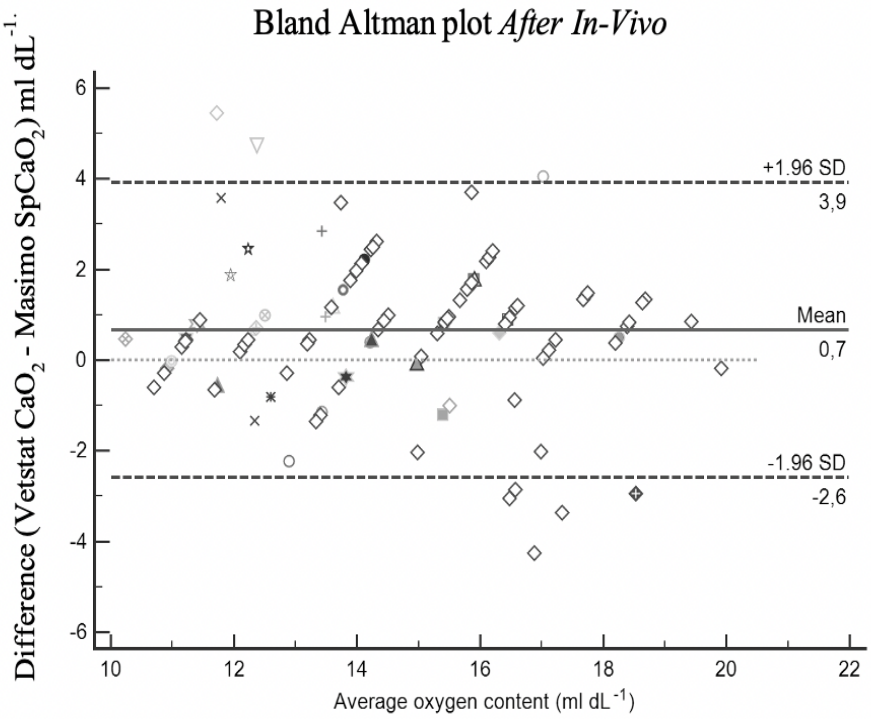

Figure 2-7 Bland-Altman's plot *after in-vivo* adjustment. Comparison of oxygen content measurements by laboratory blood gas analyser (VetStat[CaO_2]) and pulse CO-oximeter (Masimo[$SpCaO_2$]). 140 data pairs with *in-vivo* adjustment were represented with different symbols, each symbol represents a data pair. The light dashed line represents the 0-bias value. The solid horizontal line represents the bias (mean difference). The outer dark dashed lines represent the 95%limits of agreement. Bias of 0.66 ml dL^{-1} with a LoA of -2.59 to 3.91 ml dL^{-1}. CaO_2 oxygen content, SD standard deviation.

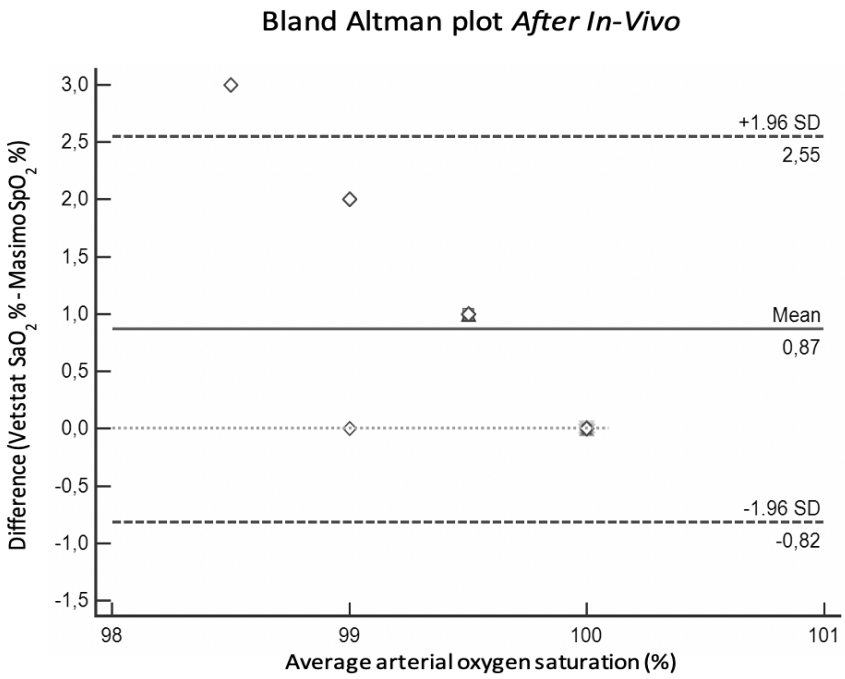

Figure 2.8 Bland-Altman's plot after *in-vivo* adjustment. Comparison of haemoglobin oxygen saturation measurements by laboratory blood gas analyser (VetStat[SaO_2]) and pulse CO-oximeter (Masimo[SpO_2]).The middle solid horizontal line represents the bias (mean difference). All data pairs were superimposed on the 5 points reported. The light dashed line represents the 0-bias value. The outer dashed lines represent the 95%limits of agreement. Bias of 0.86% and LoA of -0.81 to 2.54%. SaO_2 haemoglobin oxygen saturation, SD standard deviation

2.9.3 Regression Analysis

The results for regression analysis are shown in ***Figure 2-9***. The coefficients of determination (r^2), a statistical measure of how close the data are to the fitted regression line, was used to assess the effects of MAP, PI and tongue thickness on the difference between [Hb] and SpHb, resulting in a r^2 values of 0.016, 0.0061 and 0.0044 respectively. Values for r^2 assessing the effects of MAP, PI and tongue thickness on the difference between SaO_2 and SpO_2 were 0.008, 0.024 and 0.093 respectively. These low r^2 values are indicative of poor correlation. Nevertheless, in 35 of 104 occasions, SpHb values were recorded when the associated PI value was ≤1.4%.

Higher PI values were recorded in dogs that were given acepromazine and methadone [PI 1.5% (0.35 -4.5)] or acepromazine, dexmedetomidine, and methadone [PI 1.2% (0.62 – 5.6)] as premedication, compared with dogs to which dexmedetomidine and methadone were administered [PI 0.8% (0.34 – 1.7)].

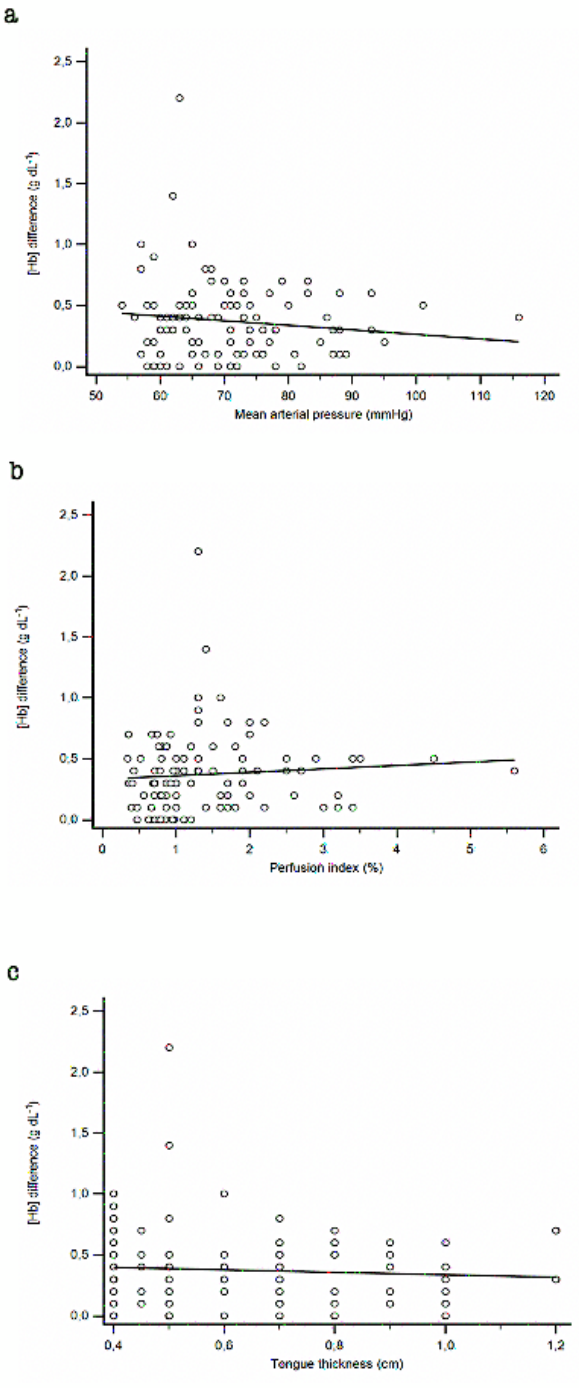

Figure 2-9 Regression analysis to assess the effect of (a.) mean arterial pressure (MAP) (mmHg), (b.) perfusion index (PI) (%), and (c.) tongue thickness (cm) on the difference in haemoglobin concentration ([Hb]) (g dL-1) measured between the 2 methods tested (VetStat [Hb] and pulse co-oximeter derived haemoglobin (SpHb)), after in-vivo adjustment. Values for r^2 for MAP, PI and tongue thickness were 0.016, 0.0061 and 0.0044, respectively.

All parameters assessed *before* and *after in-vivo* adjustment have been summarised in the following table ***[Table 2-4].***

[Hb]	Bias (95% CI) g dL^{-1}	95% LoA (95% CI) g dL^{-1}
Before *in-vivo* adjustment	-2.7 (-3.1; -2.3)	-4.9 (-5.5; -4.3) to -0.5 (-1.1; 0.1)
After *in-vivo* adjustment	- 0.2 (-0.3; -0.1)	-1.1 (-1.2; -0.9) to 0.6 (0.5; 0.8)
CaO_2	Bias (95% CI) ml dL^{-1}	95% LoA (95% CI) ml dL^{-1}
Before *in-vivo* adjustment	-2.2 (-3.2; -1.2)	-8.3 (-10.0; -6.5) to 3.8 (2.1; 5.6)
After *in-vivo* adjustment	0.6 (0.4; 1.0)	-2.6 (-3.0; -2.0) to 3.9 (3.8; 4.4)
SaO_2	Bias (95% CI) %	95% LoA (95% CI) %
Before *in-vivo* adjustment	1.3 (1.0; 1.6)	-0.8 (-1.4; -0.2) to 3.4 (2.8; 4.0)
After *in-vivo* adjustment	0.9 (0.7; 1.0)	-0.8 (-1.2; -0.6) to 2.6 (2.3; 2.9)

Table 2-4

Bland–Altman analysis comparing measurements of haemoglobin concentration [Hb], calculated arterial oxygen content (CaO_2) and arterial oxygen saturation (SaO_2), between a reference blood gas analyser (VetStat) and the Masimo Radical-7 pulse co-oximeter. Bias (mean difference), 95% limits of agreement (LoA) and associated 95% confidence intervals (CI) are reported before (39 data pairs) and after (104 data pairs) in vivo adjustment. Negative bias indicates that the pulse co-oximeter over-reads compared to the reference method.

2.9.4 Clinical Significance Analysis –Error Grid Analysis

The paired haemoglobin values provided by the Radical-7 pulse CO-oximeter (SpHb) and the haemoglobin values provided by the VetStat [Hb] after the *in-vivo* adjustment were also plotted using the Error Grid Zone Analysis as proposed by Morey and colleagues which accounts for the clinical significance of the difference. This was divided in 3 regions with clinical meaning; Zone A (green) is the maximum agreement area, Zone B (white) is better agreement area, Zone C (red) is the error area (Morey et al. 2011). Performing Morey's Error Grid analysis on all sample points, the majority of data points 102/104 (~98%) were in Zone A; in Zone B were 2/104 (1.9%); while in critical Zone C were 0/104 (0%). Considering that, it can be said that SpHb and [Hb] are in strong agreement (***Figure 2-10***). The two values within Zone B were recorded during very low PI values (<0.3). Zone A; in Zone B were 2/104 (1.9%); while in critical Zone C were 0/104 (0%). Considering that, it can be said that SpHb and [Hb] are in strong agreement.

