ASSESSMENT OF SERUM COBALT BINDING WITH DISEASES OF DOMESTIC CATS

BY

AMY NICOLE SCHNELLE

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in VMS - Veterinary Pathobiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Master’s Committee:

Associate Professor Philip F. Solter, Chair
Clinical Associate Professor Anne M. Barger
Clinical Assistant Professor Amy L. MacNeill
ABSTRACT

Ischemia-modified albumin has been investigated for use in human medicine as a clinical marker of ischemia, with particular interest geared toward detecting patients that have chest pain due to coronary artery syndrome. The modified form of circulating albumin has a decreased ability to bind transition metals, and assays are available that measure the amount of cobalt and nickel that bind to albumin in vitro. Oxidative damage has long been a suspected component of the underlying mechanism, while more recent work suggests that increased free fatty acid binding to albumin may also play a role in altering the albumin molecule binding characteristics.

This study validated a cobalt-binding assay for use in cats. Percent cobalt binding (%CB) of serum was assessed. The run-to-run coefficient of variation ranged from 2.90% to 4.37%, while within-run precision had a coefficient of variation that ranged from 2.61% to 4.04%. Day-to-day precision was 3.46%. Limited assessment of biological variation revealed minimal change. Up to three freeze-thaw cycles failed to induce clinically significant variation in cobalt binding percentages in most samples. Samples appeared stable for at least 4 hours at room temperature. Addition of conjugated bilirubin to serum was tested up to a total bilirubin concentration of 8.9 mg/dL (icteric index of 2+) and failed to have a clinically significant effect on the percent cobalt binding; however, severely hemolytic (≥4+) samples and even mildly lipemic (≥1+) samples were falsely decreased in the percent cobalt binding when compared to the unadulterated original sample. Overall, this assay appears to function reasonably well in the measurement of cobalt-binding albumin in feline serum.

Percent cobalt binding of serum from healthy and diseased cats was assessed for significant physiological and pathophysiological correlations. It did not correlate with age, sex, or breed in
the study population. Unsurprisingly, %CB was correlated with albumin concentration ($r=0.62$, $p=0.0001$). Other noted correlations included red blood cell count ($r=0.24$, $p=0.003$), hemoglobin ($r=0.29$, $p=0.0001$), hematocrit ($r=0.283$, $p=0.0001$), globulins ($r=-0.20$, $p=0.01$), calcium ($r=0.27$, $p=0.001$), total bilirubin ($r=-0.237$, $p=0.005$), and absolute lymphocytes ($r=0.16$, $p=0.05$). However, the albumin concentration was decreased in the inflammatory group, in comparison with the noninflammatory group, and, likewise, albumin was correlated with red blood cell count, hemoglobin, hematocrit, globulins, calcium, and total bilirubin. The only parameter to which %CB seemed independently linked was a very weak correlation to the absolute lymphocyte count. Percent cobalt binding and albumin concentration were decreased in cases classified as “inflammatory”, as compared with those classified as “noninflammatory”. While %CB was not correlated with any of the disease categories of this study, group numbers were small.

Limitations of this study included variable sample handling, small disease group numbers, and retrospective evaluation. The consistency of the relationship between albumin concentrations, albumin-linked correlates, and the measured %CB seems to confirm the validity of the assay in measuring ischemia-modified albumin, though this study failed to show a definitive clinical relevance of the assay in cats. Prospective studies with more controlled sample handling may yield more clinically significant findings.
For my parents,

Cathleen and David Schnelle,

For always believing in me
ACKNOWLEDGMENTS

Much gratitude goes to my research advisor, Dr. Phil Solter, for having infinite patience in walking me through the world of laboratory research. Sincerest thanks to Drs. Anne Barger and Amy MacNeill, for lending excellent clinical and research knowledge, in addition to mental and emotional support. A huge thank you goes to Dr. Mark Mitchell for performing the statistical analysis on this project. I am also much obliged to David C. Gaze for supplying several key references for this thesis.

Thank you to Drs. Amy Kubier and Sallianne Schlacks for lending time and technical skills in obtaining samples for this project. Thank you to Drs. Joanna Schmit and Caitlin DeWilde for always supporting me. Thank you to Joey Barger, Margot Schlacks, and Ella Schnelle for donating blood to help complete the validation.
# TABLE OF CONTENTS

LIST OF ABBREVIATIONS........................................................................................................vii

CHAPTER 1: INTRODUCTION..................................................................................................1

CHAPTER 2: LITERATURE REVIEW.......................................................................................4

CHAPTER 3: MATERIALS AND METHODS............................................................................30

CHAPTER 4: RESULTS.............................................................................................................41

CHAPTER 5: DISCUSSION.......................................................................................................45

CHAPTER 6: CONCLUSIONS..................................................................................................52

CHAPTER 7: FIGURES AND TABLES.....................................................................................54

REFERENCES..........................................................................................................................65
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CB</td>
<td>Percent cobalt binding</td>
</tr>
<tr>
<td>ABSU</td>
<td>Absorbance units</td>
</tr>
<tr>
<td>ACB</td>
<td>Albumin cobalt binding</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood count</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CK-MB</td>
<td>Creatine kinase isoform MB</td>
</tr>
<tr>
<td>Co(II)</td>
<td>Cobalt(II)</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>Cobalt chloride</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>cTnI</td>
<td>Cardiac troponin I</td>
</tr>
<tr>
<td>cTnT</td>
<td>Cardiac troponin T</td>
</tr>
<tr>
<td>CTP</td>
<td>Child-Turcotte-Pugh</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DK</td>
<td>Diabetic ketosis</td>
</tr>
<tr>
<td>DKA</td>
<td>Diabetic ketoacidosis</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-dithiothreitol</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>Hct</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>H-FABP</td>
<td>Heart-fatty acid binding protein</td>
</tr>
<tr>
<td>Hgb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>High sensitivity C-reactive protein</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IMA</td>
<td>Ischemia modified albumin</td>
</tr>
<tr>
<td>IMAR</td>
<td>IMA-to-serum albumin ratio</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>Max</td>
<td>Maximum</td>
</tr>
<tr>
<td>MBS</td>
<td>Multi-metal binding site</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MELD</td>
<td>Model of end-stage liver disease</td>
</tr>
<tr>
<td>Min</td>
<td>Minimum</td>
</tr>
<tr>
<td>N</td>
<td>Number</td>
</tr>
<tr>
<td>NSTE-ACS</td>
<td>Non-ST elevation acute coronary syndrome</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>Non-ST-segment elevation myocardial infarction</td>
</tr>
<tr>
<td>NTproBNP</td>
<td>Amino-terminus B-type natriuretic peptide</td>
</tr>
<tr>
<td>NTS</td>
<td>Amino-terminal binding site</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PTE</td>
<td>Pulmonary thromboembolism</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>SCB</td>
<td>Serum cobalt binding</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SMA</td>
<td>Superior mesenteric artery</td>
</tr>
<tr>
<td>STEMI</td>
<td>ST-segment elevation myocardial infarction</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UA</td>
<td>Unstable angina</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

In human medicine, there has been interest in developing biomarkers that can detect myocardial ischemia in patients with chest pain, ideally, prior to complete infarction. One proposed biomarker is the ischemia modified albumin (IMA) test, a colorimetric assay that utilizes patient serum to indirectly measure changes in the ability of albumin to reversibly bind ionized cobalt. An assay for IMA, the Albumin Cobalt Binding test, was approved by the Food and Drug Administration (FDA) in 2003\(^1\). Studies conducted over the past decade have shown that other conditions, including strenuous exercise, diabetes mellitus, and obesity, also have the potential to reduce albumin-cobalt binding\(^2\)–\(^6\). While it is likely that there are multiple mechanisms for altered serum cobalt binding with ischemia and other conditions, it is believed that free radical damage to the transition metal binding sites of albumin inhibits the protein’s ability to reversibly bind to transition metals, such as cobalt\(^7\). Increased serum free fatty acid concentrations may also competitively inhibit cobalt binding by albumin\(^8\). However, the mechanism for altered albumin-cobalt binding with disease is incompletely characterized at this time.

With the exception of limited studies utilizing laboratory animals (rabbits and rats) as experimental models of ischemia in various tissues\(^9\)–\(^12\), serum cobalt binding has not been assessed in veterinary species. It is unknown whether this marker is measurable in common companion animal species, such as cats, dogs, and horses, and if such a marker would hold any value in diagnostic testing and medical decision-making. Therefore, this study was undertaken to
validate and investigate serum cobalt binding in domestic cats, in order to evaluate for potential uses in veterinary medicine.

Between June 2011 and December 2011, residual serum from samples submitted to the University of Illinois Clinical Pathology Laboratory was collected and assayed for percent cobalt binding (\%CB). The samples were from cats, presented to the Veterinary Teaching Hospital for any reason during the study period, in which diagnostic testing on serum was part of the medical work up. The specific diagnostic testing was requested by the clinician on the case, and the author was blinded to the testing performed on the samples by the clinical pathology lab until after cobalt binding assays were complete. All serum samples submitted during the study period were considered for inclusion in the study.

Validation testing revealed that the assay successfully measures serum cobalt binding in cats. Within-run precision ranged from 2.61 to 4.04\% over 24 runs. Run-to-run precision was ranged from 2.90 to 4.37\% over 30 runs (3 samples, 10 runs each). Day-to-day precision was 3.46\% over 5 days. Limited biological variation assessment showed clinically insignificant changes. Serial dilutions (1: 2, 1: 4, and 1: 8) were linear. Temperature stability testing and freeze-thaw stability were assessed for clinically significant changes in percent cobalt binding, defined by the author as a change greater than 10\% from the baseline value. Samples were found to be stable with up to three freeze-thaw cycles, while most samples did not vary more than 10\% from baseline up to 4 hours after sample collection while at room temperature. Assessment for effects of interfering substances revealed that up to 8.9 mg/dL of conjugated bilirubin (2+ icteric index) failed to have a significant effect on \%CB, while a 1+ lipemic index or greater and 4+ hemolytic index had negative effects on \%CB.
Of 374 samples initially collected, 175 were of sufficient volume and quality to be included in the study. The median %CB was 73.9% (25-75th percentiles: 66.2-80.3%, min-max: 34.93-92.37%). Cobalt binding values were not found to be significantly different by disease state, sex, age, or breed. Cobalt binding was found to be positively correlated with red blood cell count \((r=0.24, p=0.003)\), hemoglobin \((r=0.29, p=0.0001)\), hematocrit \((r=0.283, p=0.0001)\), albumin \((r=0.62, p=0.0001)\), total calcium \((r=0.27, p=0.001)\), and absolute lymphocytes \((r=0.16, p=0.05)\).

