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**Studies exploring the potential use of
Serum Amyloid A (SAA) and other equine
acute phase proteins for the
investigation, monitoring and
prognostication of disease in horses**



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A thesis submitted for the degree of *Doctor of Philosophy* (Ph.D.)

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Abstract

A variety of inflammatory markers, coupled with changes in a number of haematological and biochemical parameters have classically been used to diagnose, monitor, and prognosticate disease in horses. Unfortunately these traditional markers respond fairly slowly to the presence of disease and inflammation and have wide normal ranges (Allen and Cold 1988; Pepys *et al.*, 1989). Serum amyloid A (SAA), and haptoglobin are acute phase proteins common to humans, cattle, sheep, mice, and several other species, including the horse. In several of these species, plasma concentration of SAA has been shown to increase 1000-fold following tissue injury, cellular necrosis, inflammation, and infection and decline rapidly in the recovery phase.

In the studies presented here the concentration of serum amyloid A (SAA), haptoglobin and fibrinogen and other indices of health were measured in a number of different groups of horses, including; normal horses, those subjected to operative surgery, horses with surgical colic, racehorses in training, some of which had evidence of gastric ulceration, and foals with respiratory disease. Acute phase protein concentration was modeled with the outcome and with other commonly measured indices of health relevant to the disease states of interest.

The study indicates that there is an association between acute and chronic inflammation and between the present of disease both overt and latent and suggests that the concentration of a number of acute phase proteins could be used to aid decision making when planning diagnostic or treatment interventions in horses.

Acknowledgements

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I am indebted to Jean Hearn, Des Leadon and Ursula Fogarty of the Irish Equine Centre for their help with the laboratory work and for the use of their facilities. I would also like to acknowledge Professor Chris Bellenger, Doctor Maureen Prendergast and Professor Pia Andersen for helping to conceive the initial ideas for the project and for supporting the many incarnations of the work.

I must recognise and thank Professor Max Murray, without whom the work would simply never have been completed, I count myself lucky to be among the many to have benefited from his enthusiasm, wit, intellect and compassion. I would also like to acknowledge Professor Tony Davies for critically assessing the project and encouraging me to resubmit. The help of Professor Sir Mark Pepys, who first described the use of acute phase proteins in clinical medicine provided valuable insights into the use of APPs in the human clinical setting and was invaluable.

Finally I would like to thank my family, my wife Kristina, our children Finnian, and Maili and my parents, Maurice and Kate, for their patience, encouragement and unwavering support over the many years that this project has taken to come to fruition.

Table of Contents

Studies exploring the potential use of Serum Amyloid A (SAA) and other equine acute phase proteins for the diagnosis, monitoring and prognostication of disease in horses.....	I
Abstract.....	II
Acknowledgements.....	III
Table of Contents.....	IV
List of Tables.....	XIII
List of Figures.....	XV
Presentations and Publications.....	XIX
Declaration.....	XX
List of Abbreviations.....	XXI
 1.0 Chapter One- Introduction and literature review.....	 1
1.1 Introduction.....	2
1.1.1 The contrast to human medicine.....	3
1.2 The use of acute phase proteins in clinical medicine and veterinary medicine.....	3
1.2.1 The acute phase response, acute phase proteins, synthesis and regulation.....	5
1.2.2 Fibrinogen.....	8
1.2.3 Serum Amyloid A- History, structure and isoforms.....	10
1.2.3.1 Synthesis & regulation of serum amyloid A (SAA)....	10
1.2.3.2 Function of Serum Amyloid A (SAA).....	12
1.2.3.3 Clinical use of Serum Amyloid A(SAA) in the horse...	12
1.2.4 Haptoglobin structure, synthesis and regulation.....	12
1.2.5. Acute phase proteins as prognostic indicators.....	13
1.3 Stress inflammation and disease.....	15
1.3.1 Where are we today?.....	17
1.3.2 Defining stress and inflammation, and its consequences in the horse.....	17
1.3.3 Physiology of the inflammatory and stress response.....	18

1.3.4 Benefits of inflammation and stress.....	20
1.3.5 Methods used to identify stress and inflammation, and associated haematological and biochemical changes	21
1.4 Evaluating diagnostic tests.....	22
1.5 Choosing horses and clinical syndromes in which to evaluate acute phase proteins.....	23
1.6 Measuring the response to surgery and the effect of disease in the horse.....	24
1.7 Equine surgical and non-surgical gastrointestinal disease (colic).....	24
1.7.1 Making the decision for intervention and determining outcome in horses affected with colic.....	25
1.8 The equine athlete-training in horses.....	26
1.9 Gastric ulceration in the horse.....	27
1.9.1 Anatomy of the equine stomach.....	28
1.9.2 Ulceration in the equine stomach.....	28
1.9.3 Prevalence and aetiology of gastric ulceration.....	29
1.9.4 Diagnosis of Gastric Ulceration.....	29
1.9.5 Treatment of Gastric Ulceration.....	30
1.9.6 Acute phase proteins and gastric ulceration.....	30
1.10 <i>Rodococcus equi</i> in Thoroughbred foals.....	31
1.10.1 Pathogenesis and virulence of <i>Rodococcus equi</i>	31
1.10.2 Development of disease and clinical signs.....	32
1.10.3 Susceptibility of the foal compared to other species.....	32
1.10.4 Diagnosis and treatment of <i>Rodococcus equi</i>	33
1.10.5 Why do we need to do better with early identification of infected foals?	33
1.11 Patient side acute phase protein testing.....	34
1.12 Summary, objectives and hypothesis.....	34
2.0 Chapter two-General materials and methods.....	36
2.1 Introduction.....	37
2.2 Animals.....	37
2.2.1 Group One - 50 Clinically normal, Thoroughbred horses.....	37
2.2.2 Group 2 - 25 horses presented for a variety of surgical procedures.....	38