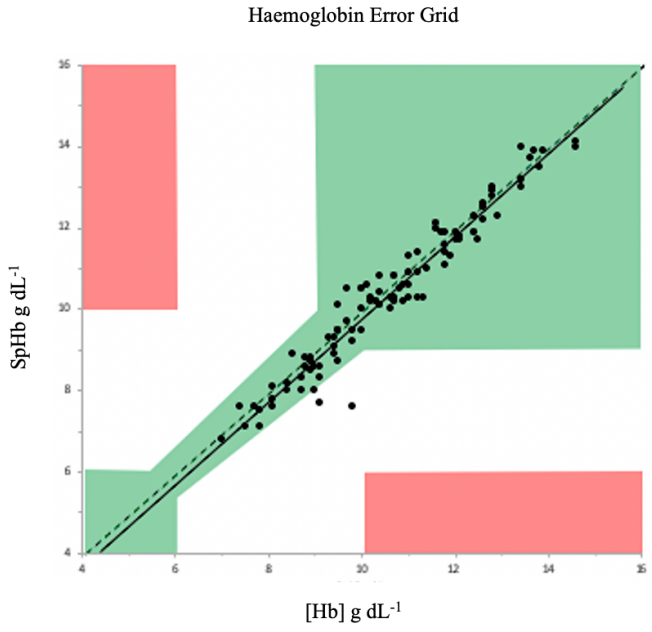

Figure 2-10 Haemoglobin error grid. Haemoglobin measured by a laboratory blood gas analyser [Hb] was plotted against haemoglobin measured by pulse CO-oximetry (SpHb). The dashed line represents a line of equality and the solid black line represents the data regression line. Zone A (green area) deviation of ± 10% of tested versus reference method, zone C (red area) major therapeutic error, zone B (white area; < 5% of all data pairs should be encompassed in Zone B) in between. The majority of data points should lie in the zone A isthmus. Hb > 10 g dL^{-1} are of little interest since this will not trigger a RBCs transfusion.

2.9.5 Trending Capability

Trending capability of the pulse co-oximeter to follow [Hb] measured by the reference method (VetStat) was assessed in addition to accuracy analysis. A modified 4Q plot was used to test the magnitude and directionality of the change in [Hb] values. A central exclusion zone for values ± 0.5 g dL^{-1} of change in [Hb] was applied to rule out pairs of data with minimal difference that may not reflect a real change in circulating haemoglobin measured by laboratory analyser [Hb] as previously reported by other authors (Applegate et al. 2020). The change in [Hb] measured by the reference method (x axis) was plotted against the change in [Hb] measured by the test method (y axis) in a regression style. Of the 104 data pairs analysed, 22 fell into the exclusion zone and were not analysed further. All the 22 pairs excluded were recorded during low PI state (≤1.4%). Once the mentioned 22 pairs were excluded, the resulting concordance rate was 92.6% (***Figure 2-11***).

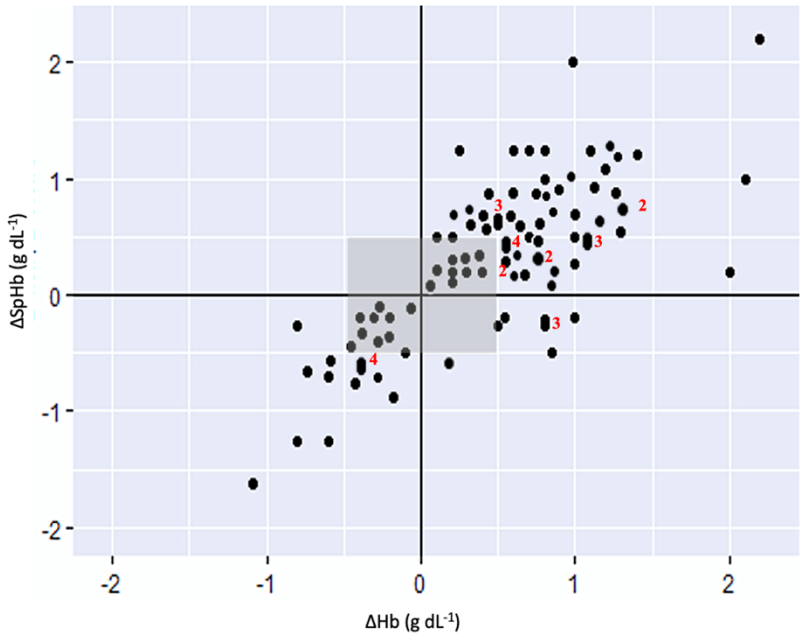

Figure 2-11 Four-quadrant plot for haemoglobin trending of the pulse CO-oximeter compares the consecutive changes in haemoglobin measured by pulse CO-oximeter (SpHb) and measured by laboratory blood gas analyser (Hb). The central exclusion zone was set at 0.5 g dL^{-1}. Of the 104 pairs 22 were excluded with a total concordance rate of 92.6%. Some of the data pairs are superimposed, the numbers of data superimposed have been reported in correspondence to the point/s in red in the picture.

Among the six change directions that did not agree, 5/6 were measured during a low PI state (≤1.4%). The concordance rate of the 4Q plot simply describes the number of points which lie within the 2 quadrants of agreement (lower left and upper right).

2.9.6 Cohen's Kappa

Cohen's Kappa value of 0.65 (substantial agreement) if used to identify time points with haemoglobin concentration less than 10 g dL^{-1} based the arterial blood gas values.

2.10 Discussion

The results of this study into pulse CO-oximeter in canine patients undergoing a general anaesthesia for spinal surgery, confirms that prior to *in-vivo* adjustment, the measurement of SpHb is not as accurate as it is reported in humans (Moore et al. 2013). In fact, the agreement resulting from the Bland-Altman analysis in the present study before *in-vivo* adjustment showed a bias of -2.7 g dL^{-1}, meaning that the pulse CO-oximeter tended to over-read compared with the VetStat and this difference was great enough to be clinically relevant.

To date, in veterinary medicine, only one other study has investigated the accuracy of SpHb in dogs (Read et al. 2016), who compared [Hb] measured by a laboratory haematology analyser with SpHb measured by pulse CO-oximetry, but without applying the *in-vivo* adjustment or trending analysis. These authors reported a bias + 3 g dL^{-1} with wide LoA (-1.55 to 7.56 g dL^{-1}), and 64.5% of the SpHb values differed from the paired [Hb] by > 2 g dL^{-1}. In the present study, we found a bias of -2.7 g dL^{-1} between the two methods and 79.5% of the SpHb values differed from the paired [Hb] by > 2 g dL^{-1}. Although the bias values reported between the two studies are similar, Read et al. (2016) showed that the pulse CO-oximeter tended to underestimate SpHb compared to [Hb] measured by the laboratory haematology analyser, which differs with our study. In regard to this, one of the possible explanations could be related to a different reference method being used to measure [Hb].

In the present study, the reference analyser (VetStat) used was a microprocessor-based instrument measuring [Hb], by using red and infrared light emitted at 3 different wavelengths (670 nm, 780 nm and 850 nm) (https://www.idexx.pl/files/vetstat-updated-operator-guide-en-gb.pdf), while the Masimo Radical-7™ pulse CO-oximeter emits +7 wavelengths of light between 500 and 1400 nm (https://techdocs.masimo.com/globalassets/techdocs/pdf/lab-5475e.pdf). Although the wavelengths of light emitted by the VetStat are included within the

Masimo Radical-7 range, the specific wavelengths may differ between the instruments and may influence the [Hb] values reported by each device. The reason behind which the VetStat blood gas analyser was chosen instead of a laboratory haematology analyser, (as in Read and colleagues' study), or a laboratory CO-Oximeter is because of the widespread spread of VetStat as a POC equipment in veterinary practices (Bell et al. 2014).

The disparity between the present study and that of Read et al. (2016) could also be related to the type of blood analysed (peripheral arterial blood in the present study vs jugular venous blood in the aforementioned study). In fact, based on previous reports [Hb] measured in arterial samples are expected to be 0.7– 1.0 g dL^{-1} less than those derived from venous samples (Yang et al. 2001).

Furthermore, while Read and colleagues used a disposable rainbow adhesive sensor (R1 25L Adult Pulse CO-oximeter Masimo Corporation, CA, USA), in the present study a reusable clip-type pulse CO-oximeter probe (Rainbow DCI-P SC 400, Masimo Corporation, CA, USA) was used.

Lastly, the use of different anticoagulants might cause a wide variation in [Hb] measurements (Fairbanks et al. 1992; Thomas & Thomas 2005). Read et al. (2016) sampled venous blood and transferred it to a vacuum tube containing ethylenediaminetetraacetic acid (EDTA), whereas in our study we sampled arterial blood directly into lyophilized calcium-balanced heparin syringes. These variations in methodology may account for some of the differences observed between the studies.

To improve the performance of SpHb, some authors have proposed to calibrate the pulse CO-oximeter based on *in-vivo* Hb values gained from other diagnostic methods. In the present study performed in dogs scheduled for decompressive hemilaminectomy under general anaesthesia, the difference between the initial value for SpHb and Hb obtained from an arterial sample

analysed by an arterial blood gas analyser was used for the *in-vivo* adjustment and also as a reference method.

In veterinary medicine, this is the first study which applies the *in-vivo* adjustment technique to SpHb values as proposed by Miyashita (2014) and Frasca (2015).

Following the application of the proposed *in-vivo* adjustment method, the bias for SpHb, was reduced to -0.2 g dL^{-1} and the LoA became narrower. Therefore *in-vivo* adjustment improved the agreement and reliability of SpHb measurements making our results in agreement to human clinical studies, where an improved accuracy and precision of SpHb after *in-vivo* adjustment has been also reported (Isosu et al. 2013; Miyashita et al. 2014; Frasca et al. 2015).

In human medicine literature, different *in-vivo* calibration methods to improve performance of SpHb have been proposed.

Isosu and colleagues (2013), examined the adjustment of SpHb based on Hb values measured by a satellite laboratory CO-Oximeter in 20 Japanese surgical patients, for a total of 92 blood samples collected. In this study, Bland-Altman showed *before in-vivo* adjustment a bias of 0.2±1.5 g dL^{-1} and LoA of -2.8 to 3.1 g dL^{-1} and *after in-vivo* adjustment a bias of 0.7±1.1 g dL^{-1} with LoA of -2.8 to 1.4 g dL^{-1}. The authors concluded that *in-vivo* adjustment may represent a significant advancement in non-invasive monitoring of Hb as it improved the bias, precision, LoA and correlation coefficient compared to satellite laboratory CO-Oximeter measurements.

Miyashita and colleagues (2014) conducted a similar study to evaluate the accuracy of SpHb after *in-vivo* adjustment, using a reference method to measure Hb in a calibrated blood gas analyser, in 19 patients undergoing elective abdominal surgical procedure. Seventy-three Hb measurements were obtained by the R2-25a SpHb sensor (pulse CO-oximeter), when compared to blood gas analyser values used to *in-vivo* adjust; it significantly reduced the percentage of

outliers (13.6% from 32.8% for SpHb), improved the correlation coefficient (0.93 from 0.83 for SpHb), improved the bias 0.16 g dL^{-1} vs 0.68 g dL^{-1} for SpHb), and LoA.

Frasca and colleagues (2015) performed a prospective observational study in patients undergoing elective major surgical procedures where significant blood loss was expected (41 patients and 173 measurements), to compare the accuracy of SpHb adjusted *in-vivo* with the mean of 3 arterial HemoCue measurements and with laboratory values. The authors of this study concluded that *in-vivo* adjusted by means of HemoCue measurements improved precision (1.4 g dL^{-1} for SpHb vs 1.1 g dL^{-1} after *in-vivo*), but without impacting significantly on bias which remained close to 0 g dL^{-1}. In Frasca and colleagues' study, pulse CO-oximeter calibration and reference methods were not the same, but interestingly they reported that also the *in-vivo* adjustment based on laboratory values provided the same results.

After the *in-vivo* adjustment was applied, the pulse CO-oximeter still tended to over-read SpHb compared with the VetStat [Hb] values, however the discrepancy between measurements was significantly reduced following *in-vivo* calibration.

In the Masimo Radical-7 pulse CO-oximeter the proprietary algorithm accounts for the presence of dyshaemoglobins (methaemoglobin and carboxyhaemoglobin), whilst the VetStat analyser used does not discriminate between functional and dysfunctional Hb species; this may have contributed to the wide LoA before *in-vivo* adjustment.

In fact, multi-wavelength CO-Oximeters such as the ABL90 FLEX, ABL800 FLEX, and RAPID Point 500 can detect most common dyshaemoglobins (HHb, O_2Hb, MetHb and COHb) by haemolyzing a small volume of sampled blood, and shining at least 4 wavelengths to calculate the optical absorbance at each wavelength. Similarly to them, the Masimo Radical-7 pulse CO-oximeter by using a multi-wavelength light that passes through living tissues, enables to detected the most common dyshaemoglobin species.

