THE EFFECT OF HAEMOLYSIS ON ANTITHROMBIN CONCENTRATION AS DETERMINED BY A CHROMOGENIC METHOD

By

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A Dissertation submitted to the Faculty of Veterinary Science, Department of Companion Animal Clinical Studies, University of Pretoria, in partial fulfilment of the requirements for the degree MMedVet (Med)

Pretoria

September 2005

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ACKNOWLEDGEMENTS

The author would like to thank the following people for their assistance during the duration of the project.

- Leila the Labrador for donating the blood used to create the haemolysate, and Antoinette Lourens for allowing her.
- Trudie Goosen from the Department of Tropical Diseases, Faculty of Veterinary Science, for all her help with centrifuging and filtering the haemolysate.
- Elsabé Myburgh from the section of Clinical Pathology, Department of Companion Animal Medicine, Faculty of Veterinary Science for all her help in the micro-plate saga.
- Dr Piet Bekker, MRC, for statistical analysis
- Linda, for her practical guidance and moral support throughout the project
- Dr Rhena Delport, Department of Telematic Education and Innovation, UP for assisting with the statistical analysis and proofreading.
- Dr Peter Thompson for having the patience to help me understand my stats.

Supervisor: Dr JP Schoeman, BVSc MMedVet (Med), Dipl ECVIM (CA)
Co-supervisor: Dr L Jacobson, BVSc MMedVet (Med)

LIST OF ABBREVIATIONS

AT = antithrombin

CPD = citrate – phoshate –dextrose anticoagulant

DIC = disseminated intravascular coagulation

Hb = haemoglobin

[Hb] = concentration of haemoglobin

IMHA = immune mediated haemolytic anaemia

NHP = normal human plasma

OVAH = Onderstepoort Veterinary Academic Hospital

PTE = pulmonary thromboembolism

PT = prothrombin Time

PTT =Partial Thromboplastin time

R^2 = correlation co-efficient (proportion of change which can be correlated to [Hb])

SIRS = systemic inflammatory response

TF = tissue Factor

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SUMMARY

The presence of free haemoglobin in serum or plasma can markedly affect the outcome of laboratory tests. Normal concentrations of plasma haemoglobin in carefully obtained specimens are less than 0.025g/l. The presence of free haemoglobin in a sample increases the spectrophotometric absorbance of tests run at wavelengths within the absorbance range of haemoglobin (400 – 440nm). Little is known about the effects of haemolysis on the determination of antithrombin levels in canine plasma samples.

Two plasma pools, designated AT 100 and AT 70 were prepared. The AT 70 pool was prepared by diluting pooled plasma with 0.9% saline in a ratio of 7:3. A unit of whole blood was collected from a healthy donor animal. The erythrocytes were lysed by freezing and thawing. The solution was centrifuged, the supernatant collected and filtered using 1.2 um and 0.22 um filters sequentially to remove residual red cell stroma. The haemoglobin concentration of the solution was determined using a modification of the automated Drabkin method. Intermediate haemoglobin solutions of decreasing known concentrations were prepared by the addition of saline. The intermediate solutions were added to the plasma pools in a 1+9 manner and a series of samples were prepared with final calculated and measured haemoglobin concentrations of 0.0; 0.5; 1.5; 2.5; 3.5; 4.5 and 5.5 g/l. The AT determinations were performed using a functional chromogenic assay and the spectrophotometric absorbances were read using a 405 nm filter, as specified.

Increasing concentrations of haemoglobin resulted in a decrease in the AT value measured. A simple linear regression analysis was performed on both AT70 and AT100 using a two-step regression analysis. The slopes up to [Hb] 1.5g/l were not significant whereas the slopes at between [Hb] 1.5 – 5.5 g/l were significant ($p < 0.001$). The slope equation for AT 100 was $y = -5.742X + 115.24$ with $R^2 = 0.794$ and for AT 70 was $y = - 4.2037X + 66.821$, with $R^2 = 0.936$. A conversion table was created by interpolation of data between these two lines.

These results show that it is possible, using a conversion equation, to perform the AT assay in haemoglobinaenic serum, thus opening the way to further evaluation of the coagulation status in patients with haemolytic disease processes.

Chapter 1

JUSTIFICATION

1.1 HAEMOGLOBIN

1.1.1 Haemoglobinaemic plasma

In the normal individual plasma contains essentially no haemoglobin. A minute amount of haemoglobin is normally released into plasma with the destruction of senescent erythrocytes. Free plasma haemoglobin is bound by haptoglobin and removed by the liver. With severe haemolytic episodes, depletion of haptoglobin occurs. The hapto-haemoglobin complex is rapidly removed by the parenchymal cells of the liver. Free haemoglobin, in excess of binding capacity, dissociates into dimers which remain in circulation (haemoglobinaemia) until they pass through the glomerular filter. Plasma haptoglobin returns to normal levels within 2-3 days[1]. Values of haemoglobin in carefully obtained plasma samples are less than 0.025g/l and can be attributed to collection trauma[1]. Measured concentrations of haemoglobin are usually higher in serum than in plasma as coagulation results in a degree of haemolysis.

The presence of free haemoglobin in serum or plasma can markedly affect the outcome of laboratory tests[2]. Haemolysis becomes grossly visible when the concentration of haemoglobin exceeds 0.3 g/l [3]. The presence of free haemoglobin in a sample increases the spectrophotometric absorbance of tests run at wavelengths within the absorbance range of haemoglobin[4]. All haeme proteins exhibit maximum absorbance in the Soret band region of 400 – 440nm but each form of haeme also has an additional characteristic absorbance range which is approximately 10-fold less than that exhibited in the Soret band: Oxyhaemoglobin at 540 nm and methaemoglobin at 630nm[1].

In the South African clinical laboratory, due to the high incidence of haemolytic disease, haemolysed samples are sufficiently common to warrant a method of salvaging the sample for analysis.[5]. It is thus essential to establish relationships between interferents, such as haemoglobin, and the results of routine screening tests as it is often impossible to obtain a sample free of interferents [6].

Haemolysis may also result from *in vitro* causes; rapid collection through narrow gauge needles, excessive suction with syringe or vacutainer causing too rapid collection or collapse of the vein, excessive agitation, forceful centrifugation and prolonged storage at extreme temperatures[3].

There are 5 different mechanisms by which haemolysis can affect laboratory results: Leakage of analytes from damaged erythrocytes, dilution of analytes in serum, colour interference by haemoglobin, increased turbidity and chemical interaction with analytes. Haemoglobin will result in colour interference in the test method for measurement of antithrombin[3].

The type of assay performed plays an important role in determining the effect of the colour interference effect of haemolysis on the assay. Endpoint assays are the most dramatically affected as a single absorbance reading is taken at the completion of the reaction. Kinetic assays are less affected as a number of readings are taken, one at the beginning of the reaction and the others at fixed points during the reaction. The rate of change between readings is used to calculate the concentration of the analyte, thus cancelling out the effect of the interference due to haemoglobin. Interference will however still occur if sufficient haemoglobin is present in the plasma to extend the absorbance beyond the analyte's range of linearity[3].

1.1.2 Haemolytic disease

Intravascular haemolysis has been shown to induce a hypercoagulable state. This state is induce by increased tissue factor (TF), which is expressed by the endothelium and monocytes during the inflammation that accompanies severe haemolysis[7-9].

Intact erythrocytes have very little effect on coagulation whereas haemolysed erythrocytes have a strong procoagulant effect[10]. Red cell stroma consists of a structurally intact phospholipid membrane and the individual constituents of the erythrocyte membrane cause no activation of the coagulation system[11]. The outer erythrocyte membrane contains a neutral phospholipid, phosphatidylcholine, which induces minimal TF function[11]. Lysed erythrocytes lead to the activation of factor Xa via TF expression and activation, bypassing the surface activation steps of the intrinsic pathway, but requiring factors VIII, IX, XI and XII. This activity is a property of the membrane stroma and not any of the individual constituents of the membrane, an observation that was already made in 1886 by Wooldridge [10,12]. Stroma, which causes induction of TF contains anionic phospholipids, especially phosphatidylserine, which is normally present on the inner surface of cell membranes and induces optimal TF function.

The effect of haemolysis on coagulation is more apparent with incompatible transfusions than haemolytic disease, probably due to the amount and rate of haemolysis. The severity of the coagulopathy, in some cases culminating in overt disseminated intravascular coagulation, has been found to correlate with the degree of haemoglobinaemia and the decrease in red cell mass[13].

1.1.2.1 Immune-mediated haemolytic anaemia

Mortality statistics of haemolytic anaemias in the veterinary literature are difficult to assess due to variations in follow up times in the various publications as well as the high number of cases euthanased. In a prospective study of 20 cases, patients presenting with immune-mediated haemolytic anaemia were found to be in a hypercoagulable state[14]. In this report AT was decreased in 50% of patients. The mortality rate for IMHA is at least 26%. AT was not shown to have prognostic significance for survival or the development of PTE, the case number was however small. [14] In a retrospective study of 72 cases, pulmonary thromboembolism (PTE) was detected antemortally in 28% and confirmed in 80% of patients on which a post mortem was performed (n=25)[15]. Serial AT determinations have however shown prognostic value in other disorders associated with coagulopathies [16,17]

1.1.2.2. Canine babesiosis

Canine babesiosis is a tickborne disease of worldwide importance. Haemolytic anaemia is the hallmark, but several variations and complications occur. In South Africa canine babesiosis is caused principally by the haemoprotozoan parasite *Babesia canis rossi*, and is highly pathogenic [18-20]The disease is a very common cause of morbidity and mortality of dogs in South Africa, accounting for 12% of cases presented at the Onderstepoort Veterinary Academic Hospital (OVAH), approximately 1/3 of which are admitted for intensive treatment[21,22]. Intra - erythrocytic parasitaemia causes both extravascular and intravascular haemolysis. Erythrocyte destruction with resultant anaemia is a major cause of clinical signs of the disease. The severity of the anaemia appears unrelated to the observed parasitaemia[23].

Activation of coagulation is a normal component of the acute inflammatory response and babesiosis has been documented to caused an inflammatory response. In a study by Welzl *et al* 87% of 91 patients fulfilled the criteria of systemic inflammatory response as defined by Hauptman [24], namely:

- white cell count < 6000/mm^3 and > 16 000/mm^{3}and / or > 3% band neutrophils plus at least one of :
  - Rectal temperature < 38.1 or > 39.2
  - Heart rate > 120 bpm
  - Respiratory rate > 20 bpm

Disseminate intravascular coagulation (DIC) in naturally occuring *B canis* infections has been described using clinical, haematological, coagulation and histopathological parameters [25].

At the OVAH a significant percentage of cases show haemoglobinaemia. Van Zyl demonstrated that a series of 350 hospitalised babesiosis cases showed a median free serum haemoglobin concentration of 2.3 g/l (range 0.4 – 18.6 g/l). The mean free serum haemoglobin concentration in non-survivors (n=36) was 4.9g/l (SD 3.33) and in survivors (n=314) was 2.94 g/l SD (2.68)[23]. A recent evaluation of 90 cases at the OVAH showed that the range of free serum haemoglobin concentration in babesiosis cases was 0.9 g/l – 10.1 g/l with a median of 1.7 g/l. Twenty five cases (28%) had values greater than 3 g/l (M Nel - unpublished data). Another babesiosis trial performed to assess carbohydrate metabolism in babesiosis (n=20) showed a free serum haemoglobin range of 1.2 – 5.2 g/l (10th – 90th percentile) [26].