2.2.3 Group 3 - 59 horses presented for the investigation and treatment of colic.....	38
2.2.4 Group 4 - 100 racehorses in training.....	38
2.2.5 Group 5 - Thoroughbred foals exposed to, and subsequently naturally infected with <i>Rhodococcus equi</i>	39
2.3 Anaesthetic Protocol.....	39
2.4 Techniques.....	40
2.4.1 Blood sampling.....	40
2.4.2 Sampling details	41
2.4.3 Routine haematology and biochemical analysis.....	41
2.4.4 Acute phase protein assays.....	41
2.4.4.1 Serum Amyloid A Assay.....	41
2.4.4.2 Haptoglobin assay.....	43
2.4.4.3 Fibrinogen Assay.....	45
2.5 Gastric endoscopy.....	46
2.6 <i>Rhodococcus equi</i> diagnostics.....	51
2.6.1 Bronchoalveolar lavage technique.....	51
2.6.2 Cytology and culture of <i>Rhodococcus equi</i>	51
2.7 Statistical Analysis.....	54
2.7.1 One-way analysis of variance (ANOVA) (Chapter 3).....	54
2.7.2 Logistic regression (Chapters 5 and 6).....	54
2.7.2.1 Clustering of data.....	56
2.7.3 ROC curves and predictive ability (Chapters 5 and 6).....	56
2.7.4 Mann Whitney u Test (Chapters 4, 6 and 7).....	57
2.7.5 <i>Post Hoc</i> Power calculations - 2 Sample t test.....	57
2.7.6 Kruskal-Wallis (Chapter, 3 and 6).....	58
2.7.7 Wilcoxin signed-rank test.....	58
3.0 Chapter three- Acute phase proteins, haematological and clinical biochemistry in Thoroughbred horses, a “snapshot” of the normal horse.....	59
3.1 Introduction.....	60
3.1.1 Variation in SAA measurement.....	61
3.2 Materials and methods.....	62
3.2.1 Ethics.....	62
3.2.2 Experimental design.....	62

3.2.3 Sample collection.....	62
3.2.3.1 Acute phase proteins.....	63
3.2.3.2 Haematological examination.....	63
3.2.4 Clinical examination.....	63
3.2.5 Data processing and statistical methods.....	63
3.2.5.1 Data processing.....	63
3.2.5.2 Statistical methods.....	64
3.2.5.2.1 Analysis of variance (ANOVA).....	64
3.2.5.2.2 Kruskal-Wallis analysis.....	64
3.2.5.3 Normality testing.....	64
3.2.5.4 Power calculations.....	64
3.3 Results.....	65
3.3.1 Initial review of data.....	65
3.3.1.1 Method.....	65
3.3.2 Normality testing.....	68
3.3.3 Statistical analysis and summary.....	68
3.4 Discussion.....	72
 4.0 Chapter four- The Effect of elective and non-elective surgery on the acute phase response in horses.....	 76
4.1 Introduction.....	77
4.1.1 Searching for the optimum biomarker.....	77
4.1.2 Acute phase proteins as biomarkers.....	78
4.2 Materials and methods.....	79
4.2.1 Study animals, case selection and experimental design.....	79
4.2.2 Ethics.....	79
4.2.3 Group 1.....	79
4.2.4 Group 2.....	79
4.2.5 Surgical procedures and anaesthetic protocol.....	79
4.2.6 Sample collection.....	80
4.2.6.1 Acute phase proteins.....	80
4.2.6.2 Haematological and clinical biochemical examination.....	83
4.2.7 Clinical examination.....	83
4.2.8 Data processing and statistical methods.....	83

4.3 Results.....	84
4.3.1 Acute phase proteins.....	84
4.3.1.1 Group 1. Elective surgery.....	84
4.3.1.2 Group 2. Non-elective surgery.....	85
4.3.1.3 Comparison between elective and non-elective surgery.....	88
4.3.1.4 Acute phase protein kinetics- Summary.....	88
4.3.1.5 Haematology.....	89
4.3.1.6 Clinical biochemistry.....	90
4.3.2 <i>Post hoc</i> power calculations.....	90
4.4 Discussion.....	94
4.4.1 Introduction.....	94
4.4.2 Developing the test, using SAA concentration in surgical practice.....	95
4.4.3 Limitations and further investigation.....	96