On the other hand, neither the IDEXX VetStat blood gas analyser, nor the non-invasive two-wavelength measurement technologies (e.g. standard pulse oximetry) are able to detect dyshaemoglobins. This will not only lead to falsely elevated oxygen saturation values in the presence of dyshaemoglobins, but will also miss the presence of MetHb and COHb. The use of the two-wavelength measurement technologies should be avoided when the presence of dyshaemoglobins is suspected. There was good agreement between the haemoglobin oxygen saturation measurements from the pulse CO-oximeter and VetStat. However, the range over which this was assessed was narrow due to the clinical nature of the study. Intentional desaturation of patients would be necessary to assess agreement at lower saturation ranges that may have an impact upon the accuracy of the pulse CO-oximeter.

Before *in-vivo* adjustment, the CaO_2 measured by the pulse CO-oximeter was significantly higher than the VetStat with wide LoA, largely due to the inclusion of a higher SpHb in the equation. However, after *in-vivo* adjustment a marked improvement of accuracy was achieved although a wide LoA remained. This means that the precision of the pulse SpOC does not improve in the same way as SpHb following *in-vivo* adjustment. Further investigation to compare SpOC with a HiCN assay or CO-Oximetry based CaO_2 calculations are needed to better understand this phenomenon.

In regard to the PI, this is an indirect indicator of peripheral circulation which results from the ratio of the amplitude of the arterial pulse detected by the pulse CO-oximeter sensor, to the amplitude of non-pulsatile factors such as the veins or subcutaneous fat. As the amplitude of the non-pulsatile factors remains almost the same regardless of the dilatation or contraction of the vessels, changes in the amplitude of the arterial pulse mostly determines the PI. Nevertheless, the PI influence on the accuracy of non-invasive SpHb monitoring is not well defined; Nguyen and colleagues (2011) reported that the SpHb bias decreased when the PI was over 2.0 in patients undergoing cardiac surgery. Similarly, Miller et al. (2012) reported a highly

accurate SpHb measurement in patients with a PI > 2.0 after the peripheral nerve block of their fingers with local anaesthetics. Chung and colleagues in 2014, also reported a significantly increased PI after spinal anaesthesia and a reduction in the discrepancy between SpHb and laboratory Hb (Chung et al. 2014). Furthermore, according to the Radical-7 manufacturer, the PI value has a definite influence on SpHb accuracy and obtaining SpHb values (Lee et al. 2014). The ability of the monitor to obtain a signal and the influence of PI on the SpHb obtained was also investigated in the present study. The lack of association between [Hb]/SpHb and SaO_2/SpO_2 differences and MAP, tongue thickness and PI values, demonstrated that these factors did not influence the agreement between the two devices. However, following *in vivo* adjustment, 35 SpHb values were recorded when the PI was ≤ 1.4%. In the present study, a higher PI value was associated with the use of acepromazine-based sedative protocols, as opposed to dexmedetomidine-based protocols. This agrees with the results of Read et al. (2016) and it is likely due to the fact that acepromazine induces peripheral vasodilation when compared to dexmedetomidine (Grasso et al. 2015).

For the sake of clarity, it should be mentioned that as we sampled dogs of ASA categories I-II, we cannot say that dogs with severe perfusion impairment can be monitored accurately with pulse CO-oximetry, and further studies including patients presented with different ASA status are needed to adequately define the influence of PI on SpHb accuracy.

The American Society of Anesthesiologists (ASA) Practice Guidelines for Perioperative Blood Transfusion (ASA guidelines) recognise that defining exactly when a perioperative blood transfusion is necessary is not obtainable from the literature, as clinical considerations other than [Hb] also influence decisions to transfuse RBCs. The ASA guidelines recommend that transfusions are mostly not needed when [Hb] >10 g dL^{-1} but should be administered when [Hb] <6 g dL^{-1} (ASA guidelines 2006). In this regard, the clinical significance was evaluated by plotting Hb accuracy against a clinically acceptable error as proposed by Morey (Morey et

al. 2011). The EGA in fact helped to better visualise the relation between SpHb and [Hb], which resulted in a higher density of data pairs lying closer to the perfect agreement line with 98% of them encompassed in Zone A, representing a 10% deviation from the reference measurement between [Hb] values of 6 and 10 g dL^{-1} (ASA guidelines 2006). These results suggest that the pulse CO-oximeter tested meets the proposed criteria for an ideal device as laid out by Morey and colleagues (2011), where an ideal device should have 95% of points within the zone A, 5% within the zone B and 0% within the zone C (98/2/0% in our study) (Morey et al. 2011).

Hence, after analysing the error grid we found that none of the SpHb readings could have contributed to wrong decision making in blood transfusions, especially if the decision was taken wisely, considering the haemodynamic state of the animal. This strongly supports the idea that pulse CO-oximetry could be a helpful tool in guiding the clinical decision to initiate a blood transfusion. Hypothetically, possible inappropriate blood transfusion decisions (which might result in harm, depending on the circumstances, but not as serious as a zone C error) could have occurred for the 2 points within zone B, if their measurements have been used as the solo way for decision making guidance. However, considering the results of the EGA within the narrow critical isthmus where Hb <10 g dL^{-1}, in conjunction with a favourable κ statistic, there is probably sufficient evidence to suggest that the pulse CO-oximetry is a reliable technology and ± 1 g dL^{-1} a reasonable degree of accuracy to provide guidance for therapeutic transfusion decisions. Additional studies of sufficient magnitude within the range of haemoglobin 6–8 g dL^{-1} are needed to confirm this assumption for all cases.

To supplant current haemoglobin monitoring practices, SpHb should accurately represent the true haemoglobin at any given in time point and the trend of Hb throughout surgery. By trend analysis, sequential changes in SpHb with sequential [Hb] changes can be tested, and a concordance rate of change defined. According to our findings, the Masimo Radical-7 Pulse

CO-oximeter acts as an acceptable trend monitor with 76/82 change direction agreements (92.6%) when compared to [Hb].

The present study is the first in veterinary medicine that has evaluated the trending and concordance rate of SpHb changes over time therefore, comparison can only be performed with prior reports of single centre studies in human medicine. In a previous study performed in 12 volunteers under general anaesthesia subjected to haemorrhage and with total Hb concentration < 10.0 g dL^{-1}, a 95.4% SpHb change agreement with a Hb laboratory CO-Oximeter base was found, however an exclusion zone of ± 1 g dL^{-1} was applied (Marques et al. 2015). Another study which used the same exclusion zone as used in the present study, resulted in 129/137 SpHb and a concordance rate of changes of 94.2% (Applegate al. 2020). In a study involving 49 patients undergoing spine surgery, change concordance was 85.1% when SpHb with low PI were excluded (Chang et al. 2019). In the present study, the measurements taken during a low PI state were not exclude, however if that should be the case, the concordance rate would rise to 97.6%.

The largest discrepancy of study design between the mentioned reports and ours, was the inability of easy comparison to be performed and in particular, it is not possible to exclude that the use of a newer SpHb probe and software version, and the choice of different refence method as reference may have played a major role.

2.11 Limitations

The current results should be interpreted within the constraints of several limitations.

2.11.1 Case Selection

The study population included a wide range ages, breeds and equal numbers of each sex, which it makes it representative of dogs seen in clinical practice. However, as the present study is a single centre observational study, with a quantitatively limited and specific patient population, it may differ from that encountered in other referral centres, therefore, the current conclusions cannot necessarily be translated to other canine populations, or different clinical conditions. Some breeds such as the French Bulldog and English Bulldog (8 cases in total) are over represented compared to others, and although this data is in accordance with the higher prevalence of intervertebral disc disease reported in these breeds (Bellumori et al. 2013; Mayousse et al. 2017), they also present other peculiarities such as significantly lower PaO_2, and higher tHb compared to meso and dolichocephalic dog breeds (Hoareau et al 2012). The higher Hb, which might be a possible compensatory mechanism to maintain normal arterial content of oxygen in brachycephalic dogs, may have had some direct influence on the results presented in our study (e.g. mean [Hb] and SpHb before *in-vivo* adjustment) and further study specifically assessing the performance of pulse CO-oximetry in brachycephalic breeds should be carried out. Furthermore, it is not possible to exclude that other physical characteristic such as a denser tongue of brachycephalic breeds compared to mesaticephalic breeds (Jones at al. 2020) may influence the ability and/or accuracy of pulse CO-oximetry reading. Although brachycephalic dogs in the present study are overrepresented compared to other breeds, their limited number does not permit further conclusion to be drawn.

In spite of the fact that only one Greyhound was included in the present study, it is not possible to exclude that the documented lower Hb P_{50} values (the partial pressure of oxygen at which

50% of Hb is saturated) that Greyhound dogs show compared to those of non-Greyhound dogs (Sullivan et al. 1994) has some influence on the pulse CO-oximeter reading, even though this technology does not rely on the ODC.

2.11.2 Sample Numbers and Clinical nature of the study

Although in line with the sample size calculation, there were limited number of dogs entered into the study and many factors were not standardised. Therefore other confounding factors cannot be entirely ruled out. Future studies in a standardised condition (e.g. same fluid therapy and rates, same anaesthetic protocols) are needed to identify other factors that may influence the accuracy of the pulse CO-oximetry in dogs undergoing general anaesthesia.

2.11.3 Spectrophotometric Interferences

Spectrophotometric analysis can be influenced by the concentration of serum bilirubin (Myers & Browne 2007) however, none of the animals included in the study had a preoperatively elevated serum bilirubin this was not measured in all of them.

Furthermore, the reference analyser that we used in the present study only emits 3 wavelengths of light and therefore cannot differentiate between Hb species. Although the present study did not aim to assess agreement between measurements of dyshaemoglobins (methaemoglobin and carboxyhaemoglobin), this would have been useful to further assess the accuracy of the pulse CO-oximeter.

2.11.4 Sample Ranges

Assessment of the pulse CO-oximeter was only conducted in healthy animals, with preoperative [Hb] within the limit range for the tested device. This is even more relevant for the accuracy trend analyses where less than 10% of change samples were with Hb ≤ 8.0 g dL^{-}

[1] (10 of 104), which may have impacted the ability to assess clinical utility at the very low Hb. Nevertheless, change direction agreement was good with Hb < 10 g dL^{-1} in > 25% of change samples (28 of 104).

In a recent pilot study, it was found that SpHb values changed significantly during preoxygenation with a high F_IO_2, suggesting that SpHb accuracy is influenced by high concentrations of O_2 (Gayat et al. 2011). In the present study, while all patients in the preparation area received 100% O_2, the same were all mechanically ventilated once arrived in the operating theatre and maintained with the same mixture of O_2 and medical air during the entire surgery (70% to 98% O_2). Therefore, it is difficult to draw firm conclusions about the influence of F_IO_2 on SpHb value. Additionally, no animals with abnormal SaO_2 and Hb values were studied. Further work is required to clarify if *in-vivo* adjustment improves the accuracy and precision of measurements in patients with [Hb] outside the validated range of the device (anaemia or polycythaemia) or with lower F_IO_2 or SaO_2.

2.11.5 Samples Tested

Arterial blood samples have been used in the present study which may be seen as a limitation for generalisation to clinical settings, where venous or capillary blood is more commonly sampled. As the studied animals were at risk of blood loss and their lungs mechanically ventilated during the entire anaesthetic period, arterial catheters were used to facilitate the ABG samples for quick testing for the appropriateness of ventilation and blood pressure care, as part of the routine anaesthetic management and the motivation behind the choice of using arterial blood samples. In spite of the fact that venous blood may be easier to obtain in some clinical situations, by using arterial blood, we removed a potential confounding point that a mix of arterial and venous blood samples could have introduced into the accuracy of the analyses (Applegate et al. 2020).

2.11.6.Repeatability

Repeatability was not carried out in the present study as a repeatability study must, for an appropriately selected sample, make at least two measurements per subject under identical conditions (Bartlett & Frost, 2018). For the clinical nature of the present study, it was not possible to take two blood samples (unless two arterial catheters per patient were placed) at the same time and two measurements of SpHb with the same pulse CO-oximeter at the same time. Moreover, as the repeatability studies should be carried out under identical condition, in a dynamic situation as during a general anaesthesia for surgical procedure, this was not feasible to achieved in a clinical setting, therefore repeatability, calculated from analysis of variance (ANOVA) was not performed. Under experimental condition, (e.g. dogs undergoing a controlled and maintained level of haemodilution), it would be interesting to quantify the agreement and reliability of measurements made by those particular methods.