Cobalt binding was negatively correlated with globulins \((r=-0.20, p=0.01)\) and total bilirubin \((r=-0.237, p=0.005)\). Cobalt binding was not correlated to white blood cell count, absolute segmented neutrophils, absolute band neutrophils, absolute monocytes, absolute eosinophils, absolute basophils, serum total protein, glucose, alkaline phosphatase (ALP), cholesterol, triglycerides, or corrected calcium. Because the correlation between albumin and %CB was the strongest of those observed, albumin was then evaluated for correlations with the other analytes described above. Absolute lymphocytes appeared to be the only analyte in which %CB was correlated, independent of albumin. The reason for this is uncertain, and the clinical significance of this weak correlation is likely negligible. Cats characterized as having inflammation \((n=20)\) had significantly lower \((p=0.002)\) cobalt binding percentages than cats without evidence of inflammation \((n=22)\). The albumin concentration of the cats in the inflammatory group also had an albumin concentration that was significantly lower than that of the noninflammatory group. Because albumin is a negative acute phase protein, this is a logical explanation for the apparent %CB correlation to inflammation in this study.
CHAPTER 2

LITERATURE REVIEW

Albumin is the most abundant protein in plasma and is synthesized by the liver. It provides colloid oncotic pressure and is a carrier for many substances, including fatty acids, bile acids, bilirubin, hormones, vitamins, calcium, and transition metals, including copper(II), nickel(II), cadmium(II) and cobalt(II). The half-life of albumin in people varies from 15-23 days, although a narrower range is sometimes used. The half-life of feline serum albumin does not seem to have been described, but it is reported as 8.2 days in dogs. Human serum albumin is 585 residues, made up of 3 homogenous domains, while feline serum albumin is reported to be 584 residues. The proteins appear 81% homologous, with similar amino-terminus residues; human albumin is Asp-Ala-His, while feline albumin is Glu-Ala-His (Figure 1).

The ability of albumin to bind transition metals, in particular Co(II), is the focus of this study and relevant principles will be reviewed here. Free Cu(II) ions and Fe(III) salts can generate free radicals via the Fenton reaction, so the ability of albumin to bind transition metal ions serves a protective role. Three binding sites for cobalt have been defined through spectroscopic and thermodynamic experiments; in order of decreasing affinity, these are the carboxylate-rich cadmium site B (site B), the multi-metal binding site (MBS or site A), and the amino-terminal binding site (NTS). Chan et al. found that human serum albumin incubated in vitro at 4°C and 37°C for 12 days experienced degradation of the Asp-Ala residues, released as a dipeptide of the NTS, but this did not appear to be due to transition metal binding itself. For a long period, the NTS was thought to be the only or the primary binding site for cobalt, and it was theorized that
this site could become damaged by free radicals secondary to ischemia and/or reperfusion injury\textsuperscript{23}. The study by Chan was used as support for the susceptibility of the NTS to degradation\textsuperscript{23}, though the cause of the \textit{in vitro} degradation in that study was not determined. Interestingly, that study did not find similar degradation with bovine, chicken, dog, goat, horse, pig, rabbit, rat, or sheep albumin. Cat albumin was not tested, though the three terminal NTS residues matched those of rat and rabbit albumin (Glu-Ala-His); however the next two residues in sequence differed from those species’ albumin (\textit{Gln-Ser vs. Lys-Ser})\textsuperscript{20,22}.

An assay has been described\textsuperscript{7,23} that measures the cobalt binding ability of a serum sample. It works by adding a known amount of Co(II) to a solution containing patient serum albumin. Normally, albumin binds a given quantity of the cobalt, leaving a small amount free in the medium. Dithiothreitol (DTT) is then added, which reacts with the free cobalt to produce a color change; this is subsequently measured spectrophotometrically. In patients experiencing ischemia, an increased fraction of the serum albumin has a decreased ability to bind the cobalt. This ischemia-modified albumin leaves increased amounts of free cobalt in solution, which consequently reacts with more DTT, producing a darker color change and a higher reading of absorbance units (ABSU); in the literature, this indirect measurement is then reported as the value of IMA in patients. Figure 2 is a cartoon illustrating the assay.

In 2000, Bar-Or et al. published a paper on the assay, terming it the albumin cobalt binding (ACB) assay\textsuperscript{23}. The authors investigated the results of this assay as an early marker of myocardial ischemia. The goal was to develop a biomarker that would improve the ability to detect myocardial ischemia in patients presenting for chest pain due to cardiac causes\textsuperscript{23}. This early study found that ACB values from a group of patients diagnosed with acute myocardial
ischemia were higher than those of patients that presented with chest pain due to noncardiac causes. The altered albumin came to be known as ischemia modified albumin (IMA). Further work in cardiac patients followed, as well as evaluation of the assay in other diseases. Evaluation of IMA levels in patients undergoing percutaneous coronary intervention (angioplasty), in which a balloon is inserted into a narrowed artery and inflated, found that this procedure causes an immediate rise in IMA, which resolves quickly after the procedure is over\(^{24-27}\). Results returned to normal by 1 hour post-procedure\(^{26}\). Garrido et al. found that IMA increased with angioplasty in patients with unstable angina, but there was less of an increase in patients that had collateral coronary circulation, compared with those that did not\(^{28}\). In one study, IMA was increased immediately after the procedure and was normal by 6 hours, while creatine kinase-MB, myoglobin, and cardiac troponin I were normal immediately after and increased at 6 hours, supporting IMA as an early marker of ischemia, with an advantage over more conventional markers\(^{27}\). Some researchers found weak correlations between IMA increase and the pressure and duration of balloon inflation\(^{25}\), but others did not\(^{29}\). IMA was also found to increase 30 minutes after cross-clamping of vessels in cardiac bypass surgery, decreasing in the 6 hours post-operatively but not returning to the pre-operative baseline\(^{30}\). Through the progression of research, it appeared that there was a strong relationship between ischemia and the development of increased proportions of IMA in circulation.

In addition to myocardial ischemia, increases in IMA have been found in volunteers experiencing skeletal muscle ischemia, in patients with conditions associated with oxidative stress, such as diabetes mellitus and obesity, in experimental models of organ torsion, in patients with thromboembolic disease, and in pregnant women, among others\(^{3,10,11,31-34}\). Several reviews
have also been published\textsuperscript{15,35–39}. The only published studies to date regarding serum cobalt binding and IMA in veterinary species have been in experimental models utilizing laboratory animals. These will be outlined below with related studies.

The exact mechanism of IMA formation remains unconfirmed. Despite initial interest in the amino terminus of albumin, a few studies have found that alteration of the NTS of albumin has little relationship to increasing IMA levels\textsuperscript{40,41}, and recent studies have convincingly shown that there are three Co(II) binding sites\textsuperscript{18,42}. This more recent work has indicated that cobalt affinity for the NTS is the lowest of the three sites. However, the early research on IMA and the ACB assay lacked this information, and the NTS was presumed to be the primary cobalt binding site and to be damaged in some way during the ischemia and/or reperfusion process. Oxidative damage by reactive oxygen species was one proposed mechanism, which found some support in an \textit{in vitro} study that evaluated the effects of hydrogen peroxide, superoxide radical, and hydroxyl radical (generated via Fenton reaction) on human serum albumin\textsuperscript{7}. Hydrogen peroxide and superoxide radical treatment were found to have negligible effects on IMA, but hydroxyl generation by the Fenton reaction with Cu(II) increased IMA up to 43.6\% after 15 minutes. Another study found similar \textit{in vitro} results, supporting the importance of the Fenton reaction and hydroxyl radical generation in causing a decrease in albumin cobalt binding\textsuperscript{43}. Studies in people have found some peripheral support for oxidative damage, likely secondary to inflammation, as a mechanism of IMA formation. Several have found IMA and C-reactive protein (CRP, a nonspecific marker of inflammation) both to be increased in patients\textsuperscript{4,5,44–49}. While some did not report correlation information\textsuperscript{4,44,46,49}, others reported a correlation between IMA and CRP\textsuperscript{5,45,48}, while one found no link\textsuperscript{47}. Others found indirect evidence of oxidative
damage via increases in malondialdehyde\textsuperscript{32,50–53}, homocysteine\textsuperscript{49,52}, protein carbonyl\textsuperscript{53,54}, and advanced oxidation protein products\textsuperscript{53}, though correlation information was not included. Two studies investigating ischemia in rat models found that administration of compounds with antioxidant properties (clotrimazole\textsuperscript{55} and N-acetylcysteine\textsuperscript{56}) blunted increases in IMA following vessel ligation.

Another theory that is gaining traction in the literature is that increased free fatty acid binding to albumin obscures the two primary binding sites for cobalt, resulting in increased levels of IMA\textsuperscript{8}. Fatty acids are known to be increased during myocardial ischemia\textsuperscript{57,58}. A study of IMA and fatty acid levels compared patients with acute myocardial infarction to a control group (in which patients with renal disease, thyroid disease, diabetes mellitus, obesity, and patients with albumin concentrations outside the reference interval were excluded)\textsuperscript{57}. They found that IMA and fatty acids were increased and correlated in patients with myocardial infarction; further evaluation determined that oleic, palmitic, linoleic, stearic, and palmitoleic acids were increased, while arachidonic acid was not. They went on to perform in vitro experiments on pooled serum to further test the association. The addition of oleate and arachidonate in vitro was found to increase the IMA levels, and the authors concluded that alterations in fatty acid binding to albumin were the source of increased IMA in patients with myocardial infarction. Another study that extracted fatty acids bound to the albumin of patients with fatty liver and normal controls found that fatty liver patients had increased amounts of oleic, myristic, and lauric acid and that this influenced cobalt binding to albumin\textsuperscript{43}. Finally, a recent in vitro study further evaluated the relationship between fatty acids and cobalt binding. The fatty acid myristate was added to bovine serum
albumin solutions in increasing concentrations and cobalt binding was assayed. With increasing myristate concentrations, cobalt binding decreased progressively\(^8\).

Unfortunately, the studies above make up the few that have reported fatty acid concentrations with IMA results. Of published reports that include other patient lipid information, triglyceride, total cholesterol, high density lipoprotein (HDL) cholesterol, and low density lipoprotein (LDL) cholesterol concentrations are those most commonly reported, and results have been variable. A study of patients with myocardial infarction found no correlation between IMA levels and total cholesterol\(^{45}\). Similarly, Kaefer et al., in a study of diabetic patients, found that IMA was not correlated to triglycerides, total cholesterol, or HDL cholesterol\(^5\). In contrast, a study of patients with hyperthyroidism or hypothyroidism found that, along with increased IMA, hypothyroid patients had increased total, HDL and LDL cholesterol levels, though none was correlated with IMA. Hyperthyroid patients, on the other hand, were found to have increased IMA and decreased total and LDL cholesterol concentrations, and both were negatively correlated with IMA.

Regardless of the mechanism of IMA formation, the overall albumin concentration has been shown to be an important factor in the IMA result\(^{50-62}\), often the only or strongest correlate—a link that is unsurprising. It is logical that IMA is negatively correlated with albumin concentrations, since a serum sample with a low albumin concentration would appear to have an increased IMA level—fewer albumin molecules would be present to bind the standardized amount of cobalt added during the cobalt binding assay, resulting in an increased amount of free cobalt and a darker colorimetric result. The negative correlation between the two factors was found to be strongest when hypoalbuminemia was present\(^{63}\), a fact that could limit the utility of the cobalt binding assay\(^{64}\).
To combat this limiting factor, several authors have proposed correction formulas or ratios to normalize a sample for the albumin concentration. In a study of patients presenting to the emergency department, use of an albumin-adjusted IMA index had an improved sensitivity (98.4%) for ruling out acute coronary syndrome compared with IMA (93.0%)\textsuperscript{65}. The formula used was: 

\[
\text{albumin-adjusted IMA index} = \text{serum albumin concentration (g/dL)} \times 23 + \text{IMA (U/mL) – 100}
\]

Another group calculated an IMA value adjusted for albumin via the following formula:

\[
[(\text{individual serum albumin concentration/median albumin concentration of the population}) \times \text{IMA value}]\textsuperscript{62}
\]

Using this, the median value increased only slightly (94 kU/L vs. 93 kU/L), while the range narrowed from 62-135 to 69-110 kU/L, and the inter-individual coefficient of variation decreased from 15.4% to 10.0%. Another study used an IMA-to-serum albumin ratio (IMAR) in comparison with IMA in patients with chronic liver disease\textsuperscript{66}. This was calculated by dividing IMA by the concentration of albumin in g/dL. Use of the IMAR allowed correlation to the model of end-stage liver disease (MELD) score and the Child-Turcotte-Pugh (CTP) score, which were not significant with IMA. The CTP score is used as an assessment of liver function, while the MELD score is used as a predictor of outcome\textsuperscript{66}. However, none of these calculations appear to have been independently validated for use.