5.0 Chapter Five - Surgical colic and the acute phase response in horses

Can we improve clinical decision making at admission?	100
5.1 Introduction.....	101
5.1.1 Developing a clinical test.....	102
5.1.2 Summary and aims.....	103
5.2 Materials and methods.....	103
5.2.1 Experimental design.....	103
5.2.2 Ethics.....	103
5.2.3 Study animals.....	104
5.2.3.1 Case selection.....	104
5.2.4 Sampling procedures.....	104
5.2.5 Anaesthesia.....	104
5.2.6 Data processing.....	105
5.2.7 Statistical methods.....	105
5.2.7.1 Univariable analysis.....	105
5.2.7.2 Multivariable analysis and model building.....	106
5.2.7.3 Receiver operating characteristic curves (ROC).....	106
5.2.7.4 Goodness of fit testing.....	107
5.2.7.5 <i>Post hoc</i> sample size calculations.....	107

5.3 Results.....	113
5.3.1 Group 1. Horses subjected to surgery which were subsequently discharged from the hospital.....	113
5.3.2 Group 2 horses that died or were euthanised.....	114
5.3.3 Graphical comparisons between groups	114
5.3.4 Statistical modelling.....	123
5.3.4.1 Horses which survived to be discharged versus horses which died or where euthanised (59 observations).....	123
5.3.5 Goodness of fit testing.....	126
5.3.6 <i>Post hoc</i> sample size calculations.....	126
5.4 Discussion.....	129
5.4.1 Review of findings.....	129
5.4.2 Comparison of these findings with previous work.....	130
5.4.3 Limitations and factors that may have influenced the results.....	130
5.4.4 What the clinician requires - Statistical significance vs biological significance.....	132
5.4.5 Future work.....	133
5.4.6 Conclusion.....	134
 6.0 Chapter Six- The effect of gastric ulceration on the acute phase response - Chronicity and the acute phase response.....	 135
6.1 Introduction.....	136
6.1.1 Pathophysiology of gastric ulceration.....	137
6.1.2 Mechanisms of gastric healing.....	138
6.1.3 Summary and aims.....	138
6.2 Materials and methods.....	139
6.2.1 Study animals.....	139
6.2.2 Ethics.....	139
6.2.3 Sampling procedures.....	140
6.2.4 Gastroscopy.....	140
6.2.5 Data processing.....	141
6.2.5.1 Statistical methods.....	141
6.2.5.2 Mann Whitney U test	141

6.2.5.3 Wilcoxin signed rank test.....	141
6.2.5.4 Univariable analysis logistic regression.....	142
6.2.5.5 Multivariable analysis and model building.....	142
6.2.5.6 Goodness of fit testing.....	143
6.2.5.7 Receiver operating characteristic curves (ROC).....	143
6.2.5.8 Sample size calculation.....	143
6.3 Results.....	145
6.3.1 Overview of gastroscopic findings.....	145
6.3.2 Gastric pathology.....	145
6.3.3 Supplementary clinical examination results.....	146
6.3.4 Acute phase protein results.....	146
6.3.5 Initial review of data.....	148
6.3.5.1 Method.....	148
6.3.6 Statistical association between explanatory and outcome variables.....	151
6.3.7 Further hypotheses.....	151
6.3.8 Model building.....	155
6.3.9 Receiver operating characteristic curves (ROC).....	162
6.3.10 Power calculations.....	165
6.4 Discussion.....	165
6.4.1 Summary of findings.....	165
6.4.2 Relevance to previous and on-going work.....	166
6.4.3 Factors that may have influenced results.....	167
6.4.4 Performance of models.....	168
6.4.5 Conclusion and future hypotheses.....	169
7.0 Chapter 7- Acute Phase Proteins: Detection of Sub-Clinical Infectious Disease.....	170
7.1 Introduction.....	171
7.1.1 Background to the study.....	172
7.2 Materials and methods.....	173
7.2.1 Experimental design and study animals.....	173
7.2.2 Ethics.....	174
7.2.3 Sampling procedures.....	174
7.2.3.1 Sample collection.....	174

7.2.3.2 Acute phase proteins.....	174
7.2.3.3 Haematological examination.....	175
7.2.3.4 Clinical examination.....	175
7.2.4 Diagnostic techniques for the detection of <i>Rhodococcus equi</i>	175
7.2.4.1 Bronchoalveolar lavage.....	175
7.2.4.2 Microbial culture.....	175
7.2.5 Data Processing and statistical methods.....	176
7.2.5.1 Data processing.....	176
7.2.5.2 Statistical methods.....	176
7.2.5.2.1 Normality testing.....	176
7.3 Results.....	176
7.3.1 Clinical findings.....	176
7.3.2 Acute phase protein profile.....	177
7.3.3 Haematology results.....	177
7.3.4 Graphical review of data.....	177
7.3.5 Statistical analysis and conclusions.....	180
7.4 Discussion.....	184
7.4.1 Developing the test, using SAA and other acute phase proteins in clinical practice.....	186
7.4.2 Limitations and further investigation.....	186
7.4.3 Conclusions and way forward.....	188
8.0 Chapter eight - General discussion.....	189
8.1 Introduction and Summary of Findings.....	190
8.2 The relationship between these studies and previous work.....	192
8.3 Factors that may have affected the outcomes and conclusions presented.....	193
8.4 Future and ongoing work - The way ahead.....	194
8.5 Mapping the results of the work presented onto the aims and general hypothesis as stated in Chapter one.....	195
8.6 Conclusion.....	197
9.0 Chapter nine - Bibliography.....	198
Appendices.....	242