2.11.7 Haemodilution

In a previous study, the SpHb after haemodilution in healthy adults showed for SpHb a bias of 0.15 g dL^{-1}± 0.92 g dL^{-1} and clinically high accuracy against reference Hb (Macknet et al. 2010). Despite no clinical evidence indicating hyper or hypohydration were recorded, it is not possible to exclude those changes in the haemoglobinemia value due to these events have happened during the surgery. To avoid this uncertainty, a Goal-Directed Fluid Therapy Protocol (GDFTP) and dedicated haemodynamic monitoring should be used. Considering the clinical nature of the present study this was not feasible on this occasion, however we advocate the need for further studies investigating the SpHb and PI interaction with a controlled GDFTP.

Furthermore, evaluation of the sensor and software version of the Pulse CO-oximeter tested here should be assessed in patients undergoing large blood loss before drawing any firm conclusions.

2.11.8 Influence of inhalational anaesthetic agents

The use of inhalational anaesthetic agents causes arteriolar dilatation which may alter PI value. However this effect was not investigated in the present study, other authors (Park et al. 2015) demonstrated that the use of sevoflurane increases the PI with an improvement of SpHb accuracy. A similar study should be conducted to investigate the influence of isoflurane on the PI value and SpHb accuracy.

2.11.9 Temperature correction

When analysing blood samples using the VetStat, we did not correct for the body temperature of the patients. Whilst this may introduce a small amount of error due to alterations in absorption spectra of haemoglobin species, it is suggested that this is not of clinical significance (Ralston et al. 1991). However, when assessing agreement between two measurement techniques, it may have minor implications.

2.11.10 Reference Methodology

The VetStat IDEXX blood analyser has been used as the reference method in the present study instead of a laboratory CO-Oximeter, as in a survey of veterinary practices POC analysers manufactured by IDEXX accounted for 85% of all in UK practice analysers (Bell et al. 2014). This also reflected the intraoperative care for many patients undergoing surgical procedures at the time this study was conducted at the Small Animals Veterinary Teaching Hospital, Veterinary Medicine School, University of Glasgow. In addition, measurement of [Hb] using

the technology used within the IDEXX VetStat (AVL Opti) showed in human adults and newborns a very good agreement and precision with routine laboratory testing (Schlebusch et al. 2001; Boonlert et al. 2003). Despite the lack of validation studies for [Hb] measurements using the VetStat in dogs, spectrophotometric characteristics of canine haemoglobin compared with human haemoglobin are almost identical (Zijlstra & Buursma 1987). Therefore, we can assume that agreement between measurements of [Hb] using the VetStat and other laboratory analysers will also be good, and it is acceptable to use the VetStat as the reference method.

2.11.11 Exclusion Zone

The four-quadrant concordance method uses exclusion zones to limit the influence of small changes in [Hb]. The exclusion zones we defined are based on 95% limits of agreement for [Hb] and suggests that in our patients, a change up to ± 0.5 g dL^{-1} may not reflect a real change in circulating haemoglobin measured. The exclusion zone may introduce random noise that may reduce statistical power and ignores potentially valuable information.

2.11.12 Study Duration

The present study took place over a long period of time and it is not possible to exclude some change in analyser performance, however the blood gas analyser used in the present study does not require periodic routine replacement. The study conditions however may represent clinical practice, where most veterinarians keep their blood gas and electrolyte analyser for many years and often service them themselves rather than by the manufacturer. In fact, in a survey of veterinary practices, POC analysers manufactured by IDEXX accounted for 85% of all in practice analysers and more than two thirds (71%) of respondents reported that they used the reference intervals supplied by the manufacturer, without further adjustment or assessment (Bell et al. 2014). Nevertheless, there was no evidence of change over time from our data,

which may have been due to the regular servicing and quality control procedures within the Glasgow University Small Animal Hospital.

2.12 Future Studies

Further studies are needed to explore the Masimo Radical-7® Pulse CO-oximeter® (Masimo Corporation, CA, USA) performances in veterinary medicine aiming to assess; the SpHb accuracy, as absolute and trend values, in patients with active blood loss or with comorbidities that affect peripheral perfusion (e.g. sepsis or cardiac disease).

Additionally, patients receiving colloids and/or vasoactive drugs as part of their anaesthetic management should be investigated to evaluate the influences of these therapies on SpHb accuracy.

As the purpose of non-invasive Hb technology in the operating room is to assist clinicians in deciding whether to transfuse, not only should non-invasive Hb devices and the reference method produce statistically similar results, but they should also lead to comparable clinical decisions (Rice et al., 2013). Future studies should explore the ability of this device to detect sudden changes in Hb as well assessing the clinical decision taken based on SpHb.

For completeness, trend data should be analysed not only with the four-quadrant plot analysis, which considers only the directionality of the change, but also with the Critchley polar plot, which includes both the directionality of the change and magnitude of the change by setting error bars to bind the data.

Furthermore, venous blood samples instead of arterial samples should be tested to assess if results are similar to our findings as venous blood samples are more widely used in daily veterinary practice than arterial blood samples.

Agreement with different reference methods of measuring Hb concentration (e.g. laboratory CO-Oximeter, other POC devices) should be also explored.

Future study would help to define if breeds characteristics (e.g. macroglossia and hyperplasia of the tongue of brachycephalic dogs) interfere with the pulse CO-oximetry accuracy.

Lastly, despite the pulse CO-oximetery technology does not rely on the ODC to measure the SaO_2 and SpHb and SpCO, it would be interesting to assess its performances in Greyhounds and other Sighthounds dogs with lower P_{50} and higher oxygen content and oxygen-binding capacity (Zaldívar-Lopez et al. 2011).

2.13 Conclusion

The proposed adjustment method for SpHb, when compared with blood gas values, reduced the numbers of outliers and bias and the SpHb showed to be consistent with the arterial haemoglobin measured by blood gas analysis. Therefore, *in-vivo* adjustment is recommended when using this device to monitor SpHb in anaesthetized dogs. Future well-designed studies are needed to confirm these findings, particularly in haemodynamically compromised patients.

2.14 Clinical Implications

Although pulse oximetry does not replace laboratory-based analysis, it provides an early warning system of decreasing oxygen saturation. In the same way, pulse CO-oximetry as a continuous non-invasive haemoglobin trend monitoring could support the laboratory measurements, providing valuable guidance regarding blood loss and the need for transfusion therapy during surgery.

Pulse CO-oximetry may help in maintaining patients within a target haemoglobin range and reducing the number of blood samples needed to control fluid therapy, as well as blood products administered intraoperatively. The CO-oximetry technology is promising, it would represent a significant advance in non-invasive monitoring of Hb and a very helpful tool in guiding the clinical decision to initiate, or not, a blood transfusion.

3 OBSERVATIONAL RETROSPECTIVE STUDY (*CASE SERIES)*

3.1 Role of retrospective observational studies

Many researchers advocate that experimental methods, such as randomised controlled trials, are always needed to address research questions however, practical implications for researchers and for funding may reduce the possibility to carry experimental studies.

The conflict between those who advocate randomised trials in all situations and those who believe observational data provide sufficient evidence is a 'false' conflict, and should be replaced with a mutual recognition of the complementary roles of the two approaches, as experimental studies may also carry some limitations (Blank, 1996).

Observational studies are widely used in veterinary medicine to address a variety of research questions, such as descriptive questions (e.g. to estimate the prevalence or incidence of a condition), to evaluate diagnostic-test accuracy, or to identify and evaluate risk or exposures (Thiese, 2014).

There are however retrospective studies, these are designed to analyse pre-existing data that might be important for expanding or narrowing the implications of established treatments, and to allow clinicians to see how interventions play in the 'real world' out of clinical medicine research (Riley 2014).

Within this context, a case series belong to a group of observational studies which do not test the hypothesis of treatment efficacy but usually follows a group of patients who have a similar diagnosis or who are undergoing the same procedure over a certain period of time (Carey & Boden 2003). The outcome from a case series may serve as initial reporting on novel diagnostic or therapeutic strategies, particularly when the option of waiting for comparative evidence is considered unacceptable, or as a tool for summarising the outcomes in a certain patient category (e.g. intraoperative bleeding patients).

Among the purposes of a case series, the generation of a hypothesis that subsequently can be tested in studies of greater methodological rigor should be primary.

Many case reports and case series have brought to the fore a hitherto unrecognized disease and played an important role in advancing medical science. For instance, HIV/AIDS was first recognised through a case report of disseminated Kaposi's sarcoma in a young homosexual man (Gottlieb et al. 1981).

In order to explore the accuracy of the pulse CO-oximetry in unhealthy canine patients undergoing general anaesthesia for different procedures, the following section, presents a retrospective observational study in the form of case series, with an aggregation of multiple cases of canine patients referred to Small Animals Veterinary Teaching Hospital, Veterinary Medicine School, University of Glasgow, between the study period of March 2017 and March 2019, for surgical procedures that suffered from acute blood loss, and/or hypotension and/or acute hypovolemic state.

3.2 Aim of this case series

The aim of this case series was to observe the ability of the Masimo pulse CO-oximeter (Radical-7®) to detect the change of Hb concentration in dogs undergoing surgical procedures that were considered based on their clinical presentation (ASA status ≥ 3), haemodynamically unstable or/and at high risk of bleeding or already presenting an active bleed. In particular, the influence of the administration of crystalloids (Hartman's solution or Plasma-Lyte®A), and/or synthetic colloids (Volulyte®) and/or blood products (PRBCs), and/or vasoactive drugs (Dopamine or Noradrenaline CRI) on the accuracy of the Masimo Radical-7 pulse CO-oximeter to measure the SpHb during general anaesthesia, compared to simultaneous discrete [Hb] obtained from ABG processed by IDEXX VetStat® analyser was investigated.

3.3 Additional Instrumentation

Due to their clinical conditions and/or the type of surgical procedures they were undergoing, all animals included in the case series were instrumented with an arterial catheter to facilitate blood pressure monitoring and/or arterial blood sampling alongside the standard anaesthesia monitoring. Furthermore, for all these cases, instead of a standard pulse oximeter, a Masimo Radical-7® pulse CO-oximeter was used for the purpose of SpHb monitoring, in addition to the SpO_2 and pulse rate. In-vivo adjustment was applied after the first ABG sample to correct the first SpHb value based on the [Hb] obtained from the VetStat.

3.4 Eligibility and Inclusion Criteria

Dogs were required to be privately owned, and to have been referred to the Small Animals Veterinary Teaching Hospital, Veterinary Medicine School, University of Glasgow, for surgical procedures or to the emergency and intensive care departments between March 2017 and March 2019, undergoing surgical procedures for which general anaesthesia was required and for whom the anaesthetic record was completed in all its parts. Based on the clinical conditions at presentation, all dogs included were required to be classified to a physical status ASA $\geq$ III. Only dogs where blood pressure was invasively monitored through a peripheral arterial catheter, and where arterial blood samples were analysed with VetStat® blood gas analyser were included. Dogs were required to have the SpO_2 as well as the SpHb values measured by a Masimo Radical-7 pulse CO-oximeter and SpHb recorded manually on the anaesthetic file. All patients included in this case series received at least one of the following treatments during general anaesthesia; one or more bolus of crystalloids, one or more bolus of synthetic colloids, vasoactive drugs and or blood products.

3.5 Exclusion Criteria

Those dogs considered outside the ASA physical status, dogs where arterial catheterisation was not performed, where arterial blood samples were analysed with a different device (e.g. i-STAT®), or whether the blood samples were either venous or not collected within dedicate syringes, or dogs for which anaesthetic records were incomplete, were excluded. All dogs that did not receive any of the mentioned treatments administered during the general anaesthesia period, were not included in the present case series.

3.6 Ethical approval and Informed consent

Due to the retrospective nature of the case series presented here ethical approval was not required, as data presented in this section were already available on the electronic database of the Small Animals Veterinary Teaching Hospital, Veterinary Medicine School, University of Glasgow. A generic consent form for clinical procedures and for data to be used for research purposes was given at the time of animal admission to the hospital. On presentation at the hospital each animal was given a six-digit hospital number used to identify the cases. In order to seek confidentiality, all data were then anonymised by removing the patient's name, owner's surname and an increasing cardinal number was assigned to each animal.

Due to their clinical presentation at the time of admission, all cases included in the present case series were at risk of developing intraoperative bleeding and/or hypovolemia and/or ventilation impairment and as per standard clinical procedure, peripheral arterial catheterisation was performed.

A pulse CO-oximeter (Masimo Radical-7™) instead of a standard pulse oximeter was used with the intent to record information that would help the intraoperative and/or postoperative case management. An arterial catheter was maintained or removed after the surgical

procedure, depending on the animal's clinical conditions and clinical management requirements.