Sample storage stability testing has been performed and published, with some variability of results between authors. A report validating the ACB assay on the Cobas MIRA Plus analyzer found that separated serum samples stored in open tubes at 4\degree\text{C} and 26\degree\text{C} displayed an increase of 3.0% and 5.7% at 4.5 hours after sample collection and a 7.9% and 13.0% increase, respectively, at 8.5 hours\textsuperscript{67}. They also found that samples stored in closed tubes with the clot at 26\degree\text{C} for 8.5 hours showed a 10.8% decrease in values. Another author found that samples stored
at 4°C and 26°C showed a 6.37% and 3.38% increase, respectively, at 2 hours after the baseline assay, while at 4 hours, the room temperature sample was increased 30% from baseline and the 4°C sample was up 21%. Another portion of that study compared sample storage on gel serum separator tubes and aliquoted samples in plain glass tubes. Interestingly, the increases with storage were less substantial here (with both storage techniques), compared with that described immediately above. At 4 hours, the samples stored on the serum separator gel increased 5.23%, while the aliquoted samples increased 9.24%. The authors of this study ultimately concluded that the samples were stable at both temperatures at least 2 hours but recommended assaying the sample within this time frame or freezing immediately to avoid any potential in vitro alterations.

Samples appear relatively stable with freezing at -20°C. While Beetham and group found a statistically significant increase in assay results following thawing, it only amounted to an increase of 3.43%, a difference that seems unlikely to have a strong influence in clinical decision-making. The Cobas MIRA paper found a 5% or less change in assay result with freezing at -20°C, with thawing at 4°C; however, a 15% increase was observed in samples thawed at room temperature.

The remainder of this review pertains to the clinical findings of IMA in particular diseases or conditions. In the majority of the studies, sample handling appears to have been relatively consistent. In many studies, the samples were frozen at -20°C or -80°C, though the period of freezing was variable and the temperature at which thawing occurred was not noted.

**Cardiac Disease**
In human medicine, diagnosis of acute myocardial ischemia in patients presenting for chest pain can be challenging and typically relies on the combination of chest pain, changes on electrocardiogram (ECG), and serum marker elevation (such as cardiac troponins). However, markers like troponins T and I, while specific for cardiac injury, are indicators of cell leakage or necrosis and therefore only become elevated once tissue damage has occurred. A reliable biomarker for early ischemia would be beneficial in minimizing cardiac damage. Bar-Or and group noticed that *in vitro* binding of cobalt by serum from patients with myocardial ischemia was decreased and in 2000 published a paper describing the cobalt binding assay as a means of indirectly measuring ischemia-modified albumin, which they proposed as a biomarker of ischemia. This initial study found that serum from patients with myocardial ischemia had greater amounts of unbound cobalt at the end of the assay, compared with patients without ischemia, suggesting that albumin was somehow stripped of its ability to bind cobalt in solution.

The Albumin Cobalt Binding test was approved by the FDA in 2003 for use in ruling out myocardial ischemia, in combination with electrocardiogram and BNP testing. This assay remains the only FDA-approved test for detecting ischemia.

In the ensuing years, many studies have been carried out to test the ACB assay. Studies have primarily centered on cardiac conditions in which ischemia or infarction is the lesion of interest. Terminology used in the medical literature includes acute coronary syndrome (ACS), acute myocardial infarction/ischemia, unstable angina pectoris, and stable angina pectoris. Myocardial ischemia, the inadequate supply of oxygen to the heart, frequently is associated with partial obstruction of coronary vessels due to atherosclerosis and typically results in angina, or chest discomfort, in the patient. Myocardial infarction is cardiac cell death following protracted
ischemia. It is further subdivided according to the presence or absence of ST-segment elevation on ECG: ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation myocardial infarction (NSTEMI). Stable angina pectoris is chest discomfort that occurs with exercise or emotional stress and resolves with rest, while unstable angina (UA) pectoris occurs at rest, as overt pain or as pain that increases in severity (“crescendo pattern”). Unstable angina and NSTEMI are sometimes grouped together as one entity (“UA/NSTEMI”). The use of the term “acute coronary syndrome” throughout the medical literature appears to be used as a general reference to myocardial ischemia and infarction. According to Aparci et al., ACS includes unstable angina, NSTEMI, and STEMI.

The assays for IMA throughout the studies described below used manual or automated albumin cobalt binding assays, and automated assays were performed on one of several chemistry analyzers, though the Cobas MIRA analyzer seems to occur most frequently. IMA values are reported as absorbance units (ABSU). Optimal cut-offs or manufacturer cut-offs were used variously in determination of sensitivity and specificity.

As referenced earlier, the use of IMA for early detection of myocardial ischemia (of any type) has been of interest to the medical community and a substantial body of literature investigating this now exists. Many studies have noted that IMA is increased in patients with myocardial ischemia, supporting the work of Bar-Or and group. Multiple studies have evaluated the sensitivity and specificity of IMA for myocardial ischemia, typically comparing patients with ischemic chest pain to those with non-ischemic chest pain. At an optimal cut-off value of 70.0 units/mL, Liyan et al. reported a sensitivity of 94.4% and a specificity of 82.6% for detection of acute coronary syndrome in 113 patients. This study measured IMA within 12 hours of onset of
chest pain, and it also compared IMA performance to that of C-reactive protein (CRP). CRP only had a sensitivity of 70.0% and specificity of 73.9% for detection of ACS\textsuperscript{44}. In a similar study, this group evaluated IMA again, this time comparing it to heart-fatty acid binding protein (H-FABP), another potential marker of cardiac ischemia that is under review\textsuperscript{75}. This study found that IMA sensitivity was 89% and specificity was 80.8% for ACS, while H-FABP was 82.9% sensitive and 80.8% specific. Combining the two markers improved sensitivity to 96.3%. The patients in this study had normal cardiac troponin concentrations upon presentation.

Overall, other studies have had similar results, finding that, in patients with chest pain at presentation to the emergency department, IMA is moderately to very sensitive for myocardial ischemia, while specificity is moderate to poor. Sensitivity of IMA in studies similar to the two described above have ranged from 80% to 94.4%, while specificity has ranged from 31% to 69%\textsuperscript{76–78}. Interestingly, a rare study has found IMA to be very specific for myocardial ischemia. Bhagavan et al. reported a sensitivity of 88% and an impressive 94% specificity in distinguishing patients with myocardial ischemia from those without. However, when they tried to use IMA to determine acute versus non-acute myocardial infarction, IMA was not very useful, with an area under the ROC curve of only 0.66\textsuperscript{41}.

IMA has also been evaluated in conditions with vascular changes that precede chest pain due to ischemia. Koc et al. measured IMA in patients determined to have slow coronary flow due to stenosis of epicardial arteries and identified no increase\textsuperscript{79}, while Kazanis et al. found IMA to be increased in patients that had atherosclerosis but no signs of acute coronary syndrome\textsuperscript{80}. The latter study did not note any correlation between IMA levels and the number of diseased vessels.
Multiple studies have compared IMA to more established biomarkers, as well as evaluating the value of combining IMA with more standard tests to diagnose myocardial ischemia. As mentioned above, it has been shown that IMA was more sensitive and specific than CRP (a positive acute phase protein) and was more sensitive than H-FABP, with similar specificity, in identifying myocardial ischemia. IMA alone was more sensitive than a panel of myoglobin, creatine kinase-MB, and cardiac troponin I, but addition of IMA to that panel resulted in a sensitivity of 97% and a negative predictive value of 92%. For detection of ACS, ECG and cardiac troponin I (cTnI) had specificities of 91% and 99% respectively, but sensitivities of only 45% and 20%, respectively. A panel of IMA, ECG, and cTnI resulted in an increased sensitivity of 95%. Collinson et al. found that in a study of patients suspected to have acute myocardial infarction, a normal cTnI result combined with a normal IMA result had a negative predictive value of 100%. In 131 patients presenting with chest pain but with normal or non-diagnostic ECGs, IMA was higher in patients with ACS compared with those with nonischemic chest pain. IMA had a sensitivity of 90.6% and specificity of 49.3% in classifying these patients. Sensitivity increased to 92.2% when combined with cardiac troponin T. From these described studies, it appears that IMA’s value primarily lies in its ability to rule out ischemia, improving the performance of more standard diagnostic tests.

A few studies have failed to find an advantage in the use of IMA assessment. A study of 185 patients with NSTEMI did not find IMA to be predictive of ACS diagnosis [odds ratio (OR) = 1.23; 95% CI = 0.87-1.81], while heart-fatty acid binding protein was predictive (OR = 4.65; 95% CI = 2.39-9.04) and specific (96.8%). A study of 367 patients with chest pain found median IMA and ranges to be almost identical between the 162 patients with nonischemic chest
pain and the 205 patients with ischemic chest pain\textsuperscript{39}. The reason for the similarity between groups in this study, in contrast with the findings of most other studies, is unknown.

Several studies have evaluated IMA’s predictive capabilities, though results describe limited utility in this capacity. A few have examined IMA’s ability to predict a serious cardiac event (including death, myocardial infarction, heart failure, and arrhythmia) within 72 hours among patients presenting for chest pain. One study of 189 patients, of which 24 experienced serious outcomes within the study period, reported that IMA was better able to predict events over time\textsuperscript{84}. Samples were taken at presentation, 3 hours, and 6 hours. With a cut-off of 80 units/mL, sensitivity was 70\% at 0 hrs, 81\% at 3 hours, and 92\% at 6 hours, while specificity was poor: 24\%, 11\%, and 6\%, respectively. Kavsak et al., in a similar study, did not find IMA to be predictive of outcomes within 72 hours\textsuperscript{85}. In patients resuscitated from cardiac arrest, IMA was increased in those that went on to have a poor outcome over those with a good outcome\textsuperscript{50}. In that study, the IMA level of the good outcome group was not increased over the control group (healthy volunteers). An evaluation of IMA relative to outcome at 1 year in patients with acute myocardial ischemia observed that the measurement was an independent predictor of death at the end of the study period, along with B-type natriuretic peptide concentration, heart failure, and age\textsuperscript{86}. In 63 patients that underwent cardiac surgery, 10 developed perioperative infarctions; the IMA levels of those 10 patients were significantly higher at 3 hours post-operatively than those that did not develop infarctions, due to a slower decrease in IMA toward baseline in the infarct group\textsuperscript{87}.