1. Signalment and details for the normal horses used in the study.....243
2. Signalment and details for the 100 Thoroughbred horses in training
.....244
3. Pollock PJ et al., 2005. Effects of surgery on the acute phase response
in clinically normal and diseased horses. Vet Rec 156:538-542

List of Tables

Table 1.1 Diseases, injuries (by body system) and disease states in which SAA concentration has been noted to rise in horses.....	6
Table 1.2 Known functions of the major acute phase proteins.....	14
25	
Table 2.1 Working Standards and Volume of Diluent used in the SAA Assay.....	44
Table 2.2 Scoring System for Gastric Ulceration Used in the 100 Thoroughbred Horses in Training.....	48
Table 3.1. Acute Phase Protein Profile for the General Population, Foals, Young Adults and Adults in Group 1.....	66
Table 4.1. 18 Horses presented for Elective Surgery.....	81
Table 4.2. Seven Horses Presented for Non-elective Surgery.....	82
Table 4.3. Group 1-Elective Surgery. Median results for Acute Phase Protein Concentrations at each time point	86
Table 4.4. Group 2- Non-Elective Surgery. Median results for Acute Phase Protein Concentrations at each time point	91
Table 5.1. Signalment, Diagnosis and Surgical Procedure Performed on the 21 Horses in Group 1.....	108
Table 5.2. Signalment, diagnosis, clinic and reason for euthanasia for the 38 horses in group 2.....	110
Table 5.3. Results of analytes collected at admission for horses in Group 1 (survivors).....	115
Table 5.4. 4 Results of analytes collected at admission for horses in Group 2 (non-survivors).....	116
Table 5.5. Results for the univariable and multivariable analyses for each of the explanatory variables.....	124
Table 5.6 Excerpt from a full report of Sensitivity and Specificity (Group 2 vs	
Table 6.1. The Scoring System used for Gastric Ulceration in Horses.....	144
Table 6.2. Gastric Ulceration Findings Over Time.....	145
Table 6.3 Acute Phase Protein Results and Ulcer Scores in 100 Thoroughbred Horses on 3 Occasions over 6 weeks.....	149
Table 6.4 Median SAA results and ulcer scores for each time point for horses affected by clinical levels of gastric ulceration (gastric ulcer score >4) and for horses not affected by gastric ulceration.....	150

Table 6.5 Single-level, multivariable logistic regression model describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus.....	157
Table 6.6 Two-level, multivariable logistic regression model describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus.....	158
Table 6.7 Two-level, multivariable logistic regression model with yard fitted as a random effect, describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus	159
Table 6.8a Two-level, multivariable logistic regression model with yard fitted as a random effect, describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus	160
Table 6.8b Alternative analysis, three-level, multivariable logistic regression model describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus.....	161
Table 7.1. Results for APPs and selected haematological parameters for the foals in groups 1 and 2.....	179

List of Figures

Figure 1.1 The Acute Phase Response in Mammals.....	9
Figure 2.1a Schematic Showing the Technique for Examination of the Stomach.....	47
Figure 2.1b Gastroscopic image showing the endoscope retroflexed in the stomach in order to image the pyloric region.....	47
Figure 2.2 Gastroscopic image of a normal stomach showing the margo plicatus, glandular and squamous regions.....	49
Figure 2.3a and b. Gastroscopic images of a stomach with Grade 4 ulceration...	49
Figure 2.4 Gastroscopic image showing moderate ulceration (Grade 6).....	50
Figure 2.5a and b. Gastroscopic images showing severe ulceration around the pylorus (Grade 8).....	50
Figure 2.6 BAL cytology showing macrophages containing multiple cocci-shaped bacteria.....	53
Figure 2.7 Agar plate showing growth on sheep blood agar at 72 hours.....	53
Figure 3.1. Mean Concentration for the Acute Phase Proteins	67
Figure 3.2 Box plot of SAA concentration for the three groups of normal horses	69
Figure 3.3 Box plot of Haptoglobin concentration for the three groups of normal horses.....	69
Figure 3.4 Box plot of Fibrinogen concentration for the three groups of normal horses.....	70
Figure 3.5 Box plot SAA concentration comparing males and females.....	70
Figure 3.6 Box plot of haptoglobin concentration comparing males and females	71
Figure 3.7 Box plot of fibrinogen concentration comparing males and females .	71
Figure 4.1 Box plot showing SAA concentration in the elective surgery group at each time point	86
Figure 4.2 Box plot showing haptoglobin concentration in the elective surgery group at each time point.....	87
Figure 4.3 Box plot showing Fibrinogen concentration in the elective surgery group at each time point.....	87
Figure 4.4 Box plot showing SAA concentration in the non-elective surgery group at each time point	91
Figure 4.5 Box plot showing haptoglobin concentration in the non-elective surgery group at each time point	92