Van Zyl also showed that patients with a higher haemoglobin concentrations were more likely to die from the disease[23]. It is these complicated cases where current research into the systemic inflammatory response to disease (SIRS) and coagulopathies is aimed, thus the need for validating assays in the presence of haemoglobinemia.

1.2 ANTITHROMBIN

1.2.1 Structure and function

Antithrombin (AT) is an α-2 -glycoprotein, which is produced by the liver and to a lesser extent the endothelium. It acts by slowly and irreversibly binding to activated coagulation factors IX, X, XI, XII, thrombin, urokinase and kallikrein and accounts for approximately 80% of all coagulation inhibition[27,28]. It has a half life of 1.7 days in the dog[27,28] The AT-heparin complex is a major inhibitor of thrombin and thus of coagulation[17]. Heparin acts as a catalyst for AT activity by forming a transient electrostatic bond and inducing a conformational change thereby increasing its activity, especially against thrombin and factor Xa, several thousand fold [28,29].

1.2.2 Clinical relevance

AT levels in animals are usually expressed as a percentage of normal human plasma (NHP). Species differences are apparent. In dogs a normal level is 104 $\pm$ 12 % (range 84 – 128%) [30]. A moderate thrombotic risk occurs with AT levels less than 75% and a severe thrombotic risk with levels less than 50%[30].

Acquired AT deficiency can occur in glomerulonephropathy, DIC, liver disease and secondary to therapy with L- asparaginase or heparin[30]. AT concentrations have been shown to be abnormal in 85% of DIC cases[31]. In a study involving DIC in dogs, AT levels were 71% $\pm$ 14 (normal controls 86% - 108%)[30,31] An increasing AT level has been shown to be a positive prognostic sign, whereas decreased AT activity or failure to return to normal correlates significantly with mortality [16,28,31].Several human studies on sepsis have shown that initial AT concentrations in sepsis may predict outcome, although this has not held true in a study of canine patients with bacterial sepsis[32].

1.2.3 Measurement

Immunological and functional assays are available to measure the concentration of AT. The functional method of AT determination is preferred as, although more costly than an immunological test, it does not measure inactivated or complexed and uncleared AT, the levels of both being increased in DIC [19,28]. Synthetic substrate assays are specific, accurate and easily automated, although expensive. Results obtained by the various available assays are comparable and reproducible even though the reagents used vary greatly[19].

In the test procedure, patient plasma is incubated with excess thrombin, in the presence of heparin. The residual thrombin is reacted with chromogenic substrate Chromozym TH®. The excess enzyme cleaves an optically active tag from the substrate which can be measured kinetically or by end point determination[19]. The amount of colour released is measured spectrophotometrically and is inversely proportional to the amount of AT in the patient plasma.

To briefly summarise:

- AT + heparin $\longrightarrow$ [AT • Heparin]

[AT • Heparin] + thrombin (excess) $\rightarrow$ [AT • Heparin • Thrombin] + residual thrombin

- Chromozym TH $\xrightarrow{\text{Thrombin}}$ [Chromozym – Optic tag] + free Optic Tag 

Higher concentrations of AT will result in less residual thrombin and therefore less optic tag will be released leading to a lower reading. The reading is therefore inversely proportional to the concentration of AT.

1.2.4 Test Kit selection

Functional synthetic substrate assays utilise either thrombin or factor Xa as substrate in the presence of heparin. Tests which utilise thrombin, instead of factor Xa, can overestimate the amount of AT due to the presence of heparin co-factor II (HCF-II) in the

plasma, which inactivates thrombin in a heparin-catalyzed reaction, but has no effect on factor Xa [33]. This is especially a problem in patients receiving heparin therapy[27,34]. The laboratory performing the assays however, routinely used an AT kit which utilised thrombin as a substrate.

1.2.5 Haemolysis as an interferent

Interferograms have been established for many routine clinical chemistry tests and some work has been done on the coagulation system but no data could be found on AT, which is an essential part of the evaluation of coagulopathy[2,3,35]. The package inserts for the AT assays all warn against interference by lipaemia, icteric plasma and haemolytic plasma in the 2-point assay.

The interference of haemoglobin is due to colour increases in the test plasma in the spectrophotometric absorbance range of the optic tag (405nm). Haemolysis will result in increased colour of the test solution thus giving an artefactually high spectrophotometric reading. Due to the inverse relationship between the excess thrombin and AT, this will be interpreted as a lower AT concentration. The package insert of the kit used in this study indicates that AT concentrations are decreased by 6% at a haemoglobin concentration of 1.5g/l (0.15 g/dl).

A study evaluating coagulation parameters in IMHA measured AT III, using a synthetic substrate method employing Factor Xa[14]. No mention was made of the degree of haemolysis in the serum or corrections made to the ATIII readings for haemolysis.

Personal communication with Chromogenix (Coamatic® Antithrombin) indicates that no trials have been performed to test the accuracy of the assay with moderate to severe haemoglobinaemia. They cite a similar assay, also using Factor Xa, which shows that haemoglobin levels of 2g/l (200mg/dl) do not affect the assay in the normal range (0 – 120%). The package insert also however, cautions about interference by haemolysis. The claims on the package inserts of the various antithrombin kits are thus vague and made in very general terms with no specific information available about the qualitative and quantitative effect of the interferent [36].

Chapter 2

STUDY OBJECTIVES

2.1 Problem

The AT assay is a chromogenic functional test read spectrophotometrically at 405nm. It was therefore anticipated that haemoglobinaemia would interfere with the results. Little is known about the effect of haemoglobinaemia on the determination of AT levels in canine plasma samples.

The dogs in which we intended determining AT concentrations had babesiosis, a haemolytic disease, thus haemoglobinaemia of varying severity was anticipated in a substantial proportion of the patients.
Haemoglobinaemia affects the ability to assess the coagulation status in dogs with babesiosis, where coagulopathy is known to occur.

2.2 Purpose of the study

The purpose of this study was to:

- Determine if haemoglobin interferes with antithrombin determination
- Determine if the interference is proportional to the concentration of haemoglobin in the serum.
- Determine whether the alteration of antithrombin is proportional to the concentration of haemoglobin and to determine if the amount of antithrombin present in the plasma will affect that proportion.
- Determine if the degree of interference can be predicted based on the concentration of haemoglobin
- Establish a factor with which to obtain the corrected antithrombin value

Chapter 3

MATERIALS AND METHODS

3.1 Ethical considerations

This study was approved by the Animal Use and Care Committee of the University of Pretoria.
Protocol reference - 36-5-399: University of Pretoria.

Owner consent was obtained for blood collection from animals presented at the Onderstepoort Veterinary Academic Hospital (OVAH) for routine sterilisation.

3.2 Preparation of Plasma

See Fig 1 (p 23) for an overview of sample preparation

3.2.1 Preparation of pooled (stock) plasma

Equal volumes of citrated plasma (5 x 4,2 ml 3.8% citrate tubes [Φ]) were collected by means of atraumatic jugular venipuncture from 10 dogs, all of which were greater than 20 kg in weight. The citrate tubes were all filled according to manufacturers recommendation. Where difficulty in collection was encountered, the tube was discarded. The blood was immediately centrifuged[♣] for 20 minutes at 3000 rpm and the plasma harvested and pooled. The pooled plasma was refrigerated at 4°C and was processed within 6 hours of collection.

3.2.2 Preparation of test plasma samples

Two subsequent plasma pools were prepared utilising the pooled plasma : one pool was designated AT100 and the other pool designated AT 70.

The AT 100% pool (AT 100) would act as the control as normal canine plasma
The AT 70% (AT 70) pool was prepared by diluting the plasma with 0.9% saline in a plasma to saline ratio of 7:3. This pool was to serve as a reflection of patients with a coagulopathy where the antithrombin levels are decreased from normal.

[Φ] Becton Dickinson Vacutainer Systems, BD Company, Franklin Lakes, NJ, USA.
[♣] Juan Benchtop Centrifuge

3.3 Preparation of haemoglobin solution

3.3.1 Preparation of the purified haemoglobin stock solution

Whole blood was collected into CPD (citrate-phosphate-dextrose) anticoagulant, with owner consent, from a healthy canine blood donor. The blood was placed into sterile glass bottles and centrifuged at 3000 rpm for 30 minutes to separate the red cells and the plasma. The plasma was aspirated and discarded. The red cells were re-suspended in 0.9% saline and once again centrifuged at 3000 rpm for 30 minutes. The supernatant was aspirated and discarded. The red cells were washed in this manner 3 times to remove any plasma and residual anticoagulant from the red cell mass. The red cells were then re-suspended in a small volume (approximately 100 ml) of saline.

The suspended red cells were placed into sterile freeze -resistant glass bottles♦ and lysed by freezing at –80°C and thawing in a waterbath at 37°C. The procedure was repeated 3 times.

The solution was then once again placed into sterile glass bottles and centrifuged at 3000rpm for 30 minutes. The supernatant was collected. The red cell stroma was discarded. The supernatant was then filtered using 1.2 μm and 0.22 μm filters ∋ sequentially to remove any residual red cell stroma.

The resultant solution was evaluated for methaemoglobin levels, which measured 3.8 %[T]. The haemoglobin concentration of the solution was determined using a modification of the automated cyanomethaemoglobin (Drabkin) method, the internationally approved standard method of haemoglobin measurement [1] (see Appendix 1). The haemoglobin concentration in the stock solution was 55g/l. The filtered haemoglobin solution was placed in a glass bottle and stored at 4°C for a period of no longer than 24 hours.

3.3.2 Preparation of the intermediate haemoglobin solutions

Intermediate haemoglobin solutions of decreasing concentrations were prepared (see table 1). This enabled the dilution of the test plasma sample by equal volumes of intermediate haemoglobin solution, thus eliminating the dilution effect on the more concentrated haemoglobinaemic plasma samples.

♦ Schott Glass Bottles, Schott Ag, Mainz, Germany

∋ Microsep Pre-filters, Millipore , Billeric, MA, USA.

T Radiometer OSM3 blood gas analyzer, Diamond diagnostics, Holliston, MA, USA

The calculated value of the haemoglobin concentrations in the intermediate solutions were confirmed using the modified cyan-methaemoglobin method.

Table 1: Creation of the intermediate haemoglobin solutions

Hb stock filtrate [55g/l]	Saline	Intermediate Hb Solution: [Hb] g/l	Creation of test plasma (AT 100 & AT 70)	Final test plasma
2 ml	0ml saline	**55g/l**	1 part 55 g/l Hb sol + 9 parts Plasma	**5.5g/l**
2 ml +	0.44 ml saline	**45g/l**	1 part 45 g/l Hb sol + 9 parts Plasma	**4.5g/l**
2 ml +	1.14 ml saline	**35g/l**	1 part 35 g/l Hb sol + 9 parts Plasma	**3.5g/l**
2 ml +	2.4 ml saline	**25g/l**	1 part 25 g/l Hb sol + 9 parts Plasma	**2.5g/l**
2 ml +	5.33 ml saline	**15g/l**	1 part 15 g/l Hb sol + 9 parts Plasma	**1.5g/l**
2 ml +	20 ml saline	**5 g/l**	1 part 5 g/l Hb sol + 9 parts Plasma	**0.5g/l**
0 ml	Saline	**0 g/l**	1 part saline + 9 parts Plasma	**0 g/l**

3.4 Preparation of haemoglobinaemic plasma test samples

The intermediate solutions were then added to the test plasma in a 1+9 manner (see table 1).