3.7 Surgical Procedures

Patients included in the case series underwent a laparotomy with a subsequent liver lobectomy, or splenectomy, or cysto-prostatectomy, or enterectomy, or thoracotomy for lung lobectomy, or pericardiectomy.

3.8 Anaesthesia Records and Protocols

The preoperative [Hb] values, medical stabilisation therapies, anaesthetic techniques as well as all drugs and therapies administered in the perioperative period, were recorded. The dogs were either sedated with dexmedetomidine (0.001-0.08 mg kg^{-1}) or acepromazine (0.005-0.01 mg kg^{-1}), combined with an opioid [methadone or morphine (0.1-0.4 mg kg^{-1}); fentanyl (0.005-0.01mg kg^{-1})] administered IV or IM, except for fentanyl that was always administered IV; or only premedicated with one of the opioids mentioned. General anaesthesia was achieved by administering IV either propofol (1-4 mg kg^{-1}), alfaxalone (1-3 mg kg^{-1}) or etomidate (0.5-2 mg kg^{-1}). Where co-induction was performed, this was achieved using IV administration of either diazepam or midazolam (0.2-0.3 mg kg^{-1}) or ketamine (0.5-1 mg kg^{-1}). Orotracheal intubation was performed to maintain anaesthesia with either isoflurane (IsoFlo, Zoetis, UK) or sevoflurane (SevoFlo, Zoetis, UK) vaporised in either oxygen or in a mixture of medical air and oxygen. The fractional inspired oxygen (F_IO_2) was maintained between 0.7 - 0.9 and was delivered via a rebreathing system (Datex Ohmeda, GE Healthcare, Chalfont St Giles, UK). Where needed, volume-controlled, mechanical ventilation was instituted (Aestiva/5 Datex Ohmeda, GE Healthcare, Chalfont St. Giles, UK) with inspiratory: expiratory ratios between 1:2.5 and 1:3, with tidal volumes and respiratory rates (f_R) adjusted based on the animal's requirements to maintain an end-tidal carbon dioxide concentration ($P_E'CO_2$) between 35 to 55 mmHg and a peak inspiratory

pressure kept below 15 cmH_2O. All dogs included in this case series were considered at an increased risk of bleeding (due to their clinical conditions or due to surgical procedures they were undergoing) or at increased risk of hypovolemia or ventilation impairment.

All dogs were instrumented with standard anaesthesia monitoring and additionally as per standard clinical procedure, peripheral arterial catheterisation was performed with a 20- or 22-gauge cannula (Biovalve Safe, Vygon, UK) depending on the size of the dog and aseptically placed in a dorsal pedal artery for measuring IBP and for the collection of arterial blood samples. During anaesthesia, HR, f_R, IBP, $PE'CO_2$, FIO_2, end-tidal isoflurane (FE´Iso) or end-tidal sevoflurane (FE´Sevo) were continuously monitored using a multiparameter monitor (S5 Compact Anaesthesia Monitor; Datex Ohmeda, Chalfont St. Giles, UK) and recorded every 5 minutes. Anaemia [Hb] < 7 gdL^{-1} (before fluid therapy and/or surgery), hypotension (MAP <60 mmHg), active intraoperative bleeding (≥20% of estimated total blood volume) were all recorded (***Table 3-1***).

All animals received either Hartmann's solution (Vetivex II Solution, Dechra, UK) or Plasma-Lyte®A (Vetivex® Veterinary pHyLyte™, Dechra, UK), administered at the initial dose of between 5 to 10 ml kg^{-1} $hour^{-1}$ throughout the anaesthetic period.

Hypotension [mean arterial blood pressure < 60 mmHg; (Tanifuji & Eger, 1976)] was treated by reducing anaesthetic delivery, treated by administering intravenous bolus of crystalloid (5-20 ml kg^{-1} h^{-1}, IV over 10-15 minutes), or treated by administering intravenous bolus (5 to 20 ml^{-1} kg^{-1}) or treated by administering dopamine (5-15 mcg kg^{-1} min^{-1}, IV) or noradrenaline (0.05-2 mcg kg^{-1} min^{-1}). Hypovolemia was treated by administering 5 to 20 ml^{-1} kg^{-1} IV of synthetic colloids (Volulyte® 6%, Fresenius Kabi, UK). Packed RBCs were transfused if; (a) Estimated blood loss was obviously > 20% of whole blood volume with mean arterial blood pressure < 60 mmHg, (b) Blood haemoglobin level was lower than 7 g dL^{-1}(c). Continuous blood loss with mean arterial blood pressure < 60 mmHg (***Table 3-1***).

Bleeding patients who did not meet the criteria for blood transfusion were resuscitated by either crystalloids solution in ratio (crystalloids solution: blood loss = 3:1) and/or colloids for a maximum of 20 ml kg^{-1} per day. A baseline haemoglobin measurement was obtained and recorded for both [Hb] and SpHb at the beginning of surgery.

Complication	Criterion	Reference
1. Hypotension	MAP <60 mmHg	Tanifuji and Eger 1976
2. Active Bleeding	≥ 20 % of tot blood volume (ml)	Linklater 2019
3. Low Haemoglobin	< 7 g dL^{-1}	
4. Packed RBCs were transfused if	(a) Blood loss > than 20% of whole blood volume with MAP < 60 mmHg (b) Hb < 7 g dL^{-1} (c) Continuous blood loss with mean arterial blood pressure < 60 mmHg	Linklater 2019

Table 3-1 Definitions of complications considered

3.9 Results

Of the 37 cases conforming to the inclusion criteria, 20 were included in the study, while 17 were excluded (in 6/17 of cases the anaesthetic record was incomplete, in 6/17 of cases *in-vivo* adjustment were not performed, in 3/17 of cases SpHb values were not always recorded and in 2/17 of cases arterial blood samples were analysed within the same anaesthetic event with two different devices). The gender distribution of animals included was; four entire males, five entire females and eleven neutered. While in regard to the breeds distribution animals were as follows; four crossbreeds; three Golden Retrievers, two Cocker Spaniels, two German Shepherds, two Labradors, two Standard Poodles, two Pugs, one French Bulldog, one Husky, and one Miniature Schnauzer. Among the 20 dogs included; 10 dogs were premedicated with a combination of dexmedetomidine and methadone IM or IV, four dogs with morphine and dexmedetomidine IM or IV, one dog with acepromazine and methadone IM, two dogs with only fentanyl IV and three with only methadone IV. In 9/20 of the dogs, induction was performed with alfaxalone, in 10/20 with propofol and one dog induction was achieved with etomidate. Among the dogs induced with propofol, four

had coinduction with midazolam and one with ketamine. Among dogs induced with alfaxalone, four had midazolam. Of the 20 dogs, general anaesthesia was maintained in 16 and in four with sevoflurane and isoflurane respectively ***Table 3-2***.

PREMEDICATION (IM or IV)					
	Dexmedetomidine & Methadone	Dexmedetomidine & Morphine	Acepromazine & Methadone	Fentanyl	Methadone
Number of dogs	10	4	1	2	3
INDUCTION of ANAESTHESIA					
	Propofol	Propofol +Midazolam	Propofol + Ketamine	Alfaxalone	Alfaxalone + Midazolam	Etomidate
Number of dogs	5	4	1	5	4	1
MAINTENANCE of GENERAL ANAESTHESIA					
	Isoflurane	Sevoflurane			
Number of dogs	4	16			

Table 3-2 Details of premedication, induction and maintenance of General Anaesthesia in 20 dogs included in the case series

Further demographic data are reported on ***Table 3-3***. Of the 20 cases included, during the general anaesthesia all bleeding and/or hypotension/hypovolaemia was recorded and treated in 17/20 instances by reducing anaesthetic delivery, 3/20 by only administering IV crystalloid as bolus (10-20 ml kg^{-1} IV), 3/20 by only administering synthetic colloids as bolus (5-20 ml kg^{-1}); 5/20 received crystalloids as bolus (5 to 30 ml kg^{-1}) and synthetic colloids as bolus (5 to 20 ml kg^{-1}); 5/20 were treated with PRBCs transfusion of which two received also synthetic colloids (5 to 10 ml kg^{-1}); 4/20 received vasoactive drugs. Of the 20 cases, two presented with anaemia before general anaesthesia. In the following sections some of the mentioned cases have been reported as examples.

	Crystalloid treatment	Synthetic Colloids	Crystalloids & Colloids	PRBCs transfusion	Vasoactive drugs
n of dogs included in each treatment	3/20	3/20	5/20	5/20	4/20
Age (mean ±SD) yrs	7.4 ± 1.9	7.5 ± 1.9	6.4 ± 2.8	10.4 ± 1.5	6.4 ± 2.6
Body mass (mean ± SD) kg	42.1 ± 4.2	23 ± 10.9	23.2 ± 11.5	31.2 ± 11.2	12.7 ± 4.3
SpHb – [Hb] before *in-vivo* (mean ±SD) g dL^{-1}	0.9 ± 0.7	2.2 ± 0.4	0.5 ± 0.2	0.8 ± 0.5	1.7 ± 0.5
*SpHb – [Hb] after *in-vivo* (mean ±SD) g dL^{-1}	0.4 ± 0.3	1.7 ± 0.4	0.4 ± 0.1	0.5 ± 0.1 1.9 ± 1.4[§]	0.6 ± 0.2[d] 1.3 ± 0.4[n]

Table 3-3 Summary of some of the demographic data of the 20 cases included in the case series. * Value after *in-vivo* adjustment and treatment. § animals treated with PRBCs and Colloids; d animals treated with dopamine; n animals treated with noradrenaline.

3.9.1 Intra-Operative bleeding: SpHb in cases treated with crystalloids

CASE 1

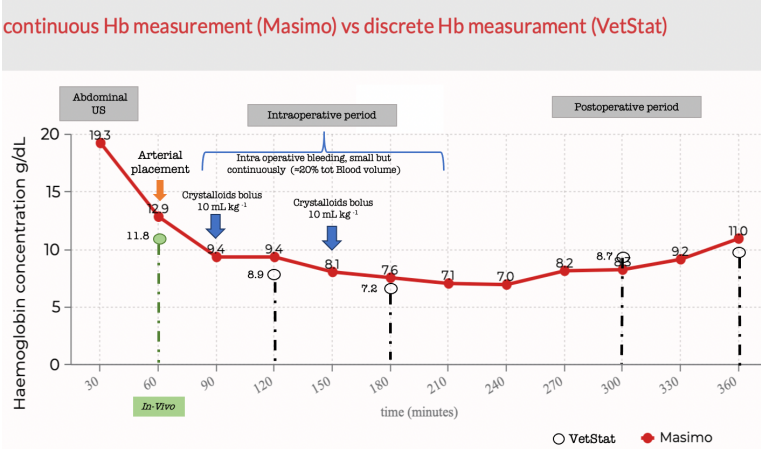

Figure 3-1 SpHb & [Hb] in spontaneous (non-traumatic) haemoabdomen due to rupture of hemangiosarcoma

A 9.5 years old neutered female Golden Retriever (44 kg), with a grade 2/6 pansystolic heart murmur, was presented after an episode of collapse that subsequently deteriorated with generalised weakness, cardiac arrhythmias and pulse deficiency. A ruptured splenic mass (probably an hemangiosarcoma) was diagnosed with abdominal ultrasound; abdominocentesis and a whole body computed tomography were also performed to exclude evidence of metastasis. The dog was then prepared for an exploratory laparotomy, which was followed by a splenectomy. During surgery mild hypotension (average 56 mmHg) and a small amount of, but constant bleeding (about 20% of total blood volume) were treated with two boluses of crystalloids (10 ml kg^{-1} Hartmann's solution IV) administered over 10 minutes.