Figure 4.6 Box plot showing Fibrinogen concentration in the non-elective surgery group at each time point	92
Figure 4.7 Graph showing SAA concentration in a horse subjected to elective surgery without complications.....	93
Figure 4.8 Graph showing SAA concentration in a horse subjected to elective surgery with complications.....	93
Figure 4.9 Graph showing SAA concentration in a horse subjected to non-elective surgery without complications.....	93
Figure 4.10 Graph showing SAA concentration in a horse subjected to non-elective surgery without complications.....	93
Figure 5.1. Box plot showing SAA Concentration at each time point for the group 1 horses that survived to discharge.....	117
Figure 5.2. Box plot showing haptoglobin concentration at each time point for the group 1 horses that survived to discharge.....	117
Figure 5.3 Box plot showing Fibrinogen Concentration at each time point for the Group 1 horses that survived to dischar.....	118
Figure 5.4 Figure 5.4 Box plot showing HR at each time point for the Group 1 horses that survived to discharge.....	119
Figure 5.5. Box plot showing PCV at each time point for the Group 1 horses that survived to discharge.....	119
Figure 5.6 Box plot showing TP Concentration at each time point for the Group 1 horses that survived to discharge.....	119
Figure 5.7 Box plot showing SAA concentration for the horses in group 1 and group 2.....	120
Figure 5.8 Box plot showing haptoglobin concentration for the horses in group 1 and group 2.....	120
Figure 5.9 Box plot showing fibrinogen concentration for the horses in group 1 and group 2.....	122
Figure 5.10 Box plot showing HR at each time point for the horses in group 1 and group 2.....	122
Figure 5.11 Box plot showing PCV at each time point for the horses in group 1 and group 2.....	122
Figure 5.12 Box plot showing total protein concentration for the horses in group 1 and group 2.....	122

Figure 5.13. ROC curve for the final model including fibrinogen concentration	127
Figure 5.14. ROC curve for the final model excluding fibrinogen concentration	127
Figure 5.15 ROC curve for SAA concentration.....	128
Figure 5.16. ROC curve for haptoglobin concentration.....	128
Figure 6.1 Box plot of ulcer score at each time point.....	147
Figure 6.2 Box plot showing SAA concentration for each time point.....	149
Figure 6.3 Box plot of SAA concentration vs Ulcer Score at time point.....	152
Figures 6.4 Box plot of SAA concentration vs Ulcer Score at time point.....	152
Figure 6.5 Box plot of SAA concentration vs Ulcer Score at time point 3.....	152
Figures 6.6 Box plot of Haptoglobin concentration vs Ulcer Score at time point 1.....	153
Figure 6.7 Box plot of Haptoglobin concentration vs Ulcer Score at time point 2.....	153
Figure 6.8 Box plot of Haptoglobin concentration vs Ulcer Score at time point 3.....	153
Figure 6.9 Box plot of Fibrinogen concentration vs Ulcer Score at time point 1.....	154
Figure 6.10 Box plot of Fibrinogen concentration vs Ulcer Score at time point 2	154
Figure 6.11 Box plot of Fibrinogen concentration vs Ulcer Score at time point 3	154
Figure 6.12 ROC Curve demonstrating the relationship between Ulcer Score 4 and above and SAA concentration at Time Point 1	163
Figure 6.13 ROC Curve demonstrating correlation between Ulcer Score 4 and above and SAA concentration.....	163
Figure 6.14 ROC Curve demonstrating correlation between ulcer score 4 and above and SAA concentration for horses trained for National Hunt racing.....	164
Figure 7.1 Lateral radiograph of the thorax of one of the foals in Group 2.....	178
Figure 7.2 Box plot of SAA concentration for the 2 groups of foals.....	181
Figure 7.3 Box plot of Haptoglobin concentration for the 2 groups of foals.....	181
Figure 7.4 Box plot of Fibrinogen concentration for the 2 groups of foals.....	182
Figure 7.5 Box plot of Platelet number for the 2 groups of foals.....	182
Figure 7.6 Box plot of Hb concentration for the 2 groups of foals.....	183

Figure 7.7 Box plot of WBC Count for the 2 groups of foals.....183

Presentations, Publications and Impact of the Research

Papers

1. Pollock PJ, Prendergast M, Schumacher J, Bellenger CR. 2005. Effects of surgery on the acute phase response in clinically normal and diseased horses. Vet Rec 156:538-542 (appendix 4)

Presentations

1. The acute phase response and what it means for healing. Presented at the Annual Meeting of the Veterinary Wound Healing Association - Cardiff - 2004
2. The Acute phase response to surgery in clinically normal and diseased horses. Presented at the 5th International Colloquium on Animal Acute Phase Proteins - Dublin - March 2005

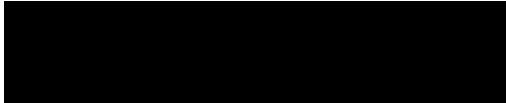
Impact

As a consequence of the work presented in this thesis, the measurement of SAA was added to the general screening panel for all foals at the three major studs in Ireland (representing the most intensive breeding area for thoroughbred horses in the world), and included on the general panel for all horses at the Irish Equine Centre.