Definite limitations have been encountered in the use of IMA in the medical literature. IMA was not different between patients with non-acute myocardial infarction, compared with acute
myocardial infarction\textsuperscript{41}. A study of chest pain patients contained a subset of stroke victims with elevated IMA and normal cTnT at presentation\textsuperscript{88}, so care must be taken in evaluating patients with chest pain and neurological signs, as IMA levels alone are not likely to distinguish these groups. Measurement of IMA did not add prognostic value to the Thrombolysis in Myocardial Infarction risk score in patients with STEMI that underwent angioplasty\textsuperscript{89}. Wudkowska et al. failed to show that IMA was able to distinguish between cardiac troponin I positive and negative groups, the underlying diagnoses of which were unstable angina and NSTEMI, respectively\textsuperscript{90}; however, elsewhere, these diagnoses are classified together as non-ST elevation acute coronary syndrome (NSTE-ACS)\textsuperscript{71}.

Exercise stress tests are used to evaluate a patient’s ECG for characteristic changes associated with myocardial ischemia that are not present while the patient is at rest. Interestingly, IMA levels in myocardial ischemia in the context of exercise stress testing appear to be less consistent than in patients at rest. Lee et al. observed that patients that developed ECG changes during exercise and had a statistically significant increase in IMA compared to baseline had a large ischemic burden, as classified during coronary angiography. Patients that had a positive exercise stress test but a negative IMA test (decrease in IMA with exercise) more often had a small ischemic burden at angiography; however, there was overlap between groups\textsuperscript{91}. Another study that similarly evaluated IMA after exercise stress testing, compared with before, did not find a difference between the two study groups, those with and without coronary artery stenosis, determined at coronary angiography within 1 week of the stress test\textsuperscript{92}. Only 14 of the 40 total patients had coronary artery stenosis, so the power of the study may have been too low to distinguish a difference. Another small study failed to identify a difference in IMA with exercise.
stress testing. Of 38 patients with chest pain, 15 had myocardial ischemia determined on myocardial perfusion scintigraphy. Both the ischemic and non-ischemic groups experienced decreased IMA levels (compared with baseline) at peak exercise. The researchers found that albumin concentration was the only determinant of IMA, with an increase in albumin (likely due to fluid shifts) and a concurrent decrease in IMA. Sbarouni et al. evaluated exercise stress testing of 40 patients with previously-diagnosed coronary artery disease; of these, 32 patients had undergone percutaneous coronary intervention (angioplasty) or coronary artery bypass surgery. The remaining 8 had at least one coronary artery with more than 80% stenosis. Of the total patient group, 25 developed ECG changes with exercise (positive result), 14 were negative, and 1 was excluded due to an equivocal ECG. IMA levels were not significantly different between the positive and negative exercise stress tests, but IMA did decrease significantly at peak exercise and return to baseline by 60 minutes post-exercise in both groups. An evaluation of 44 patients that underwent exercise stress testing did not identify a change in IMA levels from baseline in either the patients with coronary artery stenosis (n=25) or those with normal arteries (n=19). Contrasting the previously-discussed studies, there was no decrease in IMA with exercise in either group.

In a related study, exercise stress testing in patients with peripheral, rather than coronary, vascular disease reveals similar decreases in IMA levels with leg ischemia at peak exercise. These values returned to baseline when measured at 60 minutes post-exercise. A study of healthy volunteers undergoing forearm ischemia found that IMA levels were decreased at minutes 1, 3, and 5 following release of blood pressure cuff; IMA had returned to baseline by 10 minutes post-ischemia. IMA levels were negatively correlated with albumin concentrations.
In situations of exercise, regardless of underlying disease, it may be that the influence of the total albumin concentration and the fluid shifts that occur with activity mask significant variations in IMA that are caused by the underlying disease. The takeaway is that there is insufficient evidence that IMA is a reliable indicator of myocardial ischemia during exercise stress testing.

**Skeletal Muscle**

In addition to the leg and forearm ischemia studies discussed above, IMA has been further evaluated with respect to skeletal muscle, exercise, and possible ischemia in healthy volunteers, with mixed results. A study of 12 healthy volunteers performing exercise on a plantar flexion pedal to induce calf muscle ischemia reported that subjects’ IMA levels were significantly increased immediately following exercise. IMA levels returned to baseline by 30 minutes after exercise, and a negative correlation was noted between albumin and IMA. There was no association found between lactate and IMA. In another report, a comparison was made between the IMA levels of well-trained professional cyclists and sedentary controls; participants had last exercised 12 to 24 hours prior to sampling for the study. The IMA levels of the cyclists were found to be slightly but significantly elevated over that of the sedentary controls, while BNP levels were decreased. Lippi et al. evaluated the effect of high-workload and medium-workload endurance training by fit athletes, compared with light exercise by sedentary individuals, on IMA levels. They observed that athletes performing high-workload training had increased IMA levels, while they did not differ between athletes performing medium-workload exercise and sedentary individuals performing light exercise. A study of trained male athletes performing submaximal exercise failed to show any change in IMA from baseline. In another study, samples were taken from 19 marathon runners pre-race, immediately post-race, and 24-48 hours post-race. Six
runners had slight increases in IMA at baseline. IMA levels were decreased significantly from baseline immediately following the race, while a significant increase in IMA above both baseline and the immediate post-race levels was reported for the participants at the 24-48 hour mark. The researchers suggested delayed skeletal muscle ischemia or gastrointestinal ischemia as potential causes for this variability.

Evaluation of IMA in skeletal muscle ischemia in several medical scenarios has also been described. IMA appeared to be useful in monitoring the viability of muscle flaps in experimental models of rabbits, with increases observed in animals with flaps in which the vascular pedicles supplying the area had been severed. This same group performed a similar experiment using skin flaps in rats, but noted that IMA levels did not correlate with necrotic flap areas at 6 hours or 1 week post-operatively.

Regarding orthopedic surgery, two studies have been performed. The first, by Refaai et al., measured IMA levels in patients before, 15 minutes after, and before discharge (1.0 to 3.5 hours after surgery) in patients undergoing arthroscopic knee surgery with a leg tourniquet. IMA was increased both at 15 minutes post-operatively and prior to discharge. Albumin concentration was also decreased, which may be the underlying cause for the increase in IMA observed. The second study evaluated IMA levels, along with other cardiac biomarkers NT-proBNP, myoglobin, and CK-MB, in patients undergoing significant orthopedic surgery (hip arthroplasty, knee arthroplasty, or spine stabilization). IMA was increased at 4 and 72 hours post-operatively, while BNP was increased at 72 hours post-op. Myoglobin and CK-MB were increased at 4 hours post-operatively but were trending downward at 72 hours. The authors concluded that there was concern for cardiac injury in patients without pre-operative cardiac
abnormalities undergoing surgery and posited that the IV infusion of large fluid volumes may produce subclinical cardiac stress\textsuperscript{102}.

**Embolic Disease**

Given the potential value of IMA in identifying or ruling out ischemic disease, this analyte has also been evaluated in other embolic diseases, including cerebrovascular accidents and pulmonary thromboembolism. Mentioned previously, Talwalkar et al. found that several stroke patients in their study group displayed increased IMA\textsuperscript{88}. Another study examined the use of IMA, as well as a calculated IMA index, comparing stroke victims to non-stroke patients\textsuperscript{103}. They found that both IMA and IMA index were increased in stroke victims, compared with controls but that there was little overlap between groups when using the IMA index\textsuperscript{103}. Gunduz et al. found that IMA was increased in patients with intracranial hemorrhage, brain infarction and subarachnoid hemorrhage compared with controls. In that study, IMA was higher in brain infarction patients compared with subarachnoid hemorrhage, but it could not distinguish brain infarction from intracranial hemorrhage\textsuperscript{104}. Abboud et al. found that IMA was correlated with the National Institute of Health Stroke Scale in patients with brain infarction and was able to distinguish brain infarction and intracranial hemorrhage from transient ischemic attack and epileptic seizures in patients with neurologic deficits; however, IMA was not able to distinguish brain infarction from intracranial hemorrhage in the study group\textsuperscript{105}. Another group similarly was unable to use IMA to differentiate intracranial hemorrhage from stroke; additionally, IMA was not useful in predicting short-term prognosis in the study group\textsuperscript{106}.
Pulmonary thromboembolism (PTE) is another area of potential use for IMA. Turedi et al. published three sequential papers evaluating IMA in pulmonary embolism. The first, in 2007, determined that IMA was higher in people with pulmonary thromboembolism compared with healthy controls and that the marker may be useful in excluding pulmonary embolism\textsuperscript{107}. In 2008, the group reported that, in patients suspected to have PTE, IMA was higher in patients with PTE compared with those without, as determined by imaging procedures. Additionally, a comparison of IMA to D-dimer concentration in those patients with pulmonary embolism revealed that tests had similar performances (IMA sensitivity = 93\%, specificity 75\%; D-dimer sensitivity = 98.9\%, specificity 62.7\%) and that IMA was a viable alternative to D-dimer testing in suspected PTE patients\textsuperscript{108}. Finally, a paper published in 2009 reviewed IMA findings in an experimental model of pulmonary embolism in New Zealand rabbits\textsuperscript{33}. This experiment utilized 4 groups of rabbits: group 1 was made up of control animals, group 2 had ligation of the iliac vein to simulate deep venous thrombosis, group 3 had ligation of the iliac vein and induction of PTE by injection of clot material, and group 4 had only PTE. Samples were taken pre-operatively at time 0 and then again at 1, 3, and 6 hours after thrombosis. The IMA levels in the animals with PTEs were elevated by the 1 hour mark and continued to climb, while animals with deep venous thrombosis alone and control animals remained near baseline. There was no difference between groups 1 and 2 or between groups 3 and 4\textsuperscript{33}. In the discussion of this paper, the authors note that deep venous thrombosis is frequently associated with pulmonary thromboembolism and that D-dimer concentrations are typically elevated in each condition\textsuperscript{33}. IMA may be a useful adjunct in assessing a patient with deep venous thrombosis for a concurrent PTE but further work is required.
Mesenteric, Ovarian, and Testicular Ischemia

Evaluation of mesenteric ischemia has primarily occurred in experimental models with laboratory animals, though one evaluated IMA in human patients with thromboembolism of the superior mesenteric artery (SMA) and found that it was elevated, with very little overlap with the healthy control group. Two studies found elevations in IMA following experimental ligations of the SMA in rats and rabbits, which continued to increase significantly with time. However, another study in rats failed to find a difference in IMA levels between animals with vascular ligations and those that only had sham surgeries.

IMA levels were significantly increased in rat models of ovarian torsion, and a related study noted that administration of the antifungal clotrimazole, which is a potent free radical scavenger, to rats undergoing ovarian torsion decreased the magnitude of increase in IMA, malondialdehyde, and total oxidant status.

Testicular torsion has also been assessed. Three studies using rat models observed significant increases in IMA levels with testicular torsion, while one found that the increase was blunted by the administration of N-acetylcysteine. The latter study also observed that the degree of IMA increase correlated with an increasing histopathology score of the damage in the testicular tissue.

Diabetes Mellitus

There is evidence in the medical literature that ongoing oxidative damage secondary to hyperglycemia occurs in diabetic patients, which precedes overt diabetic complications.
Assessment of IMA in patients with diabetes has not only evaluated the levels of IMA in these people compared with healthy controls, but several studies have also assessed the relationship between IMA and more biochemical values than many other studies have attempted, including indicators of inflammation and oxidative damage, such as malondialdehyde, a marker of lipid peroxidation\textsuperscript{113}.