A series of 4ml samples were prepared (i.e. 3,6 ml plasma + 0,4 ml intermediate Hb solution), with final calculated and measured [Hb] of 0g/l; 0.5 g/l; 1,5g/l; 2,5g/l; 3,5g/l; 4.5 g/l and 5.5 g/l. There was no dilutional error in the test samples as the same volume of intermediate saline-haemoglobin mixture was added to each set.

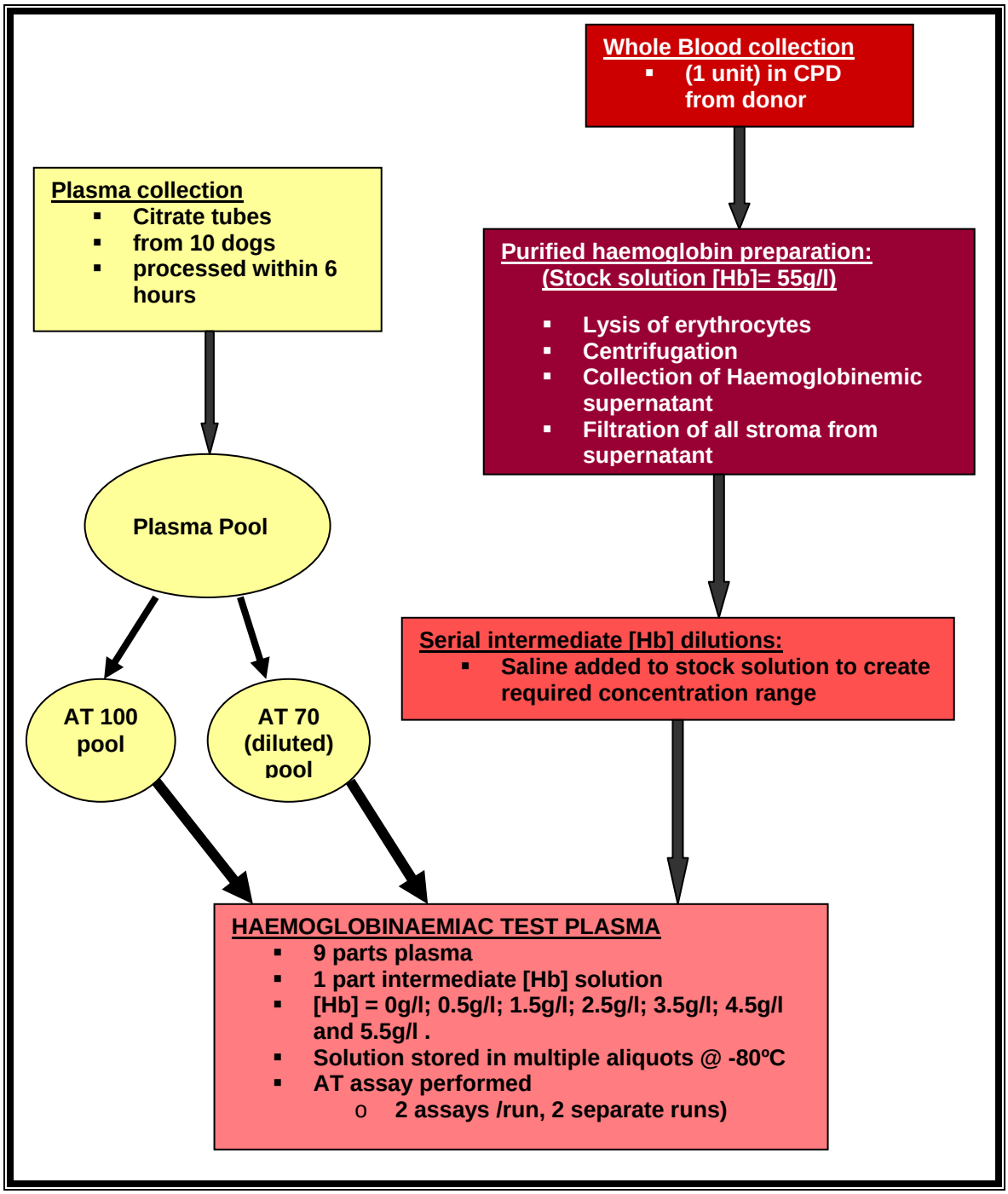
The sample of 0g/l Hb was the control plasma sample as 1 part pure saline was added to the pooled plasma (AT100 and AT70)

part saline was added. The readings from this sample provided us with the expected/normal readings.

The need for a series of dilutions of the interferent to detect any non-linear responses has been previously established[36].

The samples were aliquoted into 1 ml cryovials and frozen at –80°C. The samples were transported on icepacks to the laboratory and immediately placed in a –80°C freezer. The samples were assayed over a period of 6 weeks. Storage of canine plasma for 6 months at -70ºC has no effect on the level of antithrombin and therefore the use of frozen sample will not have affected readings [37].

Fig 1 : Diagrammatic representation of the preparation of the Test plasma



3.5 Antithrombin determination

The AT determinations were performed using a chromogenic assay[X], a functional assay which incubates patient plasma with excess thrombin in the presence of heparin (see Appendix 2 for manufacturer's sheet).

In the test procedure the residual thrombin was reacted with chromogenic substrate Chromozym TH®. The amount of colour released was measured spectrophotometrically[α] and is inversely proportional to the amount of AT in the patient plasma. A standard curve was plotted for each kit/test batch, utilising normal human plasma (NHP) with an AT content of 100%. The test was performed at 30°C, according to instructions for 2- point measurements of a semi-micro assay as indicated on the package insert. The absorbances were read using a 405 nm filter. The AT assay was performed with a 405nm (400 – 420nm) filter, with the cuvette, at a temperature of 37º C, and measured against air.

A standard curve was drawn at 50%, 100% and 200% AT using standard human plasma[©]. Results were determined from the standard curve. Each dilution was tested in duplicate. The test procedure was repeated once. Four readings were thus obtained for each dilution on each run. A fresh sample was used for each inter-assay run. Intra-assay replicates were performed on the same prepared sample. Test sample preparation was manually performed thus the possibility of technician error was present. To limit this variable as much as possible all the tests were performed by the same experienced and specialised technician. Reading of the results against the standard curve was also performed manually, off graph paper. Each 1mm block of graph paper represented an AT value of 1%. The AT results we were seeking were not required to be extremely accurate , but rather ranges (SD = 12%) therefore the effect of minor mathematical errors where a 1mm block represents only 1%, would be negligible.

[X] Boehringer Now Roche Diagnostics, Mannheim, Germany
[α] DR Lange photometer LP6. DR Lange Basingstoke, England.
[©],Cat # 3647 Standard Human Plasma Dade Behring, Deerfield Illinois, USA.

Chapter 4

RESULTS

4.1 Antithrombin Assay Results

A standard curve was drawn using standard control plasma measuring 50%, 100%, 200% AT. The Y axis representing the AT% and the X axis (+) the absorbance reading. The true absorbance reading was obtained by subtracting the absorbance obtained from that of a blank control. This value was then read off against the standard curve and the result was an AT value in %.

Table 2: Tabulation of the results of all the antithrombin assays performed
(ND = not done)

AT - 100								
	RUN 1				**RUN 2**			
[Hb] g/l	**(am)**		**(pm)**		**(am)**		**(pm)**	
	Replicate 1	**Replicate 2**	**Replicate 1**	**Replicate 2**	**Replicate 1**	**Replicate 2**	**Replicate 1**	**Replicate 2**
0	98.98	98.98	105.84	104.86	ND	ND	ND	ND
0.5	84.3	84.3	91.14	91.14	103.55	103.55	96.9	102.6
1.5	100.9	98.98	110.74	110.74	ND	ND	ND	ND
2.5	98	96	106.82	104.4	ND	ND	ND	ND
3.5	98.9	98.98	95.06	94	ND	ND	ND	ND
4.5	87.22	86.24	93	94	ND	ND	ND	ND
5.5	86.24	85.26	79	78.4	ND	ND	ND	ND
AT - 70								
	RUN 1				**RUN 2**			
[Hb] g/l	**(am)**		**(pm)**		**(am)**		**(pm)**	
	Replicate 1	**Replicate 2**	**Replicate 1**	**Replicate 2**	**Replicate 1**	**Replicate 2**	**Replicate 1**	**Replicate 2**
0	68.6	67.62	74.48	74.48	61.75	60.8	63.65	62.7
0.5	60.76	62.72	62.7	62.7	60.8	59.8	57.9	57.9
1.5	58.8	62	58.8	58.8	60.8	61.75	60.8	60.8
2.5	50.96	48	55	55	58	58	55	53.2
3.5	46	69.58	71.54	68	54.15	52.25	51.3	50.35
4.5	60.76	60.76	62.72	64.68	45.6	44.65	48.45	48.45
5.5	39.2	56.8	41.16	39.2	45.6	44.65	44.65	43.7

4.2 Data selection for statistical analysis

Replicates of AT 100 in blue (at 0.5g/l Hb) were replaced with values obtained in a second run as they were outliers based on visual assessment. The entire Run 1 of the AT 70 were excluded from analyses as the data points at [Hb] = 4.5g/l were all definite outliers based on visual assessment .

4.3 Statistical Calculations

The statistical analyses were performed using Microsoft Excel and NCSS software.

4.3.1 Co-efficients of variation (CV)

The results of the same day repetition of the AT 100 test plasma run were used to calculate the within batch analytical CV, which was 1.41% (n=88). The results of the separate repetition of the entire AT 70 test plasma run were used to determine between batch CV, which was 5.57% (n=58). These analytical CV are acceptable.[38]

4.3.2 Means and Standard deviation

Graph 1: Graphic depiction of the averages of the readings of both AT 100 and AT 70 with error bars showing the standard deviation from normal.