Declaration

I hereby declare that the work carried out in this thesis is original and was carried out by either myself or with due acknowledgement. All additional sources of information have likewise been acknowledged. This work has not been presented for the award of a degree at any other university.

Signed:

A solid black rectangular box used to redact the signature of the author.

Patrick J Pollock

November 2016

List of Abbreviations

ACTH Adrenocorticotrophin

A.D. Anno Domini

B.C. Before Christ

BAL Bronchoalveolar lavage

CCL2 Chemokine 2

COMP+ Christie, Atkins, and Munch-Peterson Test

CXCL8 Chemokine 8 (also Interleukin 8)

CRP C reactive protein

DNA Deoxyribonucleic acid

EDTA ethylenediaminetetraacetic acid

EGF Epidermal growth factor

EGUS Equine gastric ulcer syndrome

ELISA Enzyme-linked immunosorbent assay

ESR Erythrocyte sedimentation rate

Fb Fibrinogen

GERDs Gastro-oesophageal reflux disease syndrome

HDL High-density lipoprotein

HCl Hydrochloric acid

H₂ Histamine receptor

H⁺,K⁺-ATPase Hydrogen/potassium adenosine triphosphate pump

Hb Haemoglobin

Hp Haptoglobin

HPA Hypothalamic-pituitary-adrenal

IL1 Interleukin 1

IL1 Interleukin 1

IL1 Interleukin 4

IL1 Interleukin 5

IL1 Interleukin 6

IL1 Interleukin 13

IL-1Ra Interleukin 1 receptor antagonist protein

IFN- α Interferon alpha

IFN- β Interferon beta

ln Natural logarithm

MCHC mean corpuscular haemoglobin concentration

MCH Mean corpuscular haemoglobin

MCV Mean corpuscular volume

NSAIDs Non steroidal anti-inflammatory drugs

PGE₁ Prostaglandin E₂

PGE₂ Prostaglandin E₁

PCR Polymerase chain reaction

PCV Packed cell volume

PO Per os

RBC Erythrocyte count

ROC Receiver operating characteristic curves

ROS Reactive oxygen species

SAA Serum Amyloid A

TMB Tetramethylbenzidine

TNF Tumour necrosis factor

TP Total protein concentration

UK United Kingdom

USA United States of America

WBC White blood cell count

WHO World Health Organisation

Chapter 1

Introduction and literature review

1.1 Introduction

“Measurements of SAA should make a significant contribution to diagnosis and management of viral and bacterial infection in horses, and routine serial assays could provide an objective criterion for monitoring prospectively the general health of horses in training and racing” (Pepys et al., 1989).

Twenty-six years ago, Pepys and his colleagues, on the basis of a pilot study of SAA in the horse, made this statement which, as Pepys observed further in his paper, has not been implemented to a significant degree.

“The results reported here of SAA monitoring in a stable of horses in training and racing raises the exciting prospect that this procedure could become an objective criterion for assessing general health of the animals and therefore contribute to evaluation of their prospective health and performance. More extensive investigation will be necessary to confirm this suggestion, with prospective analysis of SAA results and training and racing performance”.

Since the time of Pepys’ paper things have changed. There exist, assays of SAA that can give specific and highly accurate instant readings of the serum concentration of this acute phase protein (APP). But, whilst some studies have been made of SAA in the horse as a monitor of general health and for use as an adjunct to diagnosis and early detection of disease, the adoption of SAA measurement for horses is still in its infancy.

The work presented in this thesis explored SAA concentration in various common pathological conditions in the horse in order to provide evidence that will help to move us forward to the more widespread use of SAA, in the same manner that CRP is deployed in human clinical practice, as a necessary part of clinical investigation of health status.

In this study, emphasis was placed largely on competition horses. In addition, estimations were made of both fibrinogen and haptoglobin concentrations. The former is widely used as a marker of inflammation in horses and the latter was included to act as comparator for SAA and fibrinogen. Throughout the study the standard clinical parameters that were available from routine clinical

biochemical and haematological investigation were derived and recorded to demonstrate that SAA measurement provides information to inform clinical decisions over and above that obtained using these indices alone.

The development of markers that can be used to identify disease that is fulminant and overt, latent, or subclinical has become commonplace in human medicine (van der Linden *et al.*, 2010; Tilemann *et al.*, 2011; Kiefer *et al.*, 2012) and some veterinary species. However, to date this approach has not gained widespread acceptance in the horse. The studies presented in this thesis explore the use of the acute phase protein Serum Amyloid A (SAA) and other acute phase proteins (APPs) for the diagnosis, prognostication and for monitoring response to treatment in a number of common clinical scenarios.

1.1.1 The contrast to human medicine

In human medicine, measurement of acute phase proteins in clinical medicine is routine. C-reactive protein (CRP) measurement forms part of the standard database of testing following admission to hospital across the globe. The CRP response is non-specific and CRP, and indeed all acute phase proteins, as we presently understand it, can never be diagnostic for any particular condition. They should never be used in isolation and their values can only usefully be interpreted in combination with other clinical parameters and pathological data (Pepys and Hirschfield, 2003). Used in this way, the measurement of acute phase proteins contributes powerfully to the management of patients and it is generally accepted, amongst human practitioners, that patients with a normal CRP concentration are not suffering from significant disease (Pepys and Hirschfield, 2003).