A study by Kaefer et al. noted that IMA levels in patients with type 2 diabetes mellitus (T2DM) were significantly higher when compared with controls. In addition, IMA was weakly correlated with fasting glucose concentrations and high-sensitivity C-reactive protein (hs-CRP). The correlation between IMA and glycated albumin approached significance (p=0.0561), but there was no relationship between IMA and triglycerides, creatinine, total cholesterol, HDL cholesterol, or albumin in the data\textsuperscript{5}. However, another study of patients with T2DM and diabetic ketosis (DK) failed to identify correlations between IMA and any of the biochemical data collected, including CRP, though IMA was elevated in both T2DM and DK patients and was significantly different between T2DM and DK\textsuperscript{47}. The area under the curve for distinguishing DK was 0.917, with a sensitivity of 87.0\% and specificity of 85.7\% at an optimum cut-off, and the researchers concluded that IMA was an independent risk marker for DK. Patients in that study were excluded if they had other inflammatory conditions or ECG abnormalities. Piwowar et al. noted that not only was IMA increased in T2DM compared with controls, but there was a statistically significant difference between patients with poor glycemic control and those with good glycemic control\textsuperscript{3}. In an assessment of IMA in type 1 diabetes mellitus (T1DM) patients, IMA was found to be increased in those with type 1 diabetic ketosis compared with uncomplicated T1DM and controls\textsuperscript{4}. CRP was also increased in that group. IMA, CRP, and
blood glucose all decreased following insulin treatment. Researchers in this study also concluded that IMA was an independent risk marker for diabetic ketoacidosis.

Several studies have evaluated IMA in diabetic patients with confirmed peripheral arterial disease secondary to their DM. The study by Piwowar subdivided the T2DM group into “microangiopathy”, “macroangiopathy”, and “both” groups but were unable to find a significant difference between any. However, Ma et al. observed that DM patients diagnosed with peripheral arterial disease via the ankle-brachial index had higher IMA levels than DM without vascular disease. Increased IMA levels in patients with diabetic retinopathy compared with DM patients without retinopathy is surmised to be evidence of retinal ischemia and oxidative stress-related neovascularization. These patients also had higher concentrations of malondialdehyde, though IMA was more sensitive for retinopathy.

**Obesity**

Though often present with other diseases, obesity is considered a chronic disease in its own right and is associated with an inflammatory element. With this background, some studies have evaluated IMA levels in obese individuals, measuring it concurrently with inflammatory markers, and assessing for correlations with body mass index (BMI). While a study of diabetic patients failed to find a correlation between BMI and IMA, two other reports did note links. One group identified a correlation between IMA and BMI, though there was considerable overlap between the BMI groups. Those with a BMI classified as “obese” had higher IMA, malondialdehyde, and fasting glucose concentrations, while HDL cholesterol was decreased, in comparison with individuals with a “healthy” BMI. There was no difference in total cholesterol,
LDL cholesterol, or triglycerides in the obese versus healthy groups. In a later study, this same group noted that BMI was correlated not only to IMA, but also to IL-6, nitrate/nitrite levels (as a marker of nitric oxide status), and urinary albumin. IMA, IL-6, and urinary albumin were all increased in obese individuals, while nitrate/nitrite levels were decreased. The authors considered this a marker of endothelial dysfunction in the study, and the increased urinary albumin may indicate some renal dysfunction, so it is likely that multiple factors are at work in these patients to increase the IMA. Finally, a study of overweight or obese post-menopausal women observed that IMA and the IMA: albumin ratio were increased in obese women compared with women of a healthy weight but did not differ from women with coronary artery disease (who had a range of BMI from healthy to overweight). IMA was correlated with hsCRP and insulin concentrations, consistent with reports in diabetic patients, and was negatively correlated with albumin concentration.

**Renal Disease and Effects of Dialysis**

Chronic renal disease often results in anemia due to decreased renal production of erythropoietin, which has the potential to result in tissue hypoxia. A study of 17 patients with chronic kidney disease (CKD) and associated anemia were compared with 19 healthy controls. IMA was increased in the anemic CKD patients, and IMA was correlated with hemoglobin, lactate, and creatinine. Albumin concentration was reported to be significantly lower in the CKD group, but a correlation with IMA was not reported. In combination with an elevated cTnI, an elevated IMA was predictive of mortality, but it was not an independent predictor of mortality in patients with end stage renal disease.
The majority of the studies concerning chronic kidney disease focus on the effects of hemodialysis on IMA. A study by Malindretos found that IMA, hsCRP, IL-6, and TNF-alpha all increased in post-dialysis samples, suggesting that hemodialysis induces inflammatory mediators\textsuperscript{117}. Another study identified elevated IMA levels pre-hemodialysis in 6 of 45 patients with impaired renal function; samples were adjusted for hemoconcentration in the following manner: adjusted post-dialysis value of marker = unadjusted post-dialysis value of marker x (pre-dialysis albumin/post-dialysis albumin)\textsuperscript{118}. Hemodialysis itself caused a mean increase in IMA of 38\% across the group in post-procedure sampling. Abarello et al. found that IMA and protein carbonyl groups (used as a marker of protein oxidation) both increased in patient samples following hemodialysis, with IMA correlated to protein carbonyls, and suggested that the increase in IMA was due to oxidative stress that occurred with the dialysis procedure\textsuperscript{54}. However, a study that used the IMA : albumin ratio to account for hemoconcentration observed no difference in pre- and post-hemodialysis levels, nor did they note a difference between synthetic and cellulose-based dialysis membranes\textsuperscript{119}.

**Other Conditions**

A variety of other conditions and diseases have been found to have increased levels of IMA, with ischemia and/or oxidative damage being blamed as underlying causes.

Normal pregnancy has increased IMA, with statistically significant additional elevations observed in those with complicated deliveries, women with pre-eclampsia, intrauterine growth restricted pregnancies, and women with recurrent pregnancy loss in the first trimester\textsuperscript{60,120–122}. One study did not identify a difference in IMA levels in women with pre-eclampsia\textsuperscript{34}. 

27
Children with chronic liver disease have been observed to have higher IMA and IMA: albumin ratios (IMAR), which correlated with pediatric end-stage liver disease score and fibrosis score\textsuperscript{123}. IMAR had some ability to predict the presence of severe fibrosis (sensitivity = 84%, specificity = 70%, with an optimal cut-off). Increased morbidity and/or mortality was found with an IMAR greater than 0.156, and IMAR had a sensitivity of 83% and specificity of 82% with an optimal cut-off to predict the need for transplant and/or death\textsuperscript{123}. Chen et al. found that IMA and IMAR were increased in adult patients with chronic hepatitis or cirrhosis and that both were associated with measures of liver function (indocyanine green retention, total bilirubin concentration, and total antioxidant capacity)\textsuperscript{66}. IMAR was associated with the model for end-stage liver disease (MELD) score, which is used to rank the transplant recipient waiting list. Interestingly, a 3 day intravenous infusion of human albumin did not significantly change the albumin concentration, IMA, or IMAR\textsuperscript{66}.

Among neoplasms, a few have been evaluated with respect to IMA. In two separate publications by the same group, children with soft tissue sarcomas or neuroblastomas were studied\textsuperscript{124,125}. It was revealed that IMA increased in the patients when grouped by their respective diagnosis or as a single neoplasia group and that IMA increased during treatment\textsuperscript{124}. Children with poorly-responding cancer had lower superoxide dismutase and glutathione peroxidase, but it does not appear that the researchers tried to correlate IMA with these measurements of antioxidants\textsuperscript{125}. Patients with polycythemia vera had increased IMA, and the researchers speculated that this was an indication of ischemia secondary to hyperviscosity syndrome\textsuperscript{126}. Finally, men with benign prostatic hyperplasia had statistically significant elevations in IMA over controls, while men with prostatic carcinoma had increased IMA levels that did not reach significance\textsuperscript{45}. 


Other conditions that have been found to have statistically significant elevations in IMA include psoriasis, severe sepsis, systemic sclerosis, primary angle closure glaucoma, hyperthyroidism, hypothyroidism, and beta-thalassemia major. Evaluation of IMA levels in various pleural effusions observed differences between transudates and exudates, while serum IMA levels were comparable between groups. This same study also noted some statistically significant differences between underlying disease etiologies for the effusions (cancer, tuberculosis, pulmonary thromboembolism), but a great deal of overlap was also identified. Finally, an experimental model of abdominal compartment syndrome (organ dysfunction secondary to increased intra-abdominal pressure), in which rabbit abdominal cavities were insufflated to 25 mmHg, observed that animals insufflated for 60 minutes had statistically significant elevations over animals insufflated for 15 and 30 minutes, but not the 30 minute group.
CHAPTER 3

MATERIALS AND METHODS

Objectives:

1) Validate the serum cobalt-binding assay for use in cats.

2) Evaluate serum cobalt binding in healthy and diseased cats.

3) Identify specific conditions or diseases that alter serum cobalt binding in cats.

Hypotheses:

1) Cobalt binding occurs in feline serum as reported in non-feline serum.

2) Feline serum cobalt binding reflects the serum albumin concentration and correlates with serum concentrations of other albumin-binding compounds.

3) Cobalt binding by feline serum is affected by diseases associated with increased free radical production, including tissue ischemia and systemic inflammation.

Serum Cobalt-Binding (SCB) Assay

The colorimetric assay used in this study is a modification of the original protocol published by Bar-Or et al. It uses parallel wells to measure the light absorbance with maximum and minimum cobalt-binding and normalizes for background absorbance of each sample. The theory of the SCB assay is that Co$^{32}$ bound to albumin is unavailable for reaction with dithiothreitol. Unbound cobalt ions however are free to react with dithiothreitol, resulting in a colored product that absorbs light at 450 nm. It is performed as follows:
1. Prepare CoCl$_2$ by dissolving 0.28 grams (g) CoCl$_2$·6H$_2$O (Sigma, St. Louis, MO) into 100 mLs deionized (DI) water.

2. Prepare DL-dithiothreitol (DTT) by dissolving 0.13 g DL-DTT (Sigma, St. Louis, MO) into 100 mLs DI water.

3. Add 47.5 μL serum to duplicate 300 μL wells (“Well #1” and “Well #2”) of a clear bottom 96-well microtiter plate (Thermo Fisher Scientific, Rochester, NY).

4. Add 2.5 μL CoCl$_2$ solution to Well #1.

5. Shake the plate for 9 seconds at 20 Hz and 1 mm rotation (setting #3) on an Opsys MR plate reader (Dynex Technologies, Inc., Chantilly, VA) and measure the “preincubation” (Pre Inc) light absorbance at 450 nm (A$_{450}$) immediately.

6. Incubate the plate for 10 minutes at 37°C.

7. Add 2.5 μL CoCl$_2$ to Well #2, followed immediately by 12.5 μL DTT to both wells.

8. Shake the plate for 2 minutes at 200 RPM on an IKA-Vibrax-VXR orbital shaker (IKA Works, Inc., Germany).

9. Shake the plate for 9 seconds on the plate reader, setting #3, and measure the “post incubation” (Post Inc) A$_{450}$ of Wells #1 & #2.