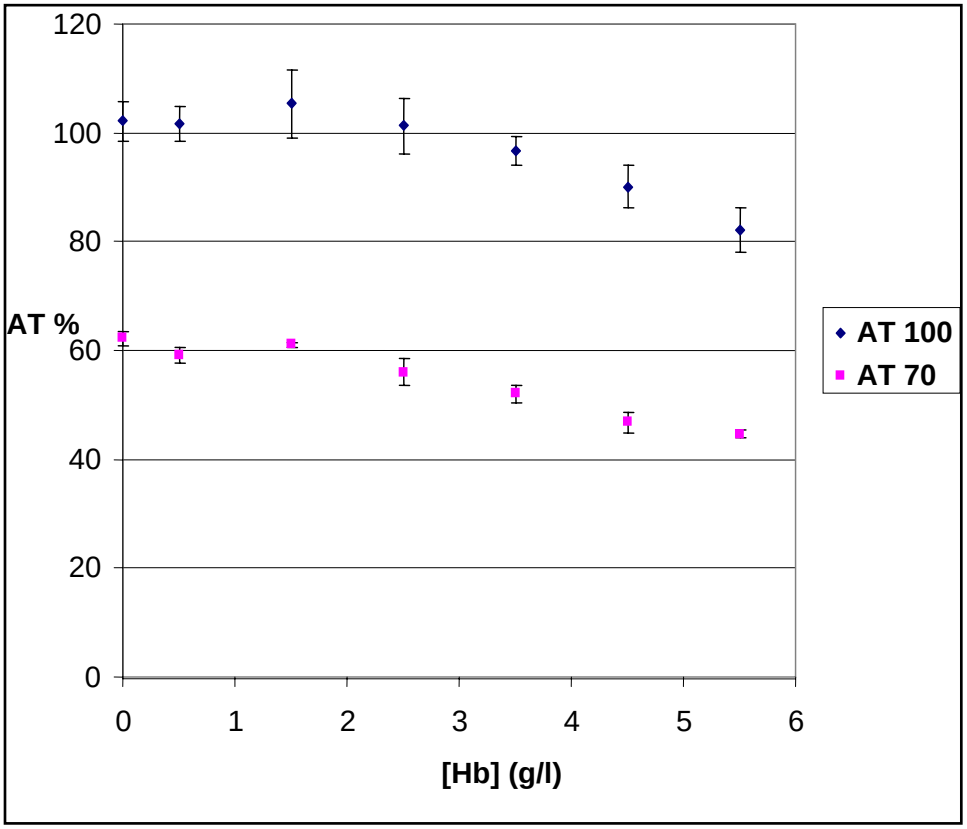


Table 3: Standard deviations and means of the two data series AT 100 and AT 70

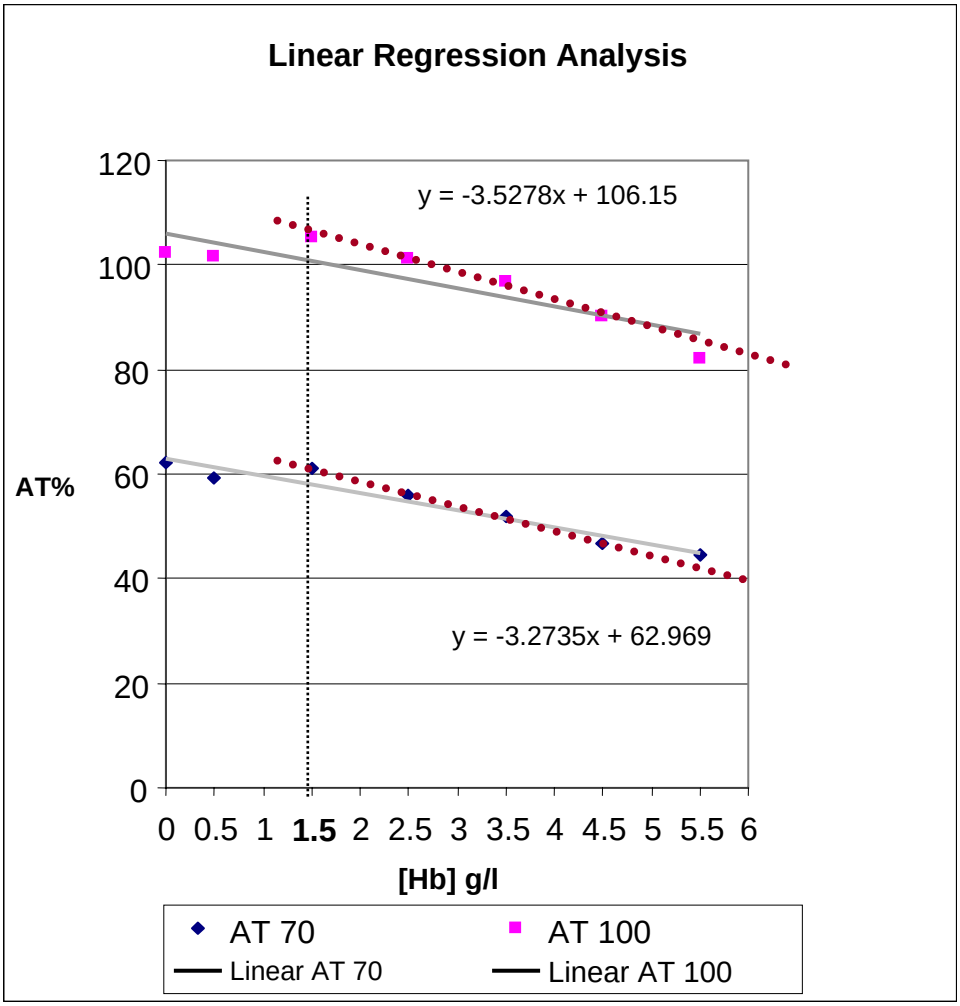
AT 100	RUN 1					
[Hb] g/l	(am)		(pm)			
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Mean	Standard deviation
0	98.98	98.98	105.84	104.86	102.165	3.699419
0.5	103.55	103.55	96.9	102.6	101.65	3.198177
1.5	100.9	98.98	110.74	110.74	105.34	6.284457
2.5	98	96	106.82	104.4	101.305	5.13356
3.5	98.9	98.98	95.06	94	96.735	2.582834
4.5	87.22	86.24	93	94	90.115	3.950236
5.5	86.24	85.26	79	78.4	82.225	4.097263

AT 70	RUN 2					
[Hb] g/l	(am)		(pm)			
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Mean	Standard Deviation
0	61.75	60.8	63.65	62.7	62.225	1.226445
0.5	60.8	59.8	57.9	57.9	59.1	1.44453
1.5	60.8	61.75	60.8	60.8	61.0375	0.457
2.5	58	58	55	53.2	56.05	2.368544
3.5	54.15	52.25	51.3	50.35	52.0125	1.622434
4.5	45.6	44.65	48.45	48.45	46.7875	1.958475
5.5	45.6	44.65	44.65	43.7	44.65	0.775672

4.3.3 Simple linear regression analysis

One of the goals of this study was to predict the AT concentrations when the haemoglobin concentration was known. To achieve this aim a simple linear regression analysis was performed (see Appendix 4 for statistical calculations).

Graph 2: Simple one-step regression analysis through all the data points starting from [Hb] 0g/l.

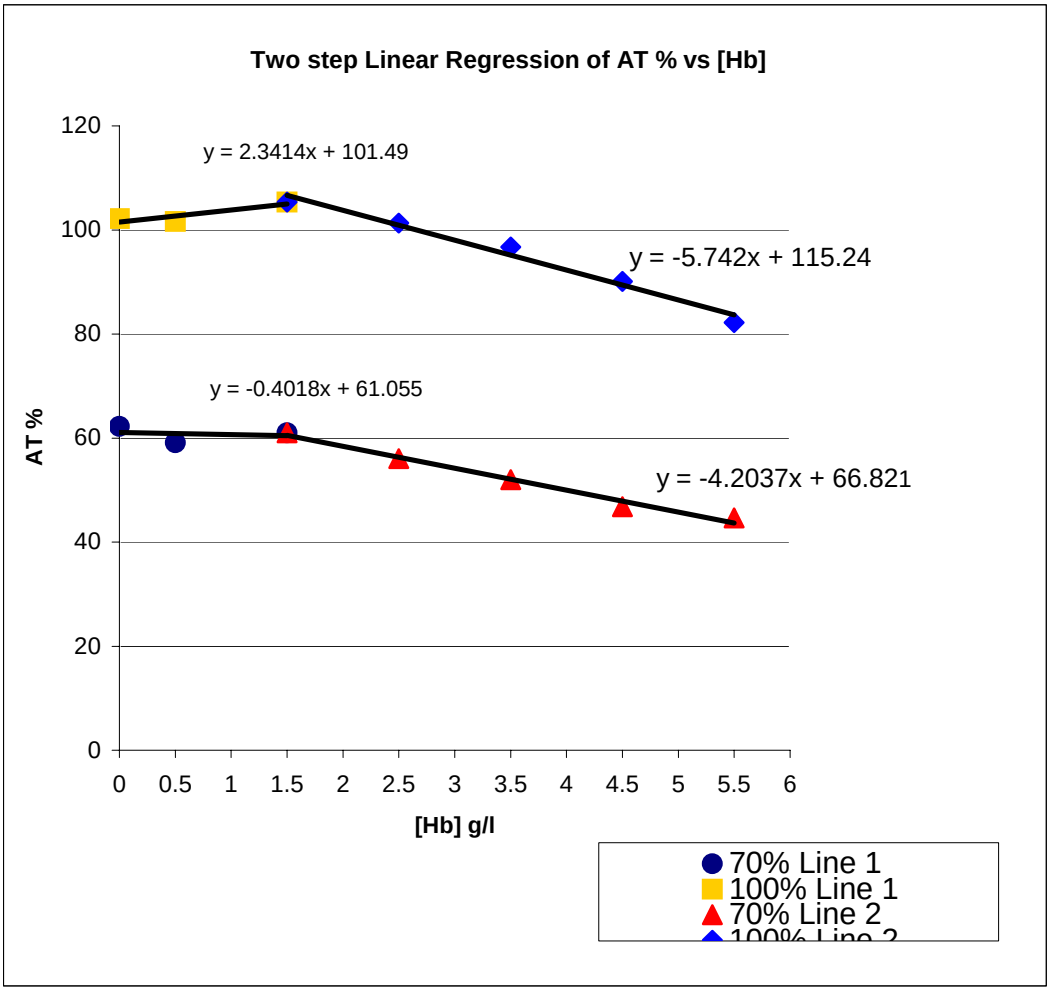


The package insert of the kit used in this study indicates that AT concentrations are decreased by 6% at a haemoglobin concentration of 0,15g/dl (1.5 g/l) and above. Visual examination of the points plotted also suggested that the slope was different and more pronounced at [Hb] ≥1.5g/l, as indicated by the red dotted lines ••••••••••• .

4.3.4 Two-step linear regression

A two-step linear regression analysis was then applied to AT 70 and AT 100 using [Hb]= 1.5g/l as the intersect point (see Appendix 3 for statistical calculations)

Graph 3: Regression analysis of the AT readings concentrations [Hb] ≥1.5g/l



Interpretation of the two step regression

- The first slope equation for AT100 is y = 2.3414x +101.49 and is statistically not significant, (p< 0.28) and the slope can be therefore be considered zero.
- The second slope equation for AT 100 is y = - 5.742X + 115.24, and is statistically significant with p < 0.0001. The correlation co-efficient is 0.79. The slope ≥ [Hb] 1.5g/l is therefore significant.
- The first slope equation for AT 70 is y = -0.4018X + 61.055 and is statistically not significant (p < 0.63) and the slope can be therefore be considered zero.

- The second slope equation for AT 70 is y = -4.2037X + 66.821 and is statistically significant with $p < 0.0001$. The correlation co-efficient is 0.93. The slope ≥ [Hb] 1.5g/l is therefore significant.