In horses, the only molecule that displays sensitivity, response speed and dynamic range comparable to CRP is Serum Amyloid A (SAA) (Pepys and Baltz, 1993).

1.2 The use of acute phase proteins in clinical medicine and veterinary medicine

The first acute phase protein to be used clinically was human C-reactive protein (CRP) (de Beer *et al.*, 1982). Increased serum concentration of CRP in humans has been associated with an increased mortality following aortic aneurysm

(Domanovits *et al.*, 2002; Schillinger *et al.*, 2002) and with increased mortality in cases of myocardial infarction (Lindahl *et al.*, 2000). It is reported to be a sensitive, non-specific and non-expensive marker of inflammation for early diagnosis of bacterial infection following orthopaedic surgery (Waleczek *et al.*, 1991). Monitoring the concentration of this protein has been shown to be superior to the simple use of erythrocyte sedimentation rate, white blood cell count, development of drainage, ultrasound, and observation of clinical signs of infection, in diagnosing post operative orthopaedic infection (Singhal *et al.*, 2011).

Since the discovery of CRP over 90 years ago (Tillett and Francis, 1930), a number of other protein markers of inflammation have been recognised, including fibrinogen (Fb), serum amyloid A (SAA), haptoglobin (Hp), α -1-acid glycoprotein, α -1-antiprotease, and ceruloplasmin (Thompson *et al.*, 1992). The production of the various acute phase proteins in response to inflammation or disease varies greatly between species. For example, CRP is a major acute phase protein marker of inflammation and disease in dogs (Conner *et al.*, 1988), pigs (Eckersall, 2000), and humans, but serum concentration of CRP is reported as not to be altered in ruminants or horses by the presence of inflammation or disease (Eckersall *et al.*, 1988). In the bovine animal, the APPs, haptoglobin, ceruloplasmin and α -1-acid glycoprotein have been shown to be sensitive indicators for mastitis (Conner *et al.*, 1986).

Serum concentration of SAA is used as an aid to diagnose, monitor, and predict the course of disease in humans and dogs. Serum amyloid A is produced rapidly in horses in response to the presence of infectious and non-infectious inflammatory conditions (Hulten *et al.*, 2002;). Studies have examined the effect of disease on production of SAA in many species (Pepys *et al.*, 1989; Hulten *et al.*, 1999; Stoneham 2001; Hulten *et al.*, 2002; Hulten *et al.*, 2002; Cohen *et al.*, 2005; Vandenplas 2005; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2006; Duggan *et al.*, 2007; Jacobsen *et al.*, 2009). Table 1.1.

In humans a number of specific disease states have either no effect or a negligible effect on the concentration of CRP, the most useful acute phase protein in this species. These diseases include systemic lupus erythematosus,

scleroderma, dermatomyositis, ulcerative colitis, leukemia and graft versus host disease (Pepys and Hirschfield, 2003). Although a number of these diseases are recognised in domestic animals, their effects on the concentration of acute phase proteins in these species has yet to be elucidated. The reasons for the lack of measurable acute phase response in response to these diseases that result in significant tissue damage, inflammation, morbidity and mortality is not known.

1.2.1 The acute phase response, acute phase proteins, synthesis and regulation

The acute phase response is a systemic, non-specific response to infectious, inflammatory, immunological and traumatic disease states and reflects a combination of local and systemic events that go hand in hand with inflammation. At a local level, vasodilation, platelet aggregation, neutrophil chemotaxis, and the production and release of histamine, kinins, oxygen, free radicals and enzymes takes place. Systemically, pyrexia, activation of the pituitary-adrenal system, alterations in metabolism and, critically, alterations of gene expression are involved, leading to the synthesis by hepatocytes of an array of APPs (Van Leewuwen *et al.*, 1994).

Acute phase proteins are defined by Kushner *et al.*, (1982) as ‘hepatic proteins that show a serum concentration increase (positive APPs) or decrease (negative APPs) of 25% or more within the first seven days following trauma, infection or other disturbances of body physiology many of which can be referred to as inflammatory processes’. The principal APPs along with their putative functions are listed in Table 1.2.

Regulation of acute phase protein production is complex; a number of workers have shown that synthesis of APPs by hepatocytes is induced by cytokines and that each cytokine exerts its effects *via* the agencies of specific genes. There is evidence that in some instances a combination of cytokines is required and that these combinations can lead to increases or decreases in concentrations of acute phase reactants (Ramadori *et al.*, 1985; Perlmutter *et al.*, 1986; Gauldie *et al.*, 1987; Bauman *et al.*, 1987). The significance of these findings is that particular pathological conditions that lead to the production of a unique combination of

Table 1.1 Diseases, injuries (by body system) and disease states in which SAA concentration has been noted to rise in horses.