Percent cobalt binding (%CB) is calculated as follows:

$$\frac{[(\text{Post Inc Well #2} - \text{Pre Inc Well #1}) - (\text{Post Inc Well #1} - \text{Pre Inc Well #1})]}{\text{Post Inc Well #2} - \text{Pre Inc Well #1}} \times 100\%$$

This formula utilizes the Post Inc Well #2 as the control for color change generated by the cobalt added in step 7 of the protocol, which is unbound by albumin and available to react with DTT,
while removing the confounding effect of the cobalt itself within the solution (Pre Inc Well #1). The results of Post Inc Well #1 represent the color change generated by the remaining unbound cobalt reacting with DTT following the 10 minute incubation period. The residual cobalt reading, reported in absorbance units (ABSU), is subtracted from the total cobalt reading (also in ABSU) to give an estimation of the cobalt that is bound by albumin. This amount is divided by the total cobalt ABSU to give a percentage of the total cobalt that is bound to albumin.

Post Inc Well #2: Represents approximately 0% cobalt binding by albumin and is the color change caused by maximum reaction of cobalt with DTT.

Pre Inc Well #1: Represents the baseline color of the solution after full addition of cobalt without DTT and without albumin binding.

Post Inc Well #1: Represents the color change obtained by DTT binding to any residual cobalt present in the solution after albumin is allowed to bind cobalt.

(Post Inc Well#2 — Pre Inc Well#1): Represents the color change from unbound cobalt binding DTT while removing any interference by addition of the cobalt itself to the solution.

(Post Inc Well#1 — Pre Inc Well#1): Represents the color change caused by residual cobalt binding DTT after albumin is allowed to bind cobalt.

The SCB assay was routinely run in duplicate on each serum sample and the %CB was reported as the average of the duplicate results.

**Sample Collection**
Clinical case samples

Animal use in this study was approved by the University of Illinois Institutional Animal Care and Use Committee. From June 2011 through December 2011, sera from cats that presented to the University of Illinois Veterinary Teaching hospital for any reason were considered for inclusion into this study. The serum samples had been submitted to the clinical pathology laboratory for clinical biochemical analysis in the course of the medical assessment. All testing performed on these samples was at the discretion of the clinician on each case and no attempt was made to seek out cats of any particular disease or status for this study. Submitted samples for which at least 250 μL of serum remained following completion of the requested testing were further considered for inclusion. Samples that had a 1+ or greater lipemic index or 3+ or greater hemolytic index as measured on the serum chemistry results from an AU680 Chemistry Analyzer (Beckman Coulter, Inc., Brea, CA) were excluded from the study. The samples were stored frozen first in a frost-free freezer at -7 to -17°C for 2 to 3 weeks before transfer to a -20°C freezer for up to 3 months before measurement of %CB.

Samples for validation of SCB stability

Five mLs of whole blood was collected into a plain glass tube, via jugular or medial saphenous venipuncture, from 3 clinically healthy cats that were volunteered by their owners. Samples were allowed to clot at room temperature for 30 minutes, after which they were centrifuged and the serum transferred into plain glass tubes and frozen at -20°C following storage at room temperature for set time periods (see below). The serum was used to test the stability of serum cobalt binding over time of storage at room temperature.
Laboratory Validation of the Serum Cobalt-binding (SCB) Assay

Randomly-chosen serum samples were used for test validation experiments. Run-to-run test imprecision was assessed using three serum samples that were divided into 12 separate aliquots and stored at -20°C. The SCB assay was performed 12 times using one aliquot of each sample, and the mean, standard deviation (SD), and percent coefficient of variation (CV) calculated for each. The within-run test imprecision of the SCB assay was tested using a pooled feline serum sample 24 times in a single run. The day-to-day test imprecision was performed by running the SCB assay on five days using aliquots of a pooled serum sample that were thawed just prior to assay. Linearity upon serial dilution (1:2, 1:4, and 1:8) of the SCB assay was tested in 3 feline serum samples that were serially diluted with pH 7.2 phosphate buffered saline (PBS; Sigma, St. Louis, MO). The biological variation of serum cobalt binding was evaluated in 2 cats that had repeat serum samples submitted to the Clinical Pathology Laboratory on different days.

Pooled serum was used to assess the effect of interfering substances. Hemolysis was induced by addition of 1 mL of PBS to a residual clot from one of the cat samples, followed by freezing and thawing; after this, the sample was thoroughly agitated, centrifuged, and the supernatant fluid removed to a new plain tube. Dilutions with pooled serum were made to create 1+, 2+, 3+, and 4+ hemolytic indices, as follows:

- One mL of the supernatant was added to 1 mL of pooled serum. From this 1:2 dilution, dilutions 1:4, 1:8, 1:16, and 1:32 were made.
- From this, further dilutions were made and tested to find the mixtures to correlate with 1+ through 4+ hemolytic index on the AU680 chemistry analyzer.
o Addition of 100 μL of the 1:32 dilution to 900 μL of pooled serum resulted in a 1+ hemolytic index.

o Addition of 100 μL of the 1:16 dilution to 900 μL of pooled serum resulted in a 2+ hemolytic index.

o Addition of 75 μL of the 1:4 dilution to 900 μL of pooled serum resulted in a 3+ hemolytic index.

o Addition of 100 μL of the 1:2 dilution to 900 μL of pooled serum resulted in a 4+ hemolytic index.

This was followed by determination of the %CB of each respective dilution and the undiluted serum.

Lipemia was simulated by the addition of Intralipid 20% (Fresenius Kabi, Uppsala, Sweden, for Baxter Healthcare Corporation, Deerfield, IL) to pooled serum to create 1+, 2+, 3+, and 4+ lipemic indices on the AU680 chemistry analyzer, as follows:

- One mL of Intralipid 20% was added to 1 mL of pooled serum. From this 1:2 dilution, dilutions 1:4, 1:8, 1:16, and 1:32 were made.

- From this, further dilutions were made and tested to find the mixtures to correlate with 1+ through 4+ lipemic index on the AU680 chemistry analyzer.

  o Addition of 100 μL of the 1:32 dilution to 900 μL of pooled serum resulted in a 1+ lipemic index.

  o Addition of 100 μL of the 1:16 dilution to 900 μL of pooled serum resulted in a 2+ lipemic index.
Addition of 100 μL of the 1:8 dilution to 900 μL of pooled serum resulted in a 3+ lipemic index.

Addition of 100 μL of the 1:2 dilution to 900 μL of pooled serum resulted in a 4+ lipemic index.

This was followed by measurement of the %CB in the respective lipemic samples.

Icterus was simulated by adding bilirubin conjugate (EMD Biosciences, Inc., La Jolla, CA) to pooled feline plasma, as follows:

- Five mg of bilirubin conjugate was reconstituted into 500 μL of distilled water for a 10 mg/mL solution.
- A 1:2 dilution was made by adding 500 μL of bilirubin solution to 500 μL of pooled serum. From this, dilutions of 1:4, 1:8, 1:16, and 1:32 were made.
  - Addition of 20 μL of the 1:2 dilution to 480 μL of pooled serum resulted in a 10.5 mg/dL solution. Actual concentration was 8.9 mg/dL at analysis.
  - Addition of 20 μL of the 1:4 dilution to 480 μL of pooled serum resulted in a 5.7 mg/dL solution. Actual concentration was 4.3 mg/dL at analysis.
  - Addition of 20 μL of the 1:8 dilution to 480 μL of pooled serum resulted in a 2.9 mg/dL solution. Actual concentration was 2.2 mg/dL at analysis.
  - Addition of 20 μL of the 1:16 dilution to 480 μL of pooled serum resulted in a 1.4 mg/dL solution. Actual concentration was 1.2 mg/dL at analysis.
  - Addition of 20 μL of the 1:32 dilution to 480 μL of pooled serum resulted in a 0.7 mg/dL solution. Actual concentration was 0.7 mg/dL at analysis.
Addition of 20 μL of pooled serum to 480 μL of pooled serum was performed for a ‘zero’ sample. Actual concentration was 0.2 mg/dL at analysis.

The total bilirubin concentration of each sample was measured on the AU680 Chemistry Analyzer. The values ranged in six samples from 0.2 mg/dL to 8.9 mg/dL. The measured icteric index of the sample with an 8.9 mg/dL total bilirubin concentration was 2+.

Fresh serum samples were divided into eight 150 μL aliquots for assessment of room temperature stability. Aliquots were labeled as 0 hr, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, 24 hr, and 48 hr and left at room temperature for the listed time periods. At each time point, the respectively-labeled aliquot was placed into a -20°C freezer until batch assay, which was performed within 1 week.

Freeze/thaw experiments were performed on waste serum from three freshly collected samples submitted for serum biochemistry panels to the Clinical Pathology Laboratory. Excess serum was divided into 3 equal aliquots, and labeled as “0”, “1”, and “2” (two samples) or “1”, “2”, and “3” (one sample). Samples labeled as “0” were assayed immediately to provide a baseline. Samples labeled as “1”, “2”, or “3” were frozen at -20°C and allowed to completely thaw at room temperature 1, 2, or 3 times before assay.

**Retrospective Evaluation**

Retrospective clinical information was obtained from the medical record of each of the 176 cases, including the signalment, and time-matched hematology, serum chemistry, and urinalysis data. Other clinical data was collected if available and included total T4 (n=131), prothrombin
time (4), activated partial thromboplastin time (4), urine protein: creatinine ratio (13),
reticulocyte count (3), fibrinogen (1), fructoasmine (6), and pre- and post-prandial bile acids (n =
1 and 2, respectively). Analytes were selected for statistical analysis based on major findings
from the human literature. Red blood cell count, hemoglobin concentration, hematocrit, white
blood cell count, absolute leukocyte counts, total protein, albumin, globulins, total calcium,
ionized calcium, alkaline phosphatase activity, total bilirubin, triglycerides, cholesterol, lactate,
and pH were selected for statistical analysis. Complete blood counts were performed on a CELL-
DYN 3700 (Abbott Laboratories, Abbott Park, IL). Some cats had heparinized blood samples
analyzed for emergency mini-panels on a Stat Profile® Critical Care Xpress machine (Nova
Biomedical Corp., Waltham, MA).

The clinical diagnosis of each case was determined from the medical record. The cases were
grouped by disease into the following categories: cardiomyopathy, dental disease, endocrine
disease (primarily hyperthyroidism and diabetes mellitus), bone fractures, infectious disease,
neoplasia, wellness visit, multiple diseases, other diagnoses, and “open” diagnosis. If possible,
cases were subclassified as inflammatory or noninflammatory based on the final diagnosis and
leukogram findings. Cases were considered noninflammatory if the leukogram had absolute
leukocyte indices within reference interval and no evidence of toxicity or “rare”/“few” toxic
changes. Patients were classified as inflammatory, 1) if a mature neutrophilia was present with
an absolute lymphocyte count within reference limits, 2) if the absolute band neutrophil count
was greater than the upper reference limit, or 3) if 1+ or greater cell toxicity was noted on
manual blood smear evaluation, or some combination of these criteria was present. Type of
toxicity was not considered, and cases with “rare” or “few” toxic changes were not included

38
unless criterions 1 or 2 were also met. Though normal cats can have signs of toxic changes on blood smear, particularly Dohle bodies, 1+ toxicity was selected as the lower limit for inclusion for higher sensitivity. Leukograms with features that did not fit these parameters were considered equivocal, and data from these patients were not included in the statistical analysis comparing inflammatory and noninflammatory %CB values. If the final diagnosis in the file was not consistent with an inflammatory disease, but the patient showed the above described signs of inflammation, it was still included in the inflammatory group. Samples in which the final diagnosis was an inflammatory condition but an inflammatory leukon was not present were considered equivocal.