CASE 2

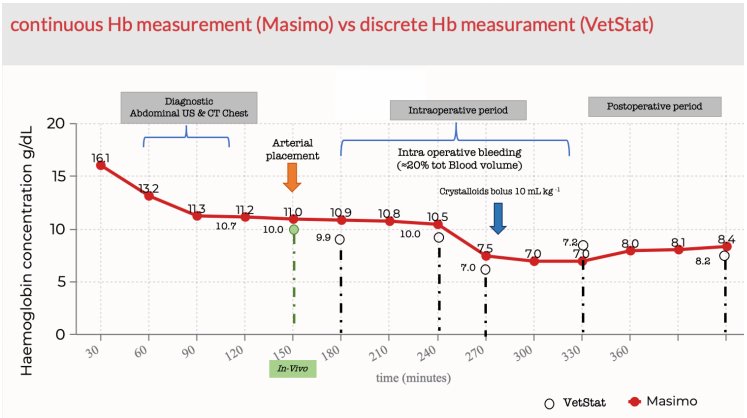

Figure 3-2 SpHb & [Hb] in acute, spontaneous (non-traumatic) haemoabdomen due to hepatocellular carcinoma

A 7.3 years old male Golden Retriever (45.1 kg), with hyperaemic mucous membranes, increased rapid capillary relief time, and moderate tachycardia. Limited fluid volume resuscitation (LFVR) was performed, before diagnostic procedures were carried out under sedation, after which general anaesthesia was induced. During surgery, a mild permissive hypotension technique was carried out to reduce the bleeding, (about 20% of total blood volume was lost) and one bolus of crystalloids (10 ml kg^{-1} Plasma Lyte®) was administered during surgery. The diagrams show the trends in SpHb values (red line) generated by the pulse CO-oximetry before and after *in-vivo* adjustment (green dot), and intermittent [Hb] values obtained from arterial blood samples analysed with VetStat blood gas analyser (empty dots).

Values of SpHb in 3 cases (2 of which have been reported in the present thesis) treated with crystalloids:

On average, crystalloid bolus administered was 16.6 ml kg^{-1} (10 to 20 ml kg^{-1} IV) and performed 20 minutes from the beginning of general anaesthesia (10 to 30 minutes); the number of arterial blood samples performed per animal was 4 (3 to 6). The mean difference between SpHb and [Hb] before *in-vivo* adjustment was 0.9 g dL^{-1} (range 0.8 to 1.1 g dL^{-1}). After *in-vivo* adjustment the mean difference SpHb –[Hb] was 0.4 g dL^{-1} (0.4 to 0.5 g dL^{-}

[1]). In all three subjects, the pulse CO-oximeter showed a consistent fall in SpHb during bleeding. After crystalloids bolus, the difference between SpHb –[Hb] did not increase.

3.9.2 Intraoperative hypovolemia: SpHb in cases treated with synthetic colloids

*CASE **3-4-5***

The diagrams on the right, show the trends in SpHb values (red line) generated by the pulse CO-oximetry before and after *in-vivo* adjustment (green dot), as well as the intermittent [Hb] values obtained from arterial blood samples analysed with VetStat blood gas analyser (empty dots).

3. A 9.7 years old male Cocker Spaniel, with an early stage B1 mitral valve disease was presented for pyrexia and anorexia, as well as pain during urination and defecation. A prostatic abscesses rupture was diagnosed and received colloids at 20 ml kg^{-1} .

4. A 7 years old neutered female Standard Poodle, with suspicious of septic peritonitis due to a perforated gastric ulcer; with pale mucous membranes and vomiting. Colloid's administration was at 10 ml kg^{-1}.

5. A 6 years old Miniature Schnauzer, with a suspicious of septic abdomen from a

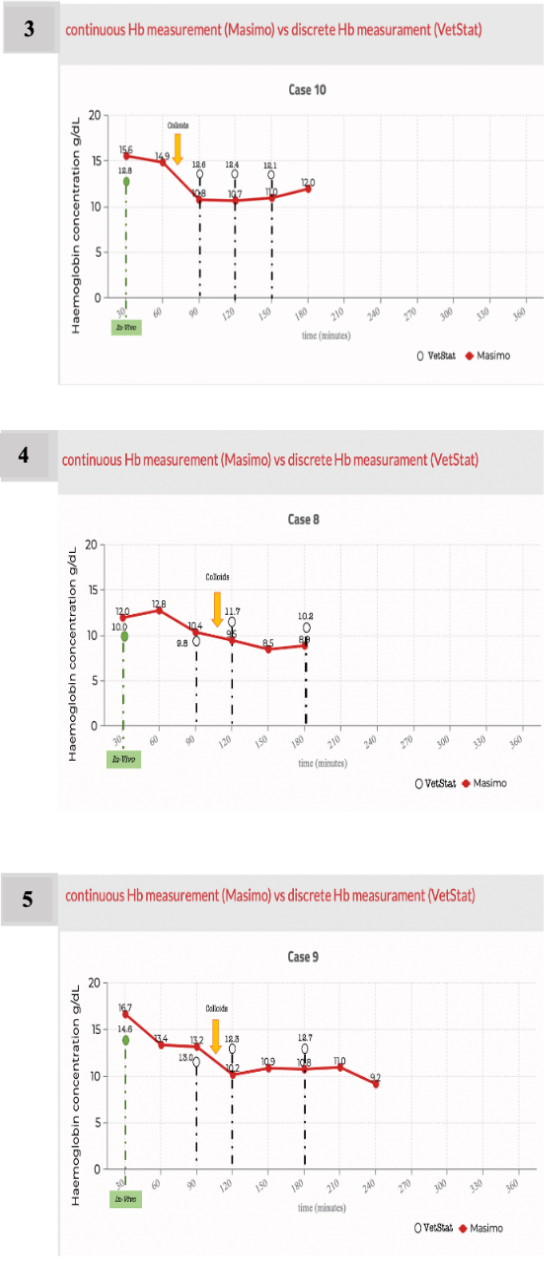

Figure 3-3-4-5 SpHb & [Hb] in hypovolemic dogs

pancreatic pseudocyst was presented with mild icterus and pain. Colloid's administration was 5 ml kg^{-1}.

All three cases presented hypovolemia, tachycardia, prolonged capillary relief time, hypalbuminaemia and hyperlactatemia (mean serum lactate of 3.2 ± 1.1 mmol L^{-1}). Before induction of the general anaesthesia, in all cases analgesic therapy, pre-surgical blood work and abdominal ultrasound were performed, followed by an administration of 10 ml kg^{-1} of fluid (Plasma Lyte® A) and by 5 ml kg^{-1} of synthetic colloids (Volulyte®).

Values of SpHb in three cases treated with synthetic colloids:

On average, intraoperatively synthetic colloids (Volulyte®) as 11.6 ml kg^{-1} (5 to 20 ml kg^{-1}) were administered at 90 minutes from the beginning of general anaesthesia (70 to 100 minutes). The average number of arterial blood samples performed per animal was three. The mean difference between SpHb and [Hb] before *in-vivo* adjustment was 2.2 g dL^{-1} (range 2 to 2.8 g dL^{-1}). After *in-vivo* adjustment and before intraoperative synthetic colloids bolus, the mean difference of SpHb –[Hb], in two over three dogs, was 0.4 g dL^{-1} (0.2 to 0.6 g dL^{-1}). After intraoperative colloids bolus the mean difference SpHb –[Hb] was 1.7 g dL^{-1} (1.3 to 2.2 g dL^{-1}). In all three subjects on average SpHb values were lower than [Hb].

3.9.3 Intra-Operative bleeding: SpHb in cases treated with crystalloids & colloids

CASE 6

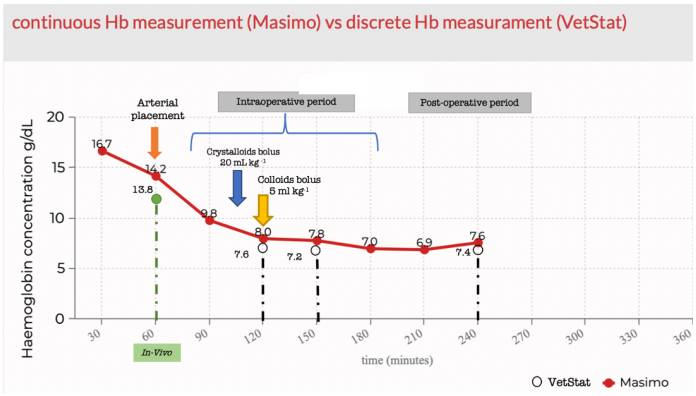

Figure 3-6 SpHb & [Hb] values in an acute traumatic hemoperitoneum for splenic rupture due to motor vehicle accident

A 4 years old neutered female German Shepherd (32.7 kg), was presented with hyperaemic mucous membranes, increased respiratory rate, and cardiac arrhythmia/tachycardia. Soft tissue swelling and bruising on different parts of the body, and a closed carpal fracture were detected. The patient was first stabilised, then LFVR and analgesic therapy were administered; once stabilised, the dog underwent an abdominal ultrasound scan that revealed a considerable amount of fluid grossly appear with bleeding and probably due to a spleen torsion. After abdominocentesis and PCV measurement of abdominal fluid, the dog was prepared for an exploratory laparotomy with subsequent splenectomy. During surgery bleeding, about 20% of total blood volume was lost and addressed with 20 ml kg^{-1} crystalloids bolus and 5 ml kg^{-1} synthetic colloids (Volulyte®).

CASE 7

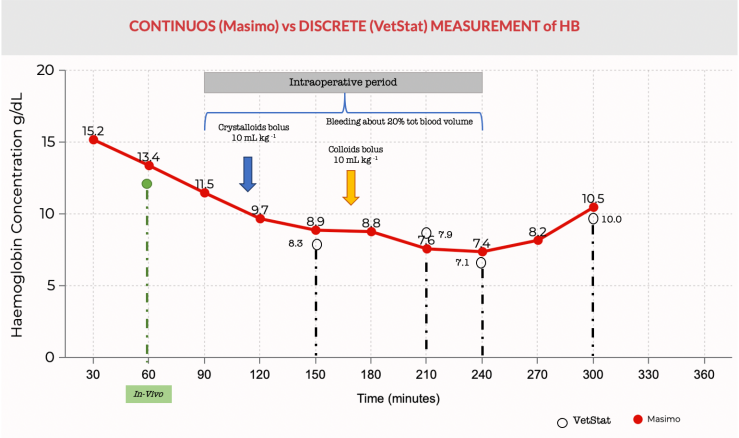

Figure 3-7 SpHb & [Hb] values during liver lobectomy surgery

A 4.3 years old neutered male Pug (14 kg) with mitral valve diseases grade 3/6 and pansystolic heart murmur was presented with pink mucous membranes, increased rapid capillary relief time, a moderate tachycardia and hypoalbuminemia. A limited fluid volume resuscitation (LFVR), 5 ml kg^{-1} of Plasma Lyte solution, before inducing of a general anaesthesia for an abdominal ultrasound was administered. During surgery, about (20% of total blood volume was lost). Blood typing but PRBCs transfusion was not carried out, however a bolus at 10 ml kg^{-1} of crystalloids and a bolus of 10 ml kg^{-1} of colloids were administered.

The two diagrams show the trends in SpHb values (red line) generated by the pulse CO-oximetry after *in-vivo* adjustment (green dot) and intermittent [Hb] performed with VetStat blood gas analyser (empty dots) taken from arterial catheter of this patient.

Values of SpHb in five cases (two of which have been reported in the present thesis) treated with crystalloids and synthetic colloids:

On average crystalloids bolus was administered as 15 ml kg^{-1} (5 to 30 ml kg^{-1}) 20 minutes from the beginning of the general anaesthesia, and a bolus of synthetic colloids (Volulyte®) as 11 ml kg^{-1} (5 to 20 ml kg^{-1}) at 41 minutes (30 to 70 minutes). The number of arterial blood samples performed was two. The mean difference between SpHb and [Hb] before *in-*

vivo adjustment was 0.5 g dL^{-1} (range 0.4 to 0.7 g dL^{-1}). After *in-vivo* adjustment and before intraoperative colloids bolus, the mean difference SpHb –[Hb], was 0.6 g dL^{-1}. After intraoperative colloids bolus the mean difference SpHb –[Hb] was 0.45 g dL^{-1}. In all three subjects on average SpHb values were higher than [Hb]. The pulse CO-oximeter showed a consistent fall in SpHb during bleeding.