4.4 Data Analysis

4.4.1 Selection of slope equation.

When the patient presents with haemolytic disease the blood concentrations of AT are an unknown quantity, although we are hypothesizing that they will be reduced. The problem is then, which equation do we select to correct for the interference; that where the AT is presumed to be at 100% or that where it is presumed to be decreased at 70%?

To simplify this situation the significance of the variation of the two slopes as depicted in graph 3 was assessed. The slopes appeared almost parallel, thus suggesting that there would be little real significance at these low concentrations of haemoglobin. The slopes also appear to converge slightly indicating that this assumption would not hold true for very high concentrations of haemoglobin.

i) Using the equation of the slopes in graph 3 to calculate the True Value of AT

The least squares regression is a way to determine the equation of a line that provides a good fit to the points.

Test sample: Assume a [Hb] of 3 g/l and a test result of AT = 60%

The regression equation is: $Y = \beta_0 + \beta_1 X + \varepsilon$

Simplified : $Y = a + b X$

Therefore : $a = Y – b X$

Where: a = True value of AT
Y = Read value of AT
b = slope co-efficient
X = haemoglobin concentration

Using slope AT 100 : $a = 60 – (-.5.742 X 3)$

$a = 60 + 17.226$

True Value = AT 77.22 % = 77%

Using slope AT 70: a = 60 – (-4.2037*3)

a = 60 + 12.611

True Value = AT 72.61% = 73%

The difference between these two values is 4%, thus the mean of the two slopes would be 75%(74.92%).

The normal range of antithrombin in dogs is wide with normal being 104 (± 12) % of NHP, therefore reading off either of the slopes obtained would provide a clinically useful result.

As the haemoglobin concentration increases the effect of the convergence of the two slopes would be expected to affect the correlation of the AT % with the haemoglobin concentration in a negative manner.

If a [Hb] of 10g/l were to be assumed with a read AT value of 60% the AT 100 slope would give a true value of 117% whereas the AT 70 slope would give a true value of 102%. The difference between these two values is 15% as compared to the difference of 4.61 at [Hb] of 3g/l.

The question now was what slope to select to create a standard slope co-efficient ?
The following options were available:

1) Average out the two slopes to allow a single slope to be used
2) Assume that the patients will have a decreased AT level and use the slope of AT 70%, which does have a better correlation coefficient (R^2).

(1.) Averaging the co-efficients of the two slopes:

$$\frac{(\text{slope AT 100}) + (\text{slope AT 70})}{2} = \frac{(-5.7240) + (-4.2037)}{2} = \frac{-9.9277}{2} = -4.9638$$

Assume once again a [Hb] of 3 g/l and a read value of AT III 60% .

a = 60 – (-4.9638 X 3)

a = 60 + 14.89

True value = AT 74.89% = 75%

This value for AT compares well with those calculated off each slope individually, viz. AT=73% for AT70 and 77% for AT100.

4.4.2 Creation of a conversion table

An alternative method of utilizing the data was to construct a table from which the corrected true reading could be read.

A table was constructed by adding data points along the regression lines to create a series of haemoglobin dilutions increasing by 0.25g/l increments to a maximum of 5.5g/l. Data points were also interpolated between the AT 100 and AT 70 lines, thus allowing the creation of a conversion table.

Table 4: Tabulation of results with interpolation of data points between measured AT100 and AT 70 values.

Calculated/Actual AT percentages	**70%**	**75%**	**80%**	**85%**	**90%**	**95%**	**100%**
Measured [Hb]g/l	**62**	**69**	**76**	**82**	**89**	**96**	**103**
[Haemoglobin] g/l in test sample	**Antithrombin % as determined from patient sample** (falsely decreased a.r.o. interference) Select value closest to value obtained in patient sample						
1.5	61	68	76	84	92	99	107
1.75	60	67	75	83	90	98	106
2	59	66	74	81	89	97	104
2.25	58	65	73	80	88	95	103
2.5	56	64	71	79	86	94	101
2.75	55	63	70	78	85	93	100
3	54	62	69	76	84	91	99
3.25	53	61	68	75	82	90	97
3.5	52	60	67	74	81	88	96
3.75	51	58	65	73	80	87	94
4	50	57	64	71	79	86	93
4.25	49	56	63	70	77	84	92
4.5	48	55	62	69	76	83	90
4.75	47	54	61	68	75	82	89
5	46	53	60	66	73	80	87
5.25	45	51	58	65	72	79	85
5.5	43	50	57	64	71	78	84

The table is used by following the row with the value closest to the patient serum/plasma [Hb] and selecting the AT value closest to that obtained for the patient. The top of the column where the selected AT value lies will show the actual AT value as calculated.

The normal range of ATIII in dogs is wide with normal being 104 (± 12) % of NHP (normal Human Plasma), therefore reading the closest value with a variation of 4-5% will not markedly affect the outcome.

Using the same example used in evaluation of the slope equation [4.4.1(i)] :

AT read value = 60 %, [Hb] = 3 g/l

The corresponding calculated value = AT 75%

This value compares well with those calculated using the equations where the range of AT% was from 73. – 77 %.

4.4.3 Practical application of data

A two-step regression was performed as the data points below a haemoglobin concentration of 1.5g/l did not satisfy the requirements of significance and the slope was statistically considered zero in both the AT 100 and AT 70 test samples.
The slope co-efficients of the data points above a haemoglobin concentration [Hb] of 1.5g/l and up until 5.5 g/l , as determined by the range of [Hb} tested in the study, were deemed significant and were used in calculations.

If we were to apply the information we have gained from the regression analyses to the data of babesiosis cases from van Zyl[23]:

Number of patients = 350

Range of [Hb] = 0.4 – 18.6 g/l

Mean [Hb] = 3.1g/l

Median [Hb] = 2.3 g/l

Patients with [Hb] $\leq$ 1.5 g/l = 28.4%

Patients with [Hb] $\geq$ 1.5g/ $\leq$ 5.5 g/l = 59.3%

Patients with [Hb] $\geq$ 5.5g/l = 12.3%

The following conclusions can be reached regarding the application of the regression analyses when assessing babesiosis cases:

- No correction of AT levels will be required in 28.4% of cases as the [Hb] has been statistically shown not to significantly interfere with the test method.
- The AT reading will need to undergo correction in 59.3% of patients.
- In 12.3 % of cases the [Hb] is beyond the range evaluated in this trial.

4.4.3.1 Slope equations

The slopes of the means of the data points in the AT 100 and AT 70 groups although appearing almost parallel with very similar co-efficients did however converge slightly, a trend which would only have more marked effect at much higher haemoglobin concentrations.

Due to financial constraints the number of replicates in this study was small. A larger study with a larger number of replicates would probably have resulted in greater congruency between the two slopes.

4.4.3.2 Assessment of tabulated measured and calculated AT values

By looking at table 3 it is apparent that the AT100% calculated control actually measured 102% in the study. The standard line against which the test readings are evaluated is calculated using standard human plasma which has an AT concentration of 100%. The range in dogs however, is 104 ±12%, it is thus not unexpected that the canine control plasma indicated as AT100 % has a value different from that of the human control.

What was not expected was that the AT 70 controls read markedly less than the calculated AT value. The same plasma pool, diluted 3:7 with saline, and the same intermediate haemoglobinaemic samples were used. It therefore follows that the readings obtained should be very close to those calculated. At AT 70 = 70% however, the measured value was only 62%. This decrease was proportionally similar across all haemoglobin dilutions at AT 70. The reason for this discrepancy is not apparent but will obviously have a minor effect on the interpolation for the table.

Chapter 5

DISCUSSION

5.1 Materials and Methods

5.1.1 Plasma Collection

Care was taken to ensure that collection of citrated plasma from healthy donors was atraumatic and that the tubes were filled to the correct ration of 1 part 3.8% citrate to 9 parts blood. Failure to collect sufficient blood into the tube results in a dilutional artefact which has been shown to cause a false increase in PT and PTT values, by a clinically significant degree, if the ratio is 1:7 or less[39]

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5.1.2 Haemolysate preparation

Creation of the hemolysate presented some problems in this study as the initial stock solution created contained 33% methaemoglobin. The normal concentration of methaemoglobin should be less than 1.5% of the total haemoglobin [1]. Within the erythrocyte, haemoglobin is oxidized slowly to the trivalent (Fe^{3+}) form methaemoglobin as the cell becomes metabolically exhausted [40]. Oxyhaemoglobin releases the superoxide radical thus utilizing the anti-oxidant defences of the red blood cell. Long -term storage of haemeprotein at –20º C, which was the temperature initially used to produce the haemolysate, induces aggregation and auto-oxidation resulting in precipitation and methaemoglobin formation. Storage at –80º C prevents methaemoglobin formation and can be used for storage between intermediate processing steps[41]. Denatured haemoglobin precipitates and is not detected by the Drabkins test [41].The absorbance range is however similar to that of haemoglobin thus it was feared that high levels of methaemoglobin would influence the test results. The initial batch of hemolysate was thus discarded and a new batch created using -80°C as the freeze temperature. Methaemoglobin measured 3.2% in the second batch.

The supernatant was then filtered sequentially using 1.2 and 0.2 μm filters to remove any residual red cell stroma. It was considered vital to filter the supernatant as red blood cell membrane and stroma may cause interference due to turbidity thus “clouding” the results of the actual interference by haemoglobin [1]. It can be argued that plasma from the patient with haemolysis will also contain red cell stroma however the concentration would be much lower than in the trial situation as the stroma is distributed throughout the entire

patient plasma volume and the reticuloendothelial system would also be actively removing stroma from circulation. This is evident when evaluating blood smears in patients with IMHA or babesiosis where increased numbers of activated monocytes with a vacuolated cytoplasm is a normal finding. The stroma contained in the plasma of the trial samples was concentrated because 200 ml of packed erythrocytes was diluted with a small volume (approximately 100ml) of saline, to create the haemolysate.

The concentration of the haemoglobin stock solution was lower than what we would have preferred and, ideally, a smaller volume of saline should have been used when the red cells were diluted prior to lysing them. This part of the haemolysate preparation was however, trial and error as no standard technique was available. The range of free haemoglobin in van Zyls' work was 0.4 – 18.6g/l. Discussion with the clinical pathologist the Faculty (pers. comm. Prof F Reyers) also indicated that most cases of babesiosis would have a free haemoglobin below 5.5g/l. It was thus decided that the range of free plasma haemoglobin concentration of 0 – 5.5 g/l would be adequate and the stock solution was used to prepare the plasma test samples.
This decision was validated by later work where the free plasma haemoglobin concentration in the data collected by Nel (unpublished data) was 0.9 – 10.1g/l (median 1.7g/l) and that of Jacobson 1.2 – 5.2 g/l[26]. Only 7 of 90 patients in the trial by Nel had a free [Hb] >5.5g/l.

5.1.3 Antithrombin determination

It would have been preferable to utilize an assay-kit using Factor Xa as the substrate, but the commercial laboratory •approached to run the tests used a thrombin based kit. This would have had no effect in the current study but will need to be considered when selecting kits to assay antithrombin levels in patients as, especially in haemolysis, it is possible that heparin may be utilized therapeutically [27,34].
Tests utilising thrombin, instead of factor Xa, are prone to overestimate the amount of AT due to the presence of heparin co-factor II (HCF-II) in the plasma, which inactivates thrombin in a heparin-catalyzed reaction, but has no effect on factor Xa [33].