**Statistical Analysis of the Retrospective Study Results**

The distribution of the data was evaluated using the Shapiro-Wilkes test, skewness, kurtosis, and q-q plots. Data that was not normally distributed was log transformed for parametric testing. Normally distributed data is reported by the mean, SD, and minimum-maximum (Min-Max), while non-normally distributed data is reported by the median, 25th-75th percentiles, and min-max. A Mann-Whitney U test was used to determine if cobalt levels differed by the presence/absence of inflammation. A general linear model was used to determine if disease status, sex, or breed influenced %CB. Pearson’s correlation was used to determine if there was an association between cobalt and the various hematology and biochemistry data. When the assumption of normality could not be met through transformation, Spearman’s rho correlation analysis was done. Multiple linear regression was used to determine if any of the hematology or biochemistry variables found to be correlated to cobalt could be used to predict cobalt values. A stepwise method was used to develop the model. A $p<0.05$ was used to determine statistical
significance. SPSS 19.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) were used to analyze the data.
RESULTS

Validation

Within-run precision ranged from 2.61% to 4.04% over 24 runs (Figure 3). Run-to-run precision ranged from 2.90% to 4.37% over 30 runs (3 samples, 10 runs each) (Figure 4). Day-to-day precision was 3.46% over 5 days (Figure 5). Limited biological variation assessment showed clinically insignificant changes (Figure 6). Results from room temperature and freeze-thaw stability experiments were examined for clinically significant alterations in the percent cobalt albumin binding (defined as a change from baseline greater than 10%). Room temperature stability testing revealed some variability. One cat’s sample had an increase of almost 16% at 4 hours after sample collection, while the two others had increases of 1% and just over 10%, respectively. The first cat, in fact, had an increase of almost 12% by 1 hour post-collection, which was not observed in the other two (Figure 7). This cat’s sample was drawn and assayed on a different day than the other two. All samples tested appeared stable with one freeze-thaw cycle, and 2 of 3 remained stable for up to three cycles (Figure 8). Even mild lipemia (lipemic index of 1+) was found to falsely decrease the percent cobalt binding, as did significant hemolysis (hemolytic index 3+ or greater; Figure 9A). Icterus, tested to 8.7 mg/dL bilirubin, did not have a significant effect on the cobalt binding measurements (Figure 9B). Percent cobalt binding appeared linear upon dilution (Figure 10).

Retrospective Evaluation

Based on the results of the validation studies, serum samples were excluded from analysis if they had a 1+ or greater lipemic index or were visibly lipemic. Samples were also excluded if 3+ or
greater hemolytic index was reported. Approximately 250 μL of sample was required to run the assay in duplicate, and, as such, samples of insufficient volume were excluded. Serum from 176 of the 374 samples obtained over the 6-month collection period was of acceptable quality and volume for inclusion in this study.

Of 176 cases, 150 had CBCs and 135 had serum biochemistry panels performed. Twenty-three had mini-panels performed on the Stat Profile® Critical Care Xpress machine. Other tests performed included renal biochemical profile, hepatic biochemical profile, urinalysis, total T4, urine protein: creatinine ratio, prothrombin time, activated partial thromboplastin time, pre- and post-prandial bile acids, fibrinogen and fructosamine.

There were 69 (39.2%) spayed female cats, 104 (59.1%) neutered males, and 3 (1.7%) intact female cats used in this study, representing 13 different breed groups (Figure 1). There were 108 domestic shorthair cats, 20 domestic longhair cats, 18 mixed breed cats, 10 domestic medium hair cats, 9 Maine Coons, 2 Abyssinians, 2 Norwegian Forest Cats, and 1 each of Birman, Himalayan, Oriental Shorthair, Ragdoll, Russian Blue, Siamese, and Tonkinese (Figure 12).

The median age of the cats in this population was 10.5 years (25th-75th percentiles: 7.4-13.3, min-max: 0.6-20.3). Health status of the cats were classified into 9 different disease states (chronic kidney disease, n=15; cardiomyopathy, n=7; dental, n=3; endocrine disease, n=13; fracture, n=3; infectious, n=5; multiple diagnoses, n=16; neoplasia, n=47; other, n=9) or characterized as having an open diagnosis (n=53) or normal health state (n=5) (Figure 13). Percent cobalt binding results for the respective disease groups can be seen in Table 1. Cats with a normal health state
were presented for routine wellness examinations and were without abnormalities on history, physical examination, complete blood count, serum biochemistry analysis, total T4, or urinalysis; their values ranged from 67.11% to 89.82%.

The median percent cobalt binding (%CB) was 73.9% \((25^{th}-75^{th} \text{ percentiles: } 66.2-80.3, \text{ min-max: } 34.93-92.37)\). Cats characterized as having inflammation \((n=20)\) had significantly lower \((p=0.002)\) %CB \((\text{median: } 64.6, 25^{th}-75^{th} \text{ percentiles: } 51.2-76.6, \text{ min-max: } 42.5-90.94)\) than cats without inflammation \((n=22)\) \((\text{median: } 77.8, 25^{th}-75^{th} \text{ percentiles: } 72.8-88.7, \text{ min-max: } 59.0-90.9)\) \((\text{Figure 14})\). Cats characterized as having inflammation also had significantly lower \((p=0.002)\) albumin concentrations \((\text{median: } 2.70 \text{ g/dL}, 25^{th}-75^{th} \text{ percentiles: } 2.125-3.100, \text{ min-max: } 1.6-3.7)\) than cats without inflammation \((\text{median: } 3.25, 25^{th}-75^{th} \text{ percentiles: } 3.0-3.4, \text{ min-max: } 2.6-3.8)\) \((\text{Figure 15})\). Surprisingly, globulin concentrations were not significantly different between the inflammatory and noninflammatory group \((p=0.6907)\).

Cobalt binding percentages were not found to be significantly different by disease state \((p=0.61)\), sex \((p=0.82)\), age \((p=0.51)\), or breed \((p=0.83)\). The cats with the lowest %CB tended to have multiple diseases or multiple affected body systems. Cobalt binding percentage was found to be correlated with red blood cell count \((r=0.24, p=0.003)\), hemoglobin \((r=0.29, p=0.0001)\), hematocrit \((r=0.283, p=0.0001)\), albumin \((r=0.62, p=0.0001)\), globulins \((r=-0.20, p=0.01)\), total calcium \((r=0.27, p=0.001)\), total bilirubin \((r=-0.237, p=0.005)\), and absolute lymphocytes \((r=0.16, p=0.05)\).

Cobalt binding percentage was not found to be correlated to white blood cell count \((p=0.32)\), absolute segmented neutrophils \((p=0.25)\), absolute bands \((p=0.75)\), absolute monocytes \((p=0.43)\),
absolute eosinophils (p=0.06), absolute basophils (p=0.86), serum total protein (p=0.86), glucose (p=0.10), ALP (p=0.91), cholesterol (p=0.26), triglycerides (p=0.72), corrected calcium (p=0.14), ionized calcium (p=0.26), lactate (p=0.27), or pH (p=0.63). The results of the stepwise linear regression model only included albumin ($R^2=0.425$). The final regression equation was: cobalt $\% = 20.8 + 16.84$ (albumin).

When albumin was compared to the same analytes, it was found to be correlated to red blood cell count ($r=0.3258$, $p<0.0001$), hemoglobin ($r=0.4465$, $p<0.0001$), hematocrit ($r=0.4339$, $p<0.0001$), total calcium ($r=0.4634$, $p<0.0001$), absolute neutrophils ($r=-0.1615$, $p=0.0484$), globulins ($r=-0.3197$, $p<0.0001$), total bilirubin ($r=-0.2251$, $p=0.0080$), total protein ($r=-0.1557$, $p=0.0486$), and cholesterol ($r=0.1852$, $p=0.0309$).

Albumin was not correlated with absolute bands (p=0.0527), absolute lymphocytes (p=0.2708), WBC (p=0.2983), absolute monocytes (p=0.3674), absolute eosinophils (p=0.4429), glucose (p=0.0552), ALP (p=0.1308), triglycerides (p=0.0704), pH (p=0.6921), lactate (p=0.4713), and ionized calcium (p=0.5841).
CHAPTER 5

DISCUSSION

Studies in the human literature report cobalt binding test results as absorbance units or absorbance units/volume unit obtained from the colorimetric assay, which are then labeled as the IMA levels. We chose to calculate and report our test results as percent cobalt binding (\%CB) in order to correct for differences in the baseline light absorbances. In our study, a decreased \%CB implies an increased IMA concentration. No correlations were observed between age, sex, or breed and \%CB; these signalment factors are not expected to be a concern in selection of study populations. Correlations between IMA and sex or age in the human literature were not noted during reviews of the literature.

The test validation experiments revealed that the cobalt binding test, using feline serum, is a high-precision assay. Coefficients of variation for run-to-run (10 to 11 runs), within-run (24 individual runs, 12 paired runs), and day-to-day (5 runs) precision were all less than 5%. This is similar to the precision of the Albumin Cobalt Binding (ACB®) test assessed on the Cobas MIRA® analyzer\(^6\). Limited assessment of biological variation was performed, but, in samples collected on two successive days from two patients, very little variation was observed (change from baseline of 4.2% and 1.1%, respectively). The assessment of serial cobalt binding in a greater number of both healthy and diseased cats, however, would be ideal to confirm a lack of biological variation with time. A review of the literature failed to find published results evaluating biological variation of IMA in people. Evaluation of the effect of interfering substances found that even mild lipemia had a significant effect, while significant hemolysis was
needed before negative interference was observed; bilirubin did not appear to significantly interfere with the assay, up to an icteric index of 2+. This is similar to reports that used human serum. Assessment of interfering substances in human samples in one study found no interference with high concentrations of hemoglobin (220.4 μmol/l) or triglycerides up to 7 mmol/l\(^{67}\). That study did find a significant effect from bilirubin; however, this did not agree with the assay manufacturer’s information. Further investigation determined that NaOH that was used for the bilirubin solution caused similar interference when added to the serum without bilirubin. However, that study did not retest bilirubin interference in another diluent.

It has been recommended that the albumin cobalt binding test be performed within 2 hours of sample collection, with most samples remaining stable at room temperature and 4\(^\circ\)C up to 4 hours post-collection\(^{68,69}\). This recommendation appears to be secondary to identification of statistically significant changes. We elected to look for clinically relevant changes, based on clinical experience, in the %CB, which was defined as a 10% or greater change from baseline. Stability testing in this study revealed that results are minimally to moderately variable from cat to cat, although overall the samples appear to be stable at room temperature for at least 2 hours. The sample that had the most variability was drawn and processed on a separate day, and sample handling may have affected results. Stability at refrigeration temperatures and with long-term freezing was not assessed, but evaluation of IMA stability at room temperature in the human literature found mild to moderate increases with storage at -20\(^\circ\)C\(^{67,68}\). Some samples from the retrospective portion of our study were likely stored up to 8 hours at room temperature before freezing, which may have affected the ultimate %CB value obtained. Since the %CB tended to increase with time spent at room temperature, it is possible that samples with decreased %CB
were missed. A prospective study with stringent sample handling requirements may uncover additional links and stronger correlations.