3.9.4 Intra-Operative bleeding: SpHb in cases treated with PRBCs

CASE 8

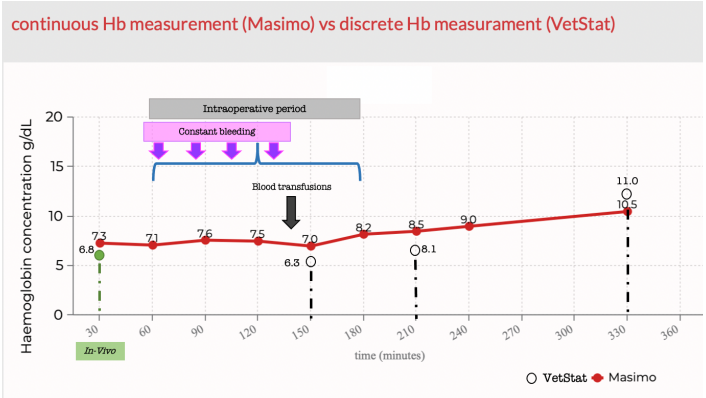

Figure 3-8 SpHb & [Hb] values in non-traumatic haemoabdomen for gastric mass

An 8.2 years old female neutered Labrador (39.4 kg), presented with depressed mentation, pale mucous membranes, prolonged capillary relief time, a weak peripheral pulse, hypothermia, tachypnoea, and tachycardia, blood tests as well as an abdominal ultrasound with paracentesis were performed. Stomach perforation by an ulcerative tumour, with the spillage of stomach contents into the abdomen was diagnosed. Before surgery PCV was 22%, VetStat based Hb was 7.1 g dL^{-1}, with mild hyperlactatemia; within the reference ranges for coagulation parameters. After an abdominal incision revealed a constant haemorrhage, gastrotomy and mass excision were performed, and once the bleeding was stopped and the dog was determined to be DEA 1.1 positive by an in-house card typing method (PCV 18%;VetStat Hb 6.3 g dL^{-1}), the PRBCs transfusion was slowly started and then maintained at 10 ml kg^{-1} h^{-1} over 3 hours. Routine monitoring showed the dog to be stable and normothermic throughout the transfusion.

CASE 9

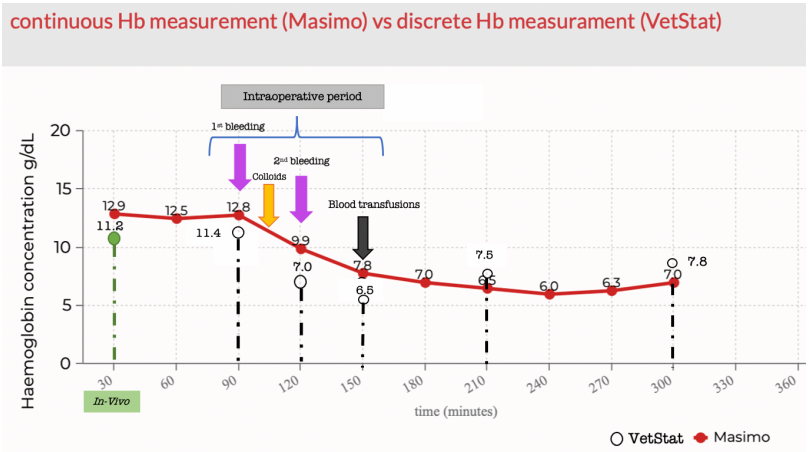

Figure 3-9 SpHb &[Hb] values in a liver lobectomy for hepatocellular carcinomas

A 10 years old male neutered Husky (37.8 kg), underwent an exploratory laparotomy with liver lobectomy to remove a hepatocellular carcinoma. Before surgery PCV, Hb, and coagulation parameters where within the reference ranges. During the surgery two episodes of bleeding were recorded; the first was mild, while the second was rapid, profuse, and difficult to arrest. The blood volume lost was estimated at about 30 % of total blood volume. The dog received after the first episode of bleeding 5 ml kg^{-1} of synthetic colloid, followed by another 5, for a total of 10 ml kg^{-1} over 15 minutes. After the second episodes of blood loss, (PCV 19%; VetStat Hb 6.5 g dL^{-1}) and dog determined to be DEA 1.1 positive by an in-house card typing method, the PRBCs transfusion was started and then maintained at 10 ml kg^{-1} h^{-1} over 4 hours. Routine monitoring showed the dog to be stable and normothermic throughout the transfusion.

Values of SpHb in five cases treated with PRBCs transfusion

On average PRBCs was administered once 25% of total blood volume was lost (20% to 30%), with a [Hb] of 6.4 g dL^{-1} (6.1 to 7 g dL^{-1}) and at 61 minutes from the beginning of general anaesthesia (35 to 120). In two of the five cases, synthetic colloids (Volulyte®) as 7.5 ml kg^{-1} (5 to 10 ml kg^{-1}) at 45 minutes (20 to 70 minutes). The number of average arterial blood samples performed for each dog was 3 (3 to 6). The mean difference between [Hb]

and SpHb before *in-vivo* adjustment was 0.8 g dL^{-1} (range 0.4 to 1.7 g dL^{-1}). After *in-vivo* adjustment the three dogs that did not receive intraoperative colloid bolus had a mean difference SpHb-[Hb] of 0.5 g dL^{-1} (0.3 to 0.8 g dL^{-1}), while in the two that received synthetic colloids and PRBCs was 1.9 g dL^{-1} (0.9 to 2.9 g dL^{-1}). In all subjects SpHb values displayed were on average higher than [Hb], however the pulse CO-oximeter showed a consistent fall in SpHb during bleeding, with rising values during the transfusion time. Post-operatively the mean difference of SpHb-[Hb] decreased for all five cases.

3.9.5 Intra-Operative hypotension: SpHb in cases treated with vasoactive drugs

CASE 10

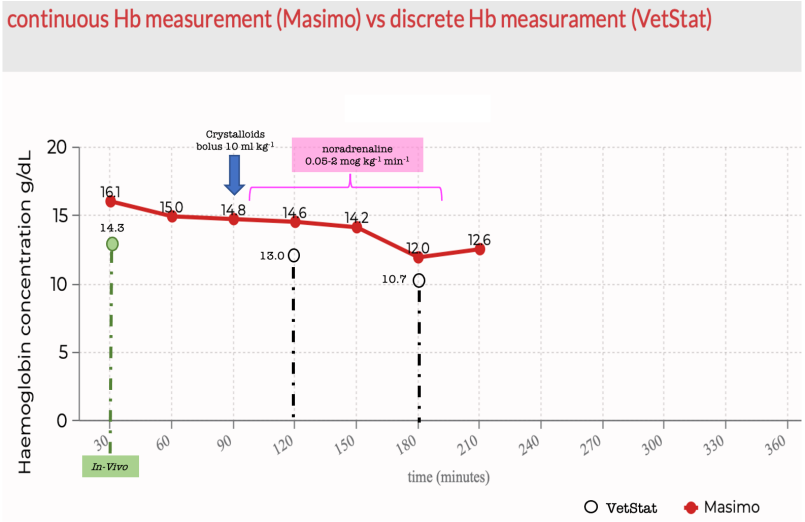

Figure 3-10 SpHb & [Hb] values during thoracotomy for lung lobe torsion

A 5 years old neutered female Pug (10.2 kg), which was presented with dyspnoea, pyrexia, lethargy, and coughing, was diagnosed with left lung lobe torsion with mild pleural effusion. On the day of surgery, she was prepared for a total lung lobectomy, performed via thoracotomy at the level of 5th intercostal space. During surgery the lung lobe was congested and enlarged, and due to a mild untwist of the torsed lobe, severe hypotension, probably due to inflammatory mediator and endotoxins release into blood stream occurred. A rapid administration of crystalloids bolus of 10 ml kg^{-1} IV, followed by a noradrenaline infusion 0.05-2 mcg kg^{-1} min^{-1} were necessary to restore the blood pressure. The dog was discharged from the hospital a few days after recovery.

CASE 11

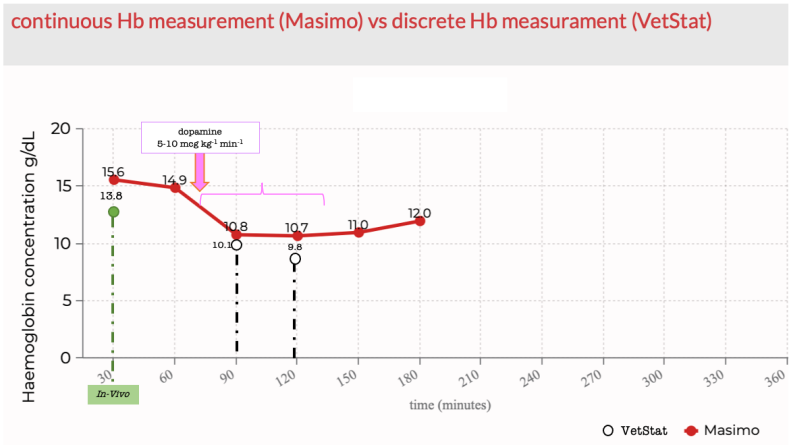

Figure 3-11 SpHb & [Hb] during thoracotomy for right auriculectomy

A 7 years old neutered female (20.4 kg) crossbreed dog was presented with collapse, lethargy, anorexia/vomiting, and recurrent pericardial effusion. Tachycardia and muffled heart sounds were detected during thoracic auscultation. A right auricular mass was detected and identified as hemangiosarcoma, with no signs of metastasis based on computed tomography and abdominal ultrasound. On the day of surgery, she was prepared for a right lateral thoracotomy at the level of 4th intercostal space. During surgery, a dopamine infusion 5-10 mcg kg^{-1} min^{-1} was necessary to maintain the blood pressure. The dog was hospitalised in the ICU for analgesia and supportive care, then discharged from the hospital a few days after.

Values of SpHb in five cases (two of which have been illustrated in the present thesis) treated with vasoactive drugs:

Five of the twenty dogs included in the case series, despite being normovolemic, developed hypotension during general anaesthesia. Of these five, three dogs received a dopamine infusion (5 to 15 mcg kg^{-1} min^{-1}) and two received noradrenaline (0.5 to 2 mcg kg^{-1} min^{-1}) during surgery. The average time of vasoactive drugs administration was 39 minutes from the beginning of general anaesthesia (30 to 65 minutes), with dopamine average infusion (7 mcg kg^{-1} min^{-1}) and noradrenaline infusion 1 mcg kg^{-1} min^{-1}. In two of the five cases crystalloids bolus was also administered before or simultaneously to the vasoactive drugs.

The number of total arterial blood samples performed per dog on average was 3 (3 to 5). The mean difference between [Hb] and SpHb before *in-vivo* adjustment was 1.7 g dL^{-1} (range 0.8 to 2.2 g dL^{-1}). After *in-vivo* adjustment among the three dogs that received intraoperative dopamine, a difference SpHb-[Hb] was 0.6 g dL^{-1} (0.4 to 0.8 g dL^{-1}), while in the two that received noradrenaline was 1.3 g dL^{-1} (0.9 to 1.7 g dL^{-1}). In all subjects SpHb values displayed were on average higher than [Hb], however the pulse CO-oximeter showed a consistent fall in SpHb during bleeding.

3.10 Discussion

In human medicine, it has been reported that intraoperative total haemoglobin are the most frequently ordered laboratory measurements, in both acute and outpatient setting (De Frances et al. 2008). In particular, acute blood loss frequently results in impaired peripheral perfusion and an acute hypovolemic state that requires rapid treatments such as blood transfusion (Adel et al. 2018).

European guidelines for the management of perioperative bleeding had suggested using non-invasive haemoglobin devices only as trend monitors (Moore et al. 2013) but more recently, in human medicine the pulse CO-oximeter has been tested in trauma patients (Gamal et al. 2017), during low perfusion states and in hypovolemic states (Adel et al. 2018), and active haemorrhage (Marques et al. 2015) with conflicting results.

In veterinary medicine, the investigations regarding the accuracy of SpHb have just started, and with this case series we aimed to report the influence of acute blood lost and/or hypovolemia and different treatments such as crystalloids, synthetic colloids, PRBCs, and vasoactive drugs, on the direction of SpHb changes and how accurately these reflected the direction of haemoglobin changes detected by an invasive [Hb] measured by VetStat® blood analyser.

Our finding highlighted the possible advantages of using pulse CO-oximetry in patients at high risk of intraoperative bleeding, in fact in all cases Masimo Radical-7 showed a consistent fall in SpHb reading during bleeding episodes, which may be form a starting point for deciding when to perform an invasive Hb measurement. Nevertheless, in all patients receiving synthetic colloids and/or noradrenaline infusion, a raise in difference between haemoglobin concentrations measured by VetStat® and by pulse CO-oximeter was recorded suggesting that values displayed by the Masimo Radical-7 under these circumstances should be considered carefully and always confirmed by an invasive blood sample.

3.10.1 Influence of hemodilution and Crystalloid administration on SpHb

It has been previously established in human and animal studies, that by using mass balance and volume kinetics, a large bolus infusion of crystalloid results in an immediate fall and then a steady decline in haemoglobin concentration that reaches its nadir at the end of the infusion (Dobrin & Hahn 1999; Vane et al.2004). In a more recent study (Marques et al. 2015), after an infusion of Ringer's lactate solution for 20 minutes, the initiation of the decrease in SpHb values occurred within a 5-minute period, and the authors reported as a possible explanation of the delay in the initial fall as a reflect time for circulatory mixing and some level of transcapillary refill.