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5.1.4 Replicates

Due to financial constraints the number of replicates in this study was small. A larger study with a larger number of replicates would probably have resulted in greater congruency between the two slopes.

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The purpose of this study was to:

- Determine if haemoglobin interferes with antithrombin determination
- Determine if the interference is proportional to the concentration of haemoglobin in the serum.
- Determine if the amount of antithrombin present in the plasma will affect that proportion of interference by haemoglobin and to determine if the amount of antithrombin present in the plasma will affect that proportion.
- Determine if the degree of interference can be predicted based on the concentration of haemoglobin
- Establish a factor with which to obtain the corrected antithrombin value

The answers obtained in the study are as follows:

- The interference of haemoglobin in test plasma on antithrombin determination is not significant, both statistically and clinically at [Hb] $\leq$ 1.5 g/l.
- The interference of haemoglobin in test plasma starts showing statistical significance once the [Hb] exceeds 1.5g/l ($p < 0.0001$).
- The degree of interference is proportional to and correlates with, the haemoglobin concentration with $R^2 = 0.7939$ for AT 100 and $R^2 = 0.9355$ for AT 70.
- The degree of interference can be predicted by the use of an equation obtained from a regression analysis
    - The equation for the AT 100 plasma at [Hb] $\geq$1.5g/l was $y = -5.742 \times [Hb] + 115.24$
    - The equation for the AT 70 plasma at [Hb] $\geq$1.5g/l was $y = -4.2037 \times [Hb] + 66.821$,
    - There is not a clinically important difference between the results gained from either slope equation: thus the AT 100 slope equation can be applied.
    - A conversion table was also created and provides results very similar to either of the slope equations
- The correction factor can be applied to patients with plasma sample haemoglobin levels of 5.5g/l. There is no evidence to support or dispute linearity of the line, and thus the slope equation, beyond this value.

- The concentration of antithrombin in the test plasma does not seem to affect the degree of interference of the haemoglobin within the range tested as the slopes for each concentration were almost parallel.

6.2 RECOMMENDATIONS

The simplified form of the regression equation in this study is:

True value of AT = Read/lab value of AT- (slope co-efficient) x [Hb]

With the example of a patient result of AT = 60%, where the plasma [Hb] = 3gl;

Using slope AT 100 : True Value = AT 77.22 % = 77%

Using slope AT 70: True Value = AT 72.61% = 73%

Using an averaged slope co-efficient from both slopes i.e.

AT = Read / lab value of AT – **(- 4.9638)** x [Hb]

True value = AT 74.89% = 75%

This value for AT compares well with those calculated off each slope individually, viz. AT=73% for AT70 and 77% for AT100 as well as that read off the conversion table viz AT = 75%. It is thus evident that a equation utilising the averaged slope co-efficient would be sufficiently accurate to determine the AT concentration of plasma in the clinical setting.

These results show that it is possible to perform the AT assay in haemoglobinaemic serum with concentrations of up to 5.5g/l of haemoglobin and, utilizing a calculation, obtain the true value despite interference by the haemoglobin. This would allow this valuable test in the evaluation of coagulopathies to be utilised in patients with haemolysed serum.

Data from patients with babesiosis and IMHA has shown that a coagulopathy exists. This study opens up the way to further evaluation of the coagulation status in these patients.

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APPENDICES

Appendix 1

Modified Drabkins Method

Test Principle:

Haemoglobin is oxidized to methaemoglobin by ferricyanide and the methaemoglobin is converted to stable cyanomethaemoglobin by the addition of KCN (potassium cyanide). The absorbance of cyanomethaemoglobin is measured at 540nm, where it exihits a broad absorbance peak. The method is standardized against a standard cyanomethaemoglobin result.

Specimen:

A minimum of 0.2ml of whole blood is required (with / without EDTA)

Reagents:

Drabkins solution: dissolve in succession, 0.20g of K_3 $Fe(CN)_6$, 0.05g of KCN and 1.0g of $NaHCO_3$ in distilled water and dilute to 1000ml. The reagent must be stored in a dark bottle in the refrigerator. Do not freeze.

Cyanomethaemoglobin standard: (usually 80mg/100ml).

Procedure:

Adapted for the Technicon RA –100 System. Tarrytown, NY, USA.

Appendix 2: Manufacturers instructions: Boehringer Mannheim AT III

Antithrombin III

759 376

for max. 60 macroassays or 120 semi-microassays

Also required:
Sodium citrate (0.11 mol/l) Cat. No. 126 420
Sodium chloride (0.9%) Cat. No. 125 326

If necessary:
Precimat® Chromogen Cat. No. 1 012 045
Acetic acid (20%) or citric acid (10%)

Method
Röka, L. (1978). Antithrombin. Published in the series *"Diagnosis Today"*, Boehringer Mannheim

Test principle
AT III + heparin ——→ [AT III · heparin]
[AT III · heparin] + thrombin (excess) ——→
[AT III · heparin · thrombin] + residual thrombin
Tos-Gly-Pro-Arg-pNA + H_2O $\xrightarrow{\text{thrombin}}$ Tos-Gly-Pro-Arg-OH + p-nitroaniline

Normal range		**80–120%** (70–130)
	10–15 IU/ml	at 25°C
	20–29 IU/ml	at 37°C

Ref.: Hesse, R. et al. (1981) *Blut* 42, 227

Sample material
Citrated plasma

Quality control
PreciChrom® I/II

Contents of pack
1 Buffer (use as supplied)
6 bottles of 22 ml each (tris/hydrochloric acid/heparin/aprotinin/sodium chloride)
1a Thrombin
12 reagent tablets each containing 0.27 units of thrombin
2 Chromozym® TH
3 bottles of lyophilisate (Tos-Gly-Pro-Arg-pNA) for 3 x 4.0 ml

Preparation, stability and concentrations of reagents

For use at 25°C/30°C
Dissolve two thrombin tablets from bottle 1a in one bottle 1 under gentle swirling.

For use at 37°C
Dissolve one thrombin tablet from bottle 1a in one bottle 1 under gentle swirling.

Leave the reagent solutions to stand for 30 min at room temperature before use!
The solution is stable for one week at +15 to 25°C
one month at +2 to 8°C
three months at –20°C
(The solution can be frozen and thawed ten times.).

Concentrations: Tris/HCl: 100 mmol/l, pH 8.1; heparin: 2 USP-U/ml; aprotinin: 6.5 IU/ml; thrombin (25°C/30°C): 0.024 U/ml; thrombin (37°C): 0.012 IU/ml; NaCl: 140 mmol/l

Solution 2
Dissolve the contents of one bottle in 4.0 ml dist. water
Stable for 3 months at +2 to 8°C
Concentration: Tos-Gly-Pro-Arg-pNA AcOH: 1.9 mmol/l

Derivation of plasma
Mix 9 vol. of freshly drawn blood with 1 vol. of sodium citrate (0.11 mol/l) and centrifuge at ca. 2000 g (ca. 3000 rpm when using conventional lab centrifuges) for 10 min. Pipette off the supernatant.
The separated supernatant may be stored up to
48 hours at +4°C
1 month at –20°C.
Thaw frozen plasma for ca. 15 min at 25°C before starting the assay and mix thoroughly.

Preparation of samples
For assays run at 25°C or 30°C, dilute 1 vol. of plasma with 50 vol. of 0.9% NaCl (e.g. 20 µl plasma + 1 ml NaCl solution).
For assays run at 37°C, dilute 1 vol. of plasma with 100 vol. of 0.9% NaCl (e.g. 20 µl plasma + 2 ml NaCl solution).

Assay instructions for 2-point measurements

Procedure
Wavelength: Hg 405 nm (400–420 nm)
Cuvette: 1 cm light path
Temperature: 25°C/30°C/37°C
Measure against air (absorbance increase).
At least one thrombin blank (TB) is required per assay series.

Pipette into the bottom of plastic tubes:		
	TB	Sample
NaCl solution, 0.9%	0.10 ml	–
diluted plasma	–	0.10 ml
reagent solution	2.00 ml	2.00 ml
Mix and incubate for 5 min at 25°C, 30°C or 37°C. Add:		
solution 2	0.20 ml	0.20 ml
Mix immediately and start stopwatch at the same time; incubate for **exactly** 2 min at 25°C, 30°C or 37°C. Add:		
acetic acid, 20% or citric acid, 10%	1.00 ml	1.00 ml
Mix immediately and read the absorbances of TB and sample within 60 min. $A_{TB} - A_{sample} = \Delta A_{AT\ III}$		

If $\Delta A_{AT\ III}$ is less than 0.034 (= 3 IU/ml for assays at 25°C/30°C, and 6 IU/ml for assays at 37°C), it is recommended to repeat the assay using plasma diluted with only half the volume of NaCl solution (result x 0.51).

Calculation

	AT III [%]	AT III [IU/ml]
25°C	713 x $\Delta A_{AT\ III}$	87 x $\Delta A_{AT\ III}$
30°C	529 x $\Delta A_{AT\ III}$	87 x $\Delta A_{AT\ III}$
37°C	730 x $\Delta A_{AT\ III}$	171,5 x $\Delta A_{AT\ III}$

Please note
It is advisable to bring all solutions to assay temperature before starting the assay in order to ensure that the measurement temperature in the cuvette is actually 25°C/30°C/37°C.
As thrombin is adsorbed by glass, do not use glass pipettes to pipette the reagent solution, and store the reagent solution only in the original bottle. If measurements cannot be made at 405 nm, use Precimat® Chromogen to perform measurements.

$$\frac{\Delta A_{sample}}{\Delta A_{Precimat^{®}\ Chromogen}} \times \text{asigned value}_{Precimat^{®}\ Chromogen} = \text{AT III [\%] or [IU/ml]}$$

Semi-microassays can be run by using half the volumes specified; the calculation factor remains the same. Special instructions for assays on **automatic analyzers** are available on request.

Interference
The following intefere with the 2-point assay:
– Lipemic plasma
– Strongly icteric plasma (for a normal plasma, a bilirubin level above 20 mg/dl introduces an error of ca. 6%)
– Hemolytic plasma (AT III values are depressed by ca. 6% at a hemoglobin concentration of 0.15 g/dl)
The kinetic assay is not affected in any of these cases.