As predicted, %CB was found to be correlated with albumin concentration and, in fact, was the strongest correlate in the study; a decreased serum albumin concentration is the most significant predictor of a decreased %CB. This follows the findings in human medicine, where a decreased albumin concentration has been associated with increased IMA values\textsuperscript{59,63}. With less circulating albumin, less protein is available to bind the finite amount of cobalt added in the test. Correction formulas have been proposed, which calculate an ischemia modified albumin index\textsuperscript{65,103}, an albumin-adjusted IMA\textsuperscript{127}, or an IMA-to-serum albumin ratio (IMAR)\textsuperscript{66}. The IMA index was found to be more sensitive than IMA as a marker for stroke, but found no difference between IMA index and IMA in patients with progressive stroke compared with nonprogressive stroke\textsuperscript{103}. Similarly, the IMA index had slightly improved sensitivity over IMA in detecting acute coronary syndrome in people\textsuperscript{65}. However, as mentioned earlier, validation for these formulas does not appear published. In future studies, care must be taken in interpreting %CB of samples from hypoalbuminemic animals, and further investigation into some type of correction formula may be interesting. Reliable evaluation of the %CB assay in patients with significant liver, gastrointestinal, or renal disease, or any other condition in which the albumin concentration is increased or decreased, may prove difficult without the use of some correction factor.

Correlations were observed between %CB and red blood cells, hemoglobin, hematocrit, globulins, total calcium, and absolute lymphocytes. However, albumin was also significantly correlated to all of these analytes, with the exception of absolute lymphocytes. Therefore, it is
likely that %CB is only independently correlated to absolute lymphocytes. The reason for the very weak correlation is uncertain and may be coincidental.

Total calcium was also correlated with %CB, but ionized calcium was not. The likely explanation for this is that approximately 45% of total calcium in blood is albumin bound\textsuperscript{13}; if albumin is decreased in concentration, then it would be unsurprising that both calcium and %CB may be decreased, as well. This connection is further support that the serum cobalt binding assay is measuring albumin-bound cobalt, other than non-specific binding to other substances.

Zapico-Muniz et al. found that IMA was negatively influenced by rising lactate concentrations both \textit{in vivo} and \textit{in vitro}, at concentrations ranging from 3 to 11 mmol/L, in healthy volunteers undergoing experimental forearm ischemia\textsuperscript{96}. Falkensammer et al. did not find such a correlation in a similar study, but lactate concentrations in that study were below the concentrations reported in the other study. We found no correlation between %CB and lactate or blood pH. However, only 22 cats in our study had lactate concentrations performed, and only 6 of these had values above 3 mmol/L, with the highest at 5.1 mmol/L. It may be that a correlation would have been found with higher numbers or more exaggerated increases.

The hypothesis that %CB will be decreased in cats with inflammatory disease compared with cats with noninflammatory disease initially appeared to be supported by the results of our study; however, we also noted that the inflammatory group had a lower albumin concentration. Given the correlation between albumin and %CB, we suspect that this is the underlying reason. Since albumin is a negative acute phase protein, decreases in the face of inflammation are expected. Additionally, animals may have conditions that cause a loss of albumin (gastrointestinal, renal)
that are also inflammatory. A relationship between %CB and segmented or band neutrophil numbers was not identified in this study, but albumin was negatively correlated with segmented neutrophil numbers, and the correlation between albumin and band neutrophils approached significance. Correlations between IMA and leukocytes do not appear to have been evaluated in the human literature. However, correlations between IMA and several markers of oxidative stress have been identified. In patients with β-thalassemia major, IMA was correlated with ferritin, ferrooxidase, malondialdehyde, and transaminases. The authors concluded that the evidence of iron-induced oxidative stress was from the continuous blood transfusions needed and increased erythrocyte turnover that occurs in these patients\textsuperscript{130}. In a study of patients with hypo- and hyperthyroidism, IMA was found to correlate positively with C-reactive protein concentrations\textsuperscript{49}. As discussed previously, several other studies found correlations between IMA and CRP in patients with a variety of conditions. While one study in people with systemic sclerosis did not find a correlation between IMA and CRP, the researchers did find correlations between IMA and carbonyl residues and advanced oxidation protein products, which the authors described as indicators of protein oxidation\textsuperscript{128}. There is sufficient evidence in the literature in \textit{in vitro} studies, as well as clinical observations, that oxidative damage, likely secondary to inflammatory processes, plays a role in the alteration of the cobalt-binding capacity of albumin.

The vast majority of the studies evaluating IMA in the human medical literature did not measure concurrent circulating fatty acid levels at the time of sampling for IMA, but a few studies convincingly demonstrate a role of fatty acid binding in the development of IMA\textsuperscript{8,43,57}. Measurement of fatty acids is not a typical component of chemistry panels in our lab and, consequently, this was not measured in any patient in our study. Closer study of free fatty acid
concentrations in inflammatory cases is needed, as this may be a significant factor in decreases in %CB. It is unknown if multiple influences are at work in the cats in this study in which %CB was decreased.

Unfortunately, the study population had very small numbers of cats in several disease groups, including cardiomyopathy and liver disease, and no cats with known thromboembolic disease, all of which had been of particular interest in the planning of this study. A prospective study recruiting cats with specific diseases of interest in adequate numbers is likely required to fully identify a relationship between disease and %CB.

Several limitations are identified within this study. First, no attempt was made to prospectively identify cases. As such, our study population included very few healthy cats to serve as controls, and several disease categories in which we had a specific interest (thromboembolic disease, cardiomyopathy, and hepatic disease) similarly had disappointing numbers. It may be that significant findings were not identified due to a lack of study power. Second, the serum cobalt binding assay lacks test standards and controls for independent test validations. We were forced to use individual and pooled serum samples from our study population to perform the validation experiments. Finally, ischemia, oxidative damage, and fatty acid binding are the main suspected factors in the formation of IMA, but the cases used in our study did not have arterial blood gas data, malondialdehyde or isoprostane levels, or fatty acid concentrations measured, as these are not tests performed routinely in basic blood work in veterinary medicine. However, they could be included in prospective studies in the future.
Overall, this study found that the serum cobalt binding assay is functional and reliable with use of cat serum. Correlations were few and almost exclusively due to the albumin concentration. This provides strong evidence that the assay is measuring the amount of cobalt bound to albumin within the tested feline serum and helps dispel concerns about cobalt binding to other serum components. Future work with this assay should require a prospective study design that targets specific diseases of interest, careful sample handling, and evaluation of correction factors or formulas for albumin concentrations in patients evaluated, in order to more clearly determine if this assay serves a practical purpose in diagnostic medicine.
CHAPTER 6

CONCLUSIONS

- Cat serum forms complexes with transition metals, such as cobalt, in a manner similar to that reported for human serum.
- Serum cobalt binding can be readily measured in cats with acceptable precision using the protocol described.
- Serum samples intended for use in the cobalt binding assay should be assayed within 4 hours; if this cannot be done, samples should immediately be frozen at -20°C until time of assay.
- Serum samples for cobalt binding appear stable with up to 3 freeze-thaw cycles but should be minimized.
- Feline serum cobalt binding is correlated with serum albumin concentration.

Future Directions

Prospective studies are needed for further evaluation of this assay and its use. Studies targeting cats with inflammatory diseases (such as pancreatitis and cholangiohepatitis), conditions associated with oxidative damage (such as diabetes mellitus and diabetic ketoacidosis), and ischemic conditions (such as heart failure, thromboembolic diseases, and severe anemia) may be of particular value. Assessment of serum cobalt binding in cats with untreated hyperthyroidism, euthyroid sick syndrome, and iatrogenic hypothyroidism would also be of interest. Because this assay has not been evaluated in common veterinary species, studies targeting other animals would be of interest, and the study designs can be positively influenced by what has been learned in the current study. Prospective studies with careful sample handling will be of the utmost
importance. A prospective study that validates the assay for use in horses and evaluates serum cobalt binding in cases of equine colic would be interesting. This study may help to discriminate medical from surgical colic cases, particularly those with ischemia secondary to intestinal torsion, or have prognostic value in such cases. There are many possible avenues of exploration with this assay in future projects involving veterinary species.
CHAPTER 7

FIGURES AND TABLES

Albumin: Homo sapiens (on top) vs. Felis catus

<table>
<thead>
<tr>
<th>Sequence 1</th>
<th>Sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>541 ekgkavndd faafveckck addketcfae egkklvaasq aalgl</td>
<td>541 ekgkavndd faafveckck addketcfae egkklvaasq aalgl</td>
</tr>
<tr>
<td>81% sequence homology</td>
<td></td>
</tr>
</tbody>
</table>


Figure 1. Comparison of human and feline albumin amino acid sequences.
Figure 2. Cartoon representation of the serum cobalt binding assay. When a greater fraction of the albumin is ischemia-modified albumin, more free Co(II) molecules are in solution. These react with dithiothreitol to produce a color reaction.
Figure 3. Within-run precision. Over 24 runs, precision ranged from 4.04% in individual runs to 2.61% in duplicate (pairs).
Figure 4. Run-to-run precision ranged from 2.90% to 4.37% over 30 runs (3 samples, 10 runs each).
Figure 5. Day-to-day precision was 3.46% over 5 days.

Figure 6. Biological variation between two days from samples from two cats. Cat A demonstrated a decrease of 4.2%. Cat B demonstrated a decrease of 1.1%.
Figure 7. Serum cobalt binding room temperature stability results, displaying mean and standard error bars from three individual cat samples. Aliquots from samples from each cat were assayed in duplicate and the results averaged. Mean: 84.55%, median: 85.61%, standard deviation: 3.311%, standard error: 1.170%.

Figure 8. Serum cobalt binding over 1 to 3 freeze-thaw cycles.
Figure 9A. Effect of increasing hemolytic index and lipemic index on serum cobalt binding. 9B. Effect of increasing concentrations of conjugated bilirubin on serum cobalt binding.
Figure 10. Test of the linearity of the %CB with dilution of feline serum with PBS. Dilutions were 1:2, 1:4, and 1:8.

Figure 11. Sex distribution of the study population. There were 59.1% neutered males, 39.2% spayed females, and 1.7% intact females.
Figure 12. Breed distribution of the study population. There were 108 domestic shorthair cats, 20 domestic longhair cats, 18 mixed breed cats, 10 domestic medium hair cats, 9 Maine Coons, 2 Abyssinians, 2 Norwegian Forest Cats, and 1 each of Birman, Himalayan, Oriental Shorthair, Ragdoll, Russian Blue, Siamese, and Tonkinese.
Figure 13. Disease distribution of the study population: chronic kidney disease (n=15), cardiomyopathy (n=7), dental (n=3), endocrine disease (n=13), fracture (n=3), infectious (n=5), multiple diagnoses (n=17), neoplasia (n=47), other (n=9), open diagnosis (n=53), and normal health state (n=5).

Table 1. Disease group %CB data. CKD: chronic kidney disease, Min: minimum, Max: maximum.
**Figure 14.** Comparison of serum cobalt binding between cases with evidence of systemic inflammation and those without. Noninflammatory: median: 77.8%, 25th-75th percentiles: 72.88-88.7%, min-max: 59.0-90.9%. Inflammatory: median: 64.6%, 25th-75th percentiles: 51.2-76.6%, min-max: 42.5-90.94%.

**Figure 15.** Comparison of albumin concentration between cases with evidence of systemic inflammation and those without. Noninflammatory: median: 3.25 g/dL, 25th-75th percentiles: 3.0-3.4 g/dL, min-max: 2.6-3.8 g/dL. Inflammatory: median: 2.70 g/dL, 25th-75th percentiles: 2.13-3.1 g/dL, min-max: 1.6-3.7 g/dL.
REFERENCES