To date, only a few studies have investigated the accuracy of SpHb in controlled hypovolemic states, unfortunately only in human medicine which limits the comparisons. Marques and colleagues reported a good precision during haemorrhage and replacement, however they suggested that the SpHb accuracy was not sufficient for a blood transfusion decision (Marques et al. 2015). Dewhirst and colleagues (Dewhirst et al. 2014) simulated a hypovolemic state by performing a preoperative phlebotomy and then assessed the accuracy of Masimo Radical-7 between Lab CO-Oximetry based Hb and SpHb, reporting that the last was not affected by acute blood loss.

Despite the limited number of the cases presented, our results showed that acute bleeding and crystalloids bolus, after *in-vivo* adjustment, did not influence the difference between pulse CO-oximetry and VetStat analyser, which was stable, on average, within an acceptable range. In fact, pulse CO-oximeter adequately followed the [Hb] curve at all the time points measured, and the differences between SpHb –[Hb] were always lower than ± 1 g dL^{-1}, which is encouraging as Δ of ±1.0 g dL^{-1} has been chosen as acceptable accuracy in many similar studies (O'Reilly 2011; Johnson et al. 2020). These results are in agreement with a previous study, where hemodilution in healthy adults showed for SpHb a bias of 0.15 g dL^{-1}± 0.92 g dL^{-1} and clinically high accuracy against reference Hb (Macknet et al. 2010).

3.10.2 Influences of hypovolaemia and Synthetic Colloids administration on SpHb

The measurement of [Hb] could be influenced by the synthetic colloids when these are administered to address hypovolemia; in fact, one of the unintended consequences of colloids therapy is the development of an acute iatrogenic haemodilution which may result in a paradoxical decrease in oxygen delivery (Bubenek-Turconi et al.2020). This event is reported to be more significant when synthetic colloids, compared to crystalloids, are administered, with a total decrease of about 5% in [Hb] for every 250 ml of colloids infused (Bubenek-Turconi et al.2020; Lobo et al. 2010). This iatrogenic haemodilution, together with intraoperative blood loss and fluid shift, may be sufficient to justify the need of serial [Hb] measurements to evaluate the fluid management strategy and/or to decide when to actually initiate the blood transfusion. A study performed in human patients undergoing hepatic resection has shown that the accuracy of SpHb measured by Masimo Radical-7 pulse CO-oximeter was influenced by colloid administration, and precisely immediately after colloids administration, in that case as 15 ml kg^{-1} IV of Voluven 6% administered for 30 minutes (Vos et al. 2012). In a recent study, the researchers reported that, <10 ml kg^{-1} *vs* >10 ml kg^{-1} colloids administration did not influence the pulse CO-oximeter accuracy, but that SpHb underestimates the [Hb] value when compared with laboratory CO-Oximeter for both groups (< and > 10 ml kg^{-1}) (De Rosa et al.2020).

In the three cases that have been reported in this case series however, Volulyte® instead of Voluven, at variable rates (5 to 20 ml kg^{-1}) was administered. These two synthetic colloids are pretty similar, in fact both contain the same hydroxyethyl starch dissolved (130 as mean molecular weight and 0.4 as degree of molar substitution) even if in different solutions; isotonic electrolyte for Volulyte and 0.9% sodium chloride for Voluven.

Despite the limited cases studied in the present case series, our data also suggests that the accuracy of SpHb decreased after rapid colloid administration (bolus) and the difference between SpHb –[Hb] was wider in cases where a higher rate of colloids was administered.

Even if a firm conclusion is not possible due to the limited data analysed, these confirm that colloids affect the SpHb accuracy in dogs under general anaesthesia in the same way that they do in human patients. Therefore, it is important to report this data, as a false assumption of too lower Hb levels may prompt unnecessary transfusion of allogenic RBCs, thereby exposing the patient to unnecessary risks (e.g. infection, allergic reactions, delayed haemolytic reaction).

The results of the present retrospective observational study regarding the accuracy of the SpHb measurement while rapidly administering colloid solution requires further elucidation to prevent unnecessary blood transfusion or, on the other hand, omission of necessary blood transfusion, as both situations are potentially harmful.

3.10.3 Influences of Blood transfusion on SpHb

Anaemia is common amongst surgical patients and independently associated with adverse outcomes, increased length of hospital and intensive care stays, postoperative complications, and increased mortality (Beattie at al. 2009). On the other hand, the administration of blood in the perioperative setting is a risk factor which also may contribute to poor outcomes (Musallam et al. 2011). According to a systematic analysis of 494 studies evaluating RBCs transfusions in humans, for actual benefit in health outcomes, it concluded that 59% of transfusions were, in fact, inappropriate (Shander et al. 2012).

The five cases studied presented in this case series showed that despite on average the SpHb displayed higher values compared to [Hb] the differences between SpHb –[Hb] were acceptable (0.5± 0.1 g dL^{-1}).

This is in disagreement with other studies (Applegate et al. 2012; Giraud et al. 2013) where the bias of SpHb and laboratory CO-Oximetry based Hb was about 0.9 ± 1 g dL^{-1} and with a high LoA. In addition to; the different blood sampled, the different software and pulse CO-oximeter probe, and reference method used, the imprecision of SpHb during rapid Hb

changes reported by the mentioned studies could be related to the frequency of blood sampling, that in the present case series were less recurrent compared to the mentioned studies. In fact, as the SpHb values displayed by the monitor are an average of measurements calculated over several minutes (2–8 min depending on the setting), if the Hb change is rapid and of large magnitude, several minutes are required before the changed value is displayed by the monitor, increasing the difference in Hb values with the reference method. This can possibly explain the reason why in our cases on average SpHb displayed higher values compared to [Hb] during active bleeding.

Naftalovich and Naftalovich (2011) explained this phenomenon as due to the fact that as SpHb is based on the microvascular Hb and macrovascular Hb, as the former is less affected during conditions of acute haemorrhage it remains high to maintain tissue oxygenation, the macrovascular Hb measured in a blood sample decreases, increasing the discrepancy between SpHb and an invasive arterial Hb derived value. This hypothesis while interesting, has not been validated with clinical evidence.

Furthermore, it is interesting that the two cases that in the present case series received also colloids bolus in addition to the PRBCs, showed a higher difference SpHb –[Hb] (1.9 ± 1.4 g dL^{-1}) which is in agreement with the cases where hypovolaemia was treated with the same synthetic colloids and with the human literature (Vos et al. 2012).

3.10.4 Influences of vasoactive drugs on SpHb

Based on the result of the present case series, the difference between SpHb –[Hb] seems to not to increase as much in patients with hypotension, who are receiving dopamine as a vasopressor, instead, the difference seems much bigger in patients receiving noradrenaline as a vasopressor. This could be related to a severe reduction of PI following noradrenaline administration in comparison to dopamine, however data related to the PI values have not been recorded and it is not possible to draw conclusions. However, Miller and colleagues (2012) using regional anaesthesia increased the perfusion to the finger wearing the SpHb

sensor, demonstrating that an increase of PI increased also the accuracy of SpHb (Miller et al. 2012). In the observational prospective study, reported in the present thesis however, we did not observe any link between the accuracy of SpHb and the value of PI, although the severity of the vasoconstriction caused by noradrenaline infusion may be severe enough to influence the pulse CO-oximeter accuracy. Nevertheless, other authors have reported that the use of norepinephrine/noradrenaline increases the likelihood of not being able to obtain a SpHb signal (Coquin et al. 2012); due to the retrospective nature of the present case series, it is not possible to exclude that the same happened for the cases analysed, as unfortunately the failures of pulse CO-oximeter in detecting signal were not recorded. Future studies are needed to investigate this hypothesis.

3.11 Limitations

Retrospective study presents several limitations that should be clarified; in fact, as previously reported, a retrospective analysis of the same data by different researchers have shown conflicting conclusions (Ward & Brier, 1999).

3.11.1 Sample Numbers and population

A very small sample size may have influenced the results. The retrospective character of this case series and the limited animal number included have surely precluded identification of important factors and limited the drawing of firm conclusions.

3.11.2 Generalisation

Data were gathered from dogs anesthetised in a University Veterinary Teaching Hospital setting, this might imply a selective population (older, unstable, or more critically ill). Due to selection bias, results of current retrospective case series may not be generalisable to the whole population.

3.11.3 Missing data

Since the data was not collected in a predesigned proforma as per the specific requirements of a study, some of the data would inevitably be missing. Furthermore, some variables that have the potential to impact the outcome (e.g. perfusion index) may not have been recorded at all.

3.11.4 Lack of Homogeneity

Different persons have recorded the data in different ways, and at different times during the procedure. Moreover, animals included underwent different surgical procedures and did not undergo uniform anaesthetic protocols, which may have influenced some of the results. It is also not possible to guarantee that SpHb value displayed by the Masimo Radical-7 and the arterial samples were taken at the same time. Future studies with a standardised design are needed to confirm or deny the results here presented.

3.11.5 Estimation of Total Blood loss

The volume of total blood loss was estimated based on the amount in the suction chamber, and by the gravimetric method (weighing of the pre- and post-procedure gauze). However, currently there is no 'gold standard' method to quantify intraoperative blood loss, and its estimation might be more difficult if most of the blood is absorbed by surgical gauze and not collected in the suction bottle. Despite other methodologies for blood loss estimation existing, the most are not in routine use either due to their unavailability or time-consuming nature during surgical procedures. Therefore, it is not possible to exclude that other methods for estimating blood loss would result in different treatment or blood transfusion timing, even if the amount of blood loss was not the only end point to start a PRBCs administration.

3.11.6 Transfusion trigger

The term "transfusion trigger" is used to describe a set of conditions under which transfusion is considered to be indicated and for which no further justification is required. One of the earliest transfusion triggers was the 10/30 rule used in both human and veterinary patients, which stated that presurgical patients should be transfused if their Hb concentration was less than 10 g dL^{-1} or their haematocrit was less than 30% (Adams & Lundy, 1942). Further support for the 10/30 rule comes from the observations in animal models (Chapler &Carn, 1986; McFarland, 1999). Nevertheless, it was also recognised that healthy anesthetised animals could tolerate very low [Hb] and Hct (5 g dL^{-1}; 15%) as long as intravascular volume is maintained (Weiskopf et al. 1998). The result reported in the present case series could be influenced by the transfusion trigger chosen; and different transfusion trigger would produce probably different results. Patient Blood Management (PBM) would help to reduce the discrepancy among future studies, as this clinical concept, if implemented, has the primary goal of avoiding unnecessary blood transfusions and improving patient outcome and safety (Thakrar et al. 2017).

3.11.7 Monitoring of Fluid therapy

Despite the impressive ability of the body to adjust 'wrong' fluid therapy administration, fluid requirements should be re-evaluated and adjusted regularly based on the patient requirements, rather than based on a general recommendation. To avoid this uncertainty, a GDFTP and a dedicated haemodynamic monitoring should be used, however due to the retrospective and clinical nature of the data present in this case series, it was not feasible in this occasion. Future prospective studies should use a controlled GDFTP.

3.12 Conclusion and Clinical Relevance

The non-invasive measurement of blood constituents such as total Hb concentration has been a highly desired and largely unachieved goal of medical bioengineering until the introduction of the pulse CO-oximeter, which can be used for continuous haemoglobin (SpHb) monitoring.

Although SpHb monitoring is not sufficiently accurate to completely replace invasive measurements, the results of the present retrospective study are very encouraging and showed the utility of this device in the surgical theatre. Future well-designed prospective studies (experimental and/clinical) are needed to confirm or contradict these findings.

3.13 Future Studies

The influences of the type of colloids administered (synthetic vs natural), timing, rate, duration, and rapidity of administration on SpHb values needs future investigation.

Nevertheless, it would be interesting to investigate if the SpHb may be able to inform the clinician of decrease in [Hb] in a timely and accurate manner, preventing unnecessary diagnostic blood draw, while offering detailed clinical evidence for transfusion decision as shown in human patients (Tang et al. 2019).

Additionally, as PRBCs transfusion is costly and a significant contributor to the expense of surgical care, it would be interesting to study if SpHb will shortening the time to start transfusion and decreased the number of blood sampling to test Hb values and/or the transfusion events during surgery and the total expense of the surgical procedure and/or postoperative hospitalisation.

Lastly, the influences of different vasoactive drugs on the PI and the pulse CO-oximeter signal extraction/quality and SpHb accuracy should be investigated.

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