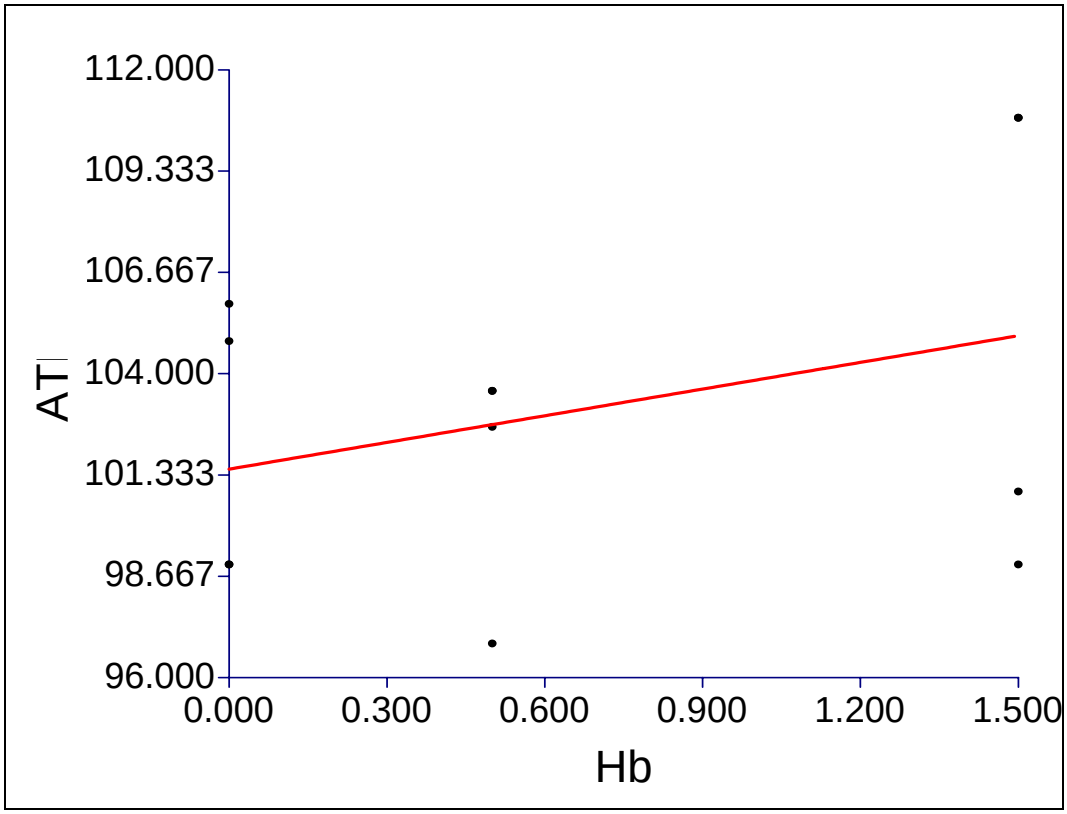
BOEHRINGER MANNHEIM

Appendix 3

Two step regression Analysis: original graphs

A) Statistical calculations for AT 100

Graph 1: The regression line from [Hb] 0g/l to 1.5g/l with AT 100



Run Summary Section

Parameter	Value	Parameter	Value
Dependent Variable	ATIII	Rows Processed	84
Independent Variable	Hb	Rows Used in Estimation	12
Frequency Variable	None	Rows with X Missing	0
Weight Variable	None	Rows with Freq Missing	0
Intercept	101.4907	Rows Prediction Only	0
Slope	2.3414	Sum of Frequencies	12
R-Squared	0.1152	Sum of Weights	12.0000
Correlation	0.3393	Coefficient of Variation	0.0430
Mean Square Error	19.65906	Square Root of MSE	4.433853

Appendix 3 cont…

Linear Regression Report
Y = ATIII X = Hb

Summary Statement
The equation of the straight line relating ATIII and Hb is estimated as: ATIII = (101.4907) + (2.3414) Hb using the 12 observations in this dataset. The y-intercept, the estimated value of ATIII when Hb is zero, is 101.4907 with a standard error of 1.8736. The slope, the estimated change in ATIII per unit change in Hb, is 2.3414 with a standard error of 2.0525. The value of R-Squared, the proportion of the variation in ATIII that can be accounted for by variation in Hb, is 0.1152. The correlation between ATIII and Hb is 0.3393.

A significance test that the slope is zero resulted in a t-value of 1.1408. The significance level of this t-test is 0.2805. Since 0.2805 > 0.0500, the hypothesis that the slope is zero is not rejected.

The estimated slope is 2.3414. The lower limit of the 95% confidence interval for the slope is -2.2318 and the upper limit is 6.9146. The estimated intercept is 101.4907. The lower limit of the 95% confidence interval for the intercept is 97.3160 and the upper limit is 105.6655.

Descriptive Statistics Section

Parameter	**Dependent**	**Independent**
Variable	ATIII	Hb
Count	12	12
Mean	103.0517	0.6667
Standard Deviation	4.4942	0.6513
Minimum	96.9000	0.0000
Maximum	110.7400	1.5000

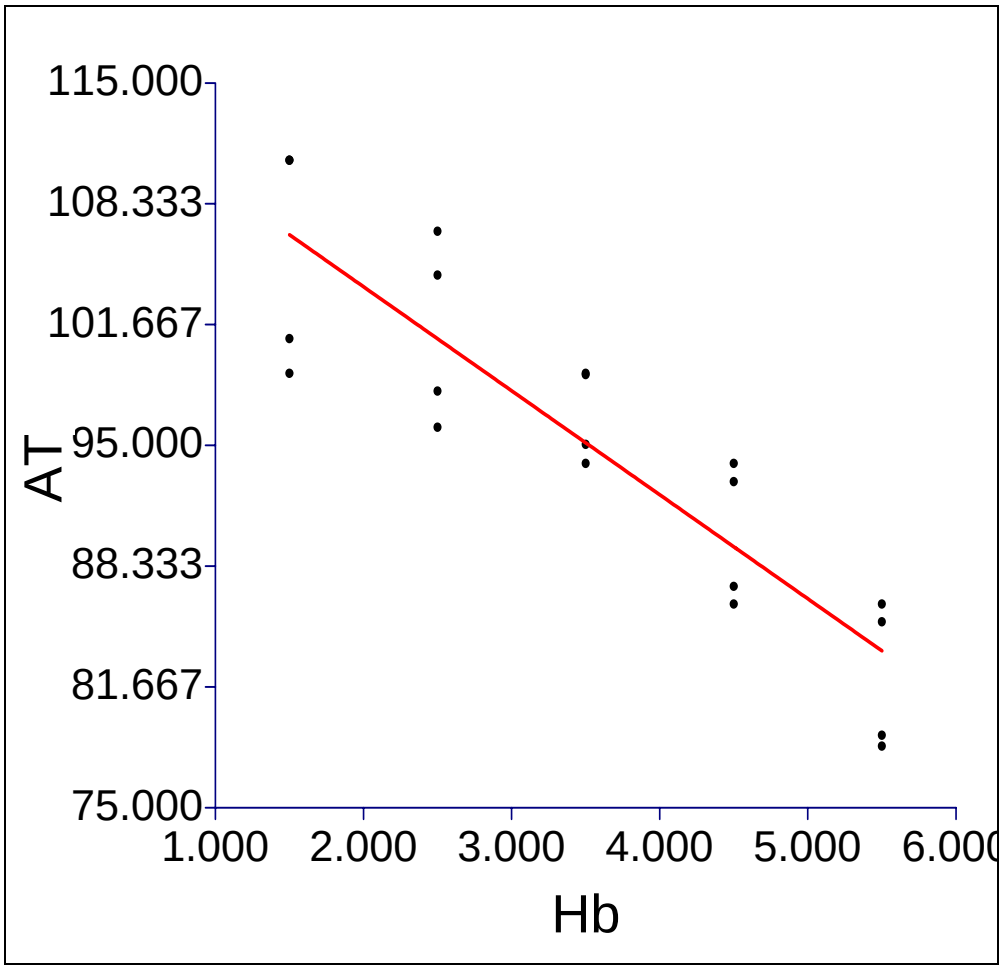
Regression Estimation Section

Parameter	**Intercept B(0)**	**Slope B(1)**
Regression Coefficients	101.4907	2.3414
Lower 95% Confidence Limit	97.3160	-2.2318
Upper 95% Confidence Limit	105.6655	6.9146
Standard Error	1.8736	2.0525
Standardized Coefficient	0.0000	0.3393
T Value	54.1675	1.1408
Prob Level (T Test)	0.0000	0.2805
Reject H0 (Alpha = 0.0500)	Yes	No
Power (Alpha = 0.0500)	1.0000	0.1786
Regression of Y on X	101.4907	2.3414
Inverse Regression from X on Y	89.4962	20.3332
Orthogonal Regression of Y and X	89.7475	19.9563

Estimated Model
(101.490714285714) + (2.34142857142854) * (Hb)

Appendix 3 (cont)

Graph2: Linear regression [Hb] ≥ 1.5g/l ≤5.5 g/l with AT 100



Run Summary Section

Parameter	Value	Parameter	Value
Dependent Variable	ATIII	Rows Processed	84
Independent Variable	Hb	Rows Used in Estimation	20
Frequency Variable	None	Rows with X Missing	0
Weight Variable	None	Rows with Freq Missing	0
Intercept	115.2410	Rows Prediction Only	0
Slope	-5.7420	Sum of Frequencies	20
R-Squared	0.7939	Sum of Weights	20.0000
Correlation	-0.8910	Coefficient of Variation	0.0458
Mean Square Error	19.02588	Square Root of MSE	4.361867

Appendix 3 cont….

Linear Regression Report
Y = ATIII X = Hb

Summary Statement
The equation of the straight line relating ATIII and Hb is estimated as: ATIII = (115.2410) + (-5.7420) Hb using the 20 observations in this dataset. The y-intercept, the estimated value of ATIII when Hb is zero, is 115.2410 with a standard error of 2.6035. The slope, the estimated change in ATIII per unit change in Hb, is -5.7420 with a standard error of 0.6897. The value of R-Squared, the proportion of the variation in ATIII that can be accounted for by variation in Hb, is 0.7939. The correlation between ATIII and Hb is -0.8910.

A significance test that the slope is zero resulted in a t-value of -8.3257. The significance level of this t-test is 0.0000. Since 0.0000 < 0.0500, the hypothesis that the slope is zero is rejected.

The estimated slope is -5.7420. The lower limit of the 95% confidence interval for the slope is -7.1909 and the upper limit is -4.2931. The estimated intercept is 115.2410. The lower limit of the 95% confidence interval for the intercept is 109.7713 and the upper limit is 120.7107.

Descriptive Statistics Section

Parameter	**Dependent**	**Independent**
Variable	ATIII	Hb
Count	20	20
Mean	95.1440	3.5000
Standard Deviation	9.3507	1.4510
Minimum	78.4000	1.5000
Maximum	110.7400	5.5000

Regression Estimation Section

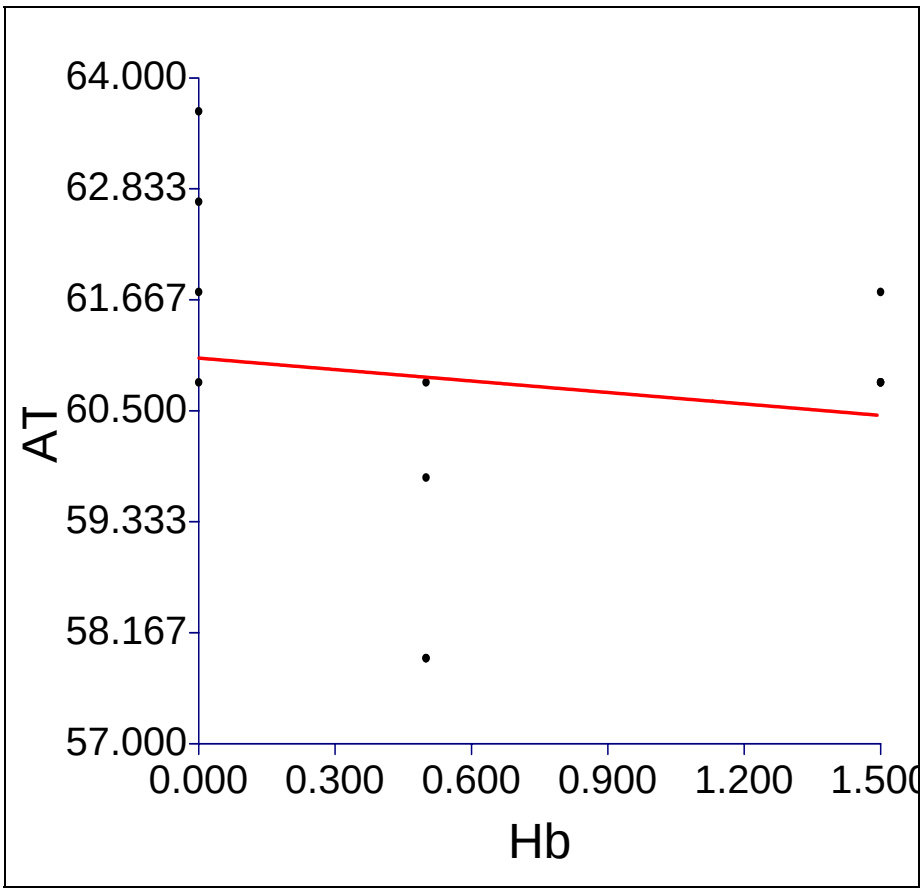
Parameter	**Intercept B(0)**	**Slope B(1)**
Regression Coefficients	115.2410	-5.7420
Lower 95% Confidence Limit	109.7713	-7.1909
Upper 95% Confidence Limit	120.7107	-4.2931
Standard Error	2.6035	0.6897
Standardized Coefficient	0.0000	-0.8910
T Value	44.2647	-8.3257
Prob Level (T Test)	0.0000	0.0000
Reject H0 (Alpha = 0.0500)	Yes	Yes
Power (Alpha = 0.0500)	1.0000	1.0000
Regression of Y on X	115.2410	-5.7420
Inverse Regression from X on Y	120.4597	-7.2331
Orthogonal Regression of Y and X	120.3364	-7.1978

Estimated Model
(115.241) + (-5.742) * (Hb)

Appendix 3 cont…

B) Statistical calculations for AT 70%

Graph 3: Regression analysis between [Hb] 0g/l and [Hb] 1.5 g/l for AT 70



Run Summary Section

Parameter	Value	Parameter	Value
Dependent Variable	ATIII	Rows Processed	84
Independent Variable	Hb	Rows Used in Estimation	12
Frequency Variable	None	Rows with X Missing	0
Weight Variable	None	Rows with Freq Missing	0
Intercept	61.0554	Rows Prediction Only	12
Slope	-0.4018	Sum of Frequencies	12
R-Squared	0.0240	Sum of Weights	12.0000
Correlation	-0.1550	Coefficient of Variation	0.0288
Mean Square Error	3.060228	Square Root of MSE	1.749351

Appendix 3 cont….

Linear Regression Report
Y = ATIII X = Hb

Summary Statement
The equation of the straight line relating ATIII and Hb is estimated as: ATIII = (61.0554) + (-0.4018) Hb using the 12 observations in this dataset. The y-intercept, the estimated value of ATIII when Hb is zero, is 61.0554 with a standard error of 0.7392. The slope, the estimated change in ATIII per unit change in Hb, is -0.4018 with a standard error of 0.8098. The value of R-Squared, the proportion of the variation in ATIII that can be accounted for by variation in Hb, is 0.0240. The correlation between ATIII and Hb is -0.1550.

A significance test that the slope is zero resulted in a t-value of -0.4962. The significance level of this t-test is 0.6305. Since 0.6305 > 0.0500, the hypothesis that the slope is zero is not rejected.

The estimated slope is -0.4018. The lower limit of the 95% confidence interval for the slope is -2.2061 and the upper limit is 1.4025. The estimated intercept is 61.0554. The lower limit of the 95% confidence interval for the intercept is 59.4082 and the upper limit is 62.7025.

Descriptive Statistics Section

Parameter	**Dependent**	**Independent**
Variable	ATIII	Hb
Count	12	12
Mean	60.7875	0.6667
Standard Deviation	1.6883	0.6513
Minimum	57.9000	0.0000
Maximum	63.6500	1.5000

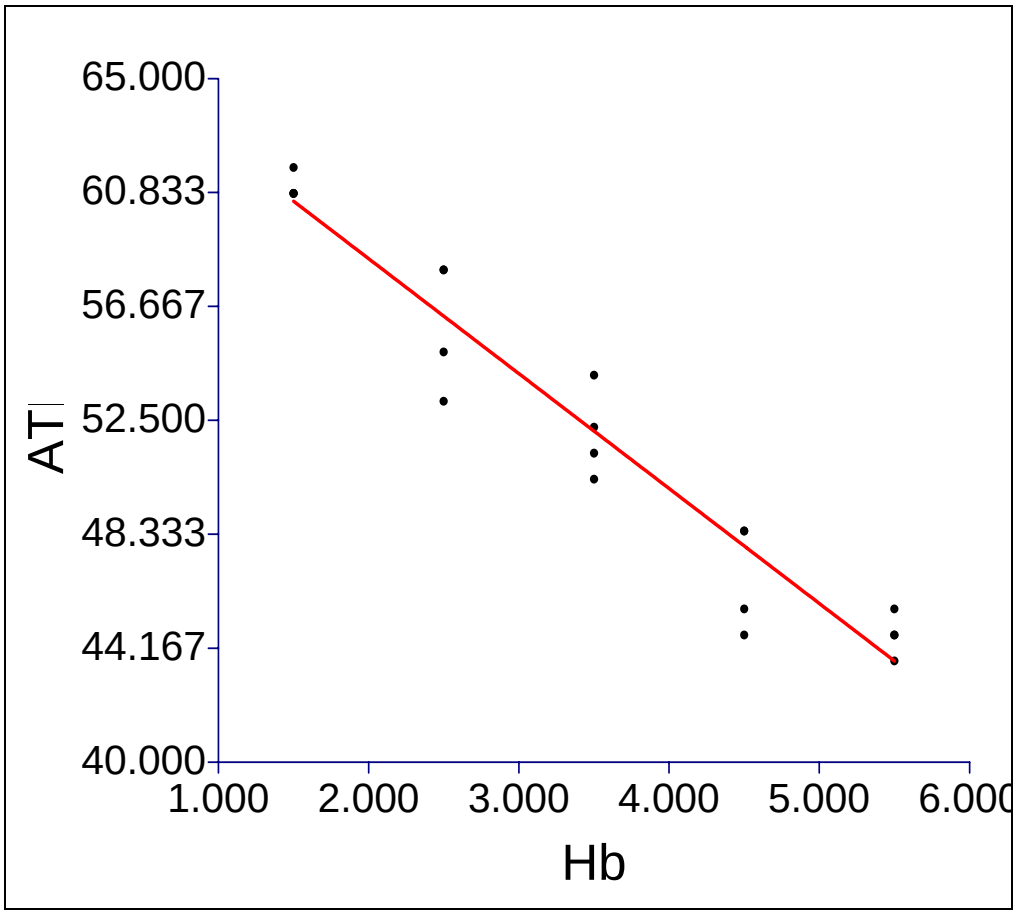
Regression Estimation Section

Parameter	**Intercept B(0)**	**Slope B(1)**
Regression Coefficients	61.0554	-0.4018
Lower 95% Confidence Limit	59.4082	-2.2061
Upper 95% Confidence Limit	62.7025	1.4025
Standard Error	0.7392	0.8098
Standardized Coefficient	0.0000	-0.1550
T Value	82.5926	-0.4962
Prob Level (T Test)	0.0000	0.6305
Reject H0 (Alpha = 0.0500)	Yes	No
Power (Alpha = 0.0500)	1.0000	0.0735
Regression of Y on X	61.0554	-0.4018
Inverse Regression from X on Y	71.9362	-16.7230
Orthogonal Regression of Y and X	70.3235	-14.3040

Estimated Model
(61.0553571428571) + (-.401785714285714) * (Hb)

Appendix 3 cont…

Graph 4: Linear regression [Hb] ≥ 1.5g/l ≤ 5.5 g/l for AT 70



Run Summary Section

Parameter	Value	Parameter	Value
Dependent Variable	ATIII	Rows Processed	84
Independent Variable	Hb	Rows Used in Estimation	20
Frequency Variable	None	Rows with X Missing	0
Weight Variable	None	Rows with Freq Missing	0
Intercept	66.8206	Rows Prediction Only	20
Slope	-4.2037	Sum of Frequencies	20
R-Squared	0.9355	Sum of Weights	20.0000
Correlation	-0.9672	Coefficient of Variation	0.0316
Mean Square Error	2.706156	Square Root of MSE	1.64504

Appendix 3 cont…

Linear Regression Report
Y = ATIII X = Hb

Summary Statement
The equation of the straight line relating ATIII and Hb is estimated as: ATIII = (66.8206) + (-4.2037) Hb using the 20 observations in this dataset. The y-intercept, the estimated value of ATIII when Hb is zero, is 66.8206 with a standard error of 0.9819. The slope, the estimated change in ATIII per unit change in Hb, is -4.2037 with a standard error of 0.2601. The value of R-Squared, the proportion of the variation in ATIII that can be accounted for by variation in Hb, is 0.9355. The correlation between ATIII and Hb is -0.9672.

A significance test that the slope is zero resulted in a t-value of -16.1618. The significance level of this t-test is 0.0000. Since 0.0000 < 0.0500, the hypothesis that the slope is zero is rejected.

The estimated slope is -4.2037. The lower limit of the 95% confidence interval for the slope is -4.7502 and the upper limit is -3.6573. The estimated intercept is 66.8206. The lower limit of the 95% confidence interval for the intercept is 64.7578 and the upper limit is 68.8835.

Descriptive Statistics Section

Parameter	**Dependent**	**Independent**
Variable	ATIII	Hb
Count	20	20
Mean	52.1075	3.5000
Standard Deviation	6.3061	1.4510
Minimum	43.7000	1.5000
Maximum	61.7500	5.5000

Regression Estimation Section

Parameter	**Intercept B(0)**	**Slope B(1)**
Regression Coefficients	66.8206	-4.2037
Lower 95% Confidence Limit	64.7578	-4.7502
Upper 95% Confidence Limit	68.8835	-3.6573
Standard Error	0.9819	0.2601
Standardized Coefficient	0.0000	-0.9672
T Value	68.0545	-16.1618
Prob Level (T Test)	0.0000	0.0000
Reject H0 (Alpha = 0.0500)	Yes	Yes
Power (Alpha = 0.0500)	1.0000	1.0000
Regression of Y on X	66.8206	-4.2037
Inverse Regression from X on Y	67.8345	-4.4934
Orthogonal Regression of Y and X	67.7834	-4.4788

Estimated Model
(66.820625) + (-4.20374999999999) * (Hb)