Disease	Description	Reference(s)
Surgery	Miscellaneous procedures	Pepys <i>et al.</i> , 1989
	Ex-Laparotomy	Jacobsen <i>et al.</i> , 2009
	Castration	Busk <i>et al.</i> , 2010
	Colic	Pollock <i>et al.</i> , 2005
		Nunokawa <i>et al.</i> , 1993
		Hulten <i>et al.</i> , 1999
		Jacobsen <i>et al.</i> , 2005 Pihl <i>et al.</i> , 2015
Synovial	Induced arthritis	Jacobsen <i>et al.</i> , 2006a
	Naturally occurring arthritis	Jacobsen <i>et al.</i> , 2006b
		Pepys <i>et al.</i> , 1989
		Hulten <i>et al.</i> , 2002
Inflammation	IM injection of turpentine	Nunokawa <i>et al.</i> , 1993
Vaccination		Andersen <i>et al.</i> , 2012
Gastrointestinal disease	Grass sickness	Copas <i>et al.</i> , 2013
	Colic	Pihl <i>et al.</i> , 2015
	Diarrhoea	Vandenplas <i>et al.</i> , 2005
		Nunokawa <i>et al.</i> , 1993
Reproductive disease	Placentitis	Canisso <i>et al.</i> , 2014
	Abortion	Coutinho da Silva <i>et al.</i> , 2013
	Parturition	Christoffersen <i>et al.</i> , 2010
	Endometritis	Tuppits <i>et al.</i> , 2014
	AI	
Respiratory disease	<i>Rhodococcus equi</i> pneumonia	Hulten and Demmers 2002
	Strangles	Cohen <i>et al.</i> , 2005
	Heaves	Chavatte <i>et al.</i> , 1991
		Lavoie-Lamoureux <i>et al.</i> , 2012
		Hobo <i>et al.</i> , 2007
Musculoskeletal/exercise	Laminitis	Menzies-Gow <i>et al.</i> , 2014
	Exercise	Cywińska <i>et al.</i> , 2012
Parasitic disease	Cyathostomiasis and strongyles	Andersen <i>et al.</i> , 2014
Neonatal	Prematurity	Chavatte <i>et al.</i> , 1991
	Neonatal maladjustment syndrome	Husebekk <i>et al.</i> , 1986
		Pepys <i>et al.</i> , 1989
	Sepsis	Nunokawa <i>et al.</i> , 1993
	EED	Paltrinieri <i>et al.</i> , 2008
		Stoneham <i>et al.</i> , 2001 Hulten and Demmers 2002 Krakowski <i>et al.</i> , 2011

cytokines may, in turn, have a unique acute phase profile (Ganapathi *et al.*, 1991) and this may be a key diagnostic target for the future.

Figure 1.1. illustrates the relative complexity of physiological systems that interact in the activation of the APP responses.

The supposed purpose of the acute phase response is far from fully understood. It has been suggested that it facilitates the inflammatory process and protects the organism from the potentially detrimental effects of inflammatory mediators (Jensen and Whitehead, 1998). The activities of a number of the APPs have, however, precisely been defined: for example, haptoglobin is a scavenger of free haemoglobin (Nyman, 1959), while α_1 -antitrypsin antagonises the activities of a number of proteolytic enzymes.

The first APP to be discovered was the pentraxin (evolutionary conserved family of proteins containing a calcium dependent ligand), C-reactive protein (CRP). The serum concentration of CRP and other APPs are now well established as non specific diagnostic general tests for organic disease, and as a means of monitoring the course of disease and response to treatment in humans (Kushner *et al.*, 1987; Lindahl *et al.*, 2000). CRP concentration, and that of other APPs have also been utilised as sensitive tests for intercurrent microbial infection (Hind *et al.*, 1985; Conner *et al.*, 1989; Pepys *et al.*, 1989). Haptoglobin and fibrinogen concentration are currently routinely used as markers of inflammation in veterinary medicine, although these compounds have a number of disadvantages, including slow response to inflammation and wide “normal” ranges (Pollock *et al.*, 2005).

In humans and a number of other domestic animals including cattle, dogs (Conner *et al.*, 1988) and horses, SAA concentration has been demonstrated to have the following clinically useful characteristics in the diagnosis, monitoring of response to treatment and prediction of outcome in disease and tissue injury:

- (1) Consistently low ‘normal’ serum concentrations.
- (2) Rapid change in concentration in response to changes in disease status and tissue injury.

(3) Synthesis rate is the only important determinant of serum concentration. (Gorevic *et al.*, 1976; McAdam *et al.*, 1978; deBeer *et al.*, 1982a; deBeeer *et al.*, 1982b; Pepys *et al.*, 1983; Hulten *et al.*, 1997).

Recently, several workers have demonstrated that SAA concentration in equine serum increases in response to a number of infectious disease conditions and in response to trauma, both accidental and surgical (Hulten *et al.*, 1999; Stoneham *et al.*, 2001; Hulten *et al.*, 2002; Cohen *et al.*, 2005; Pollock *et al.*, 2005; Vandenplas 2005; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2006; Duggan *et al.*, 2007; Jacobsen *et al.*, 2009).

To date, the major bodies of work regarding SAA and APPs, including haptoglobin and fibrinogen, in horses, have focused on their identification and description of their behaviour in response to a variety of diseases, injuries and infestations rather than testing for clinical utility or to inform treatment planning or monitor recovery and or disease progression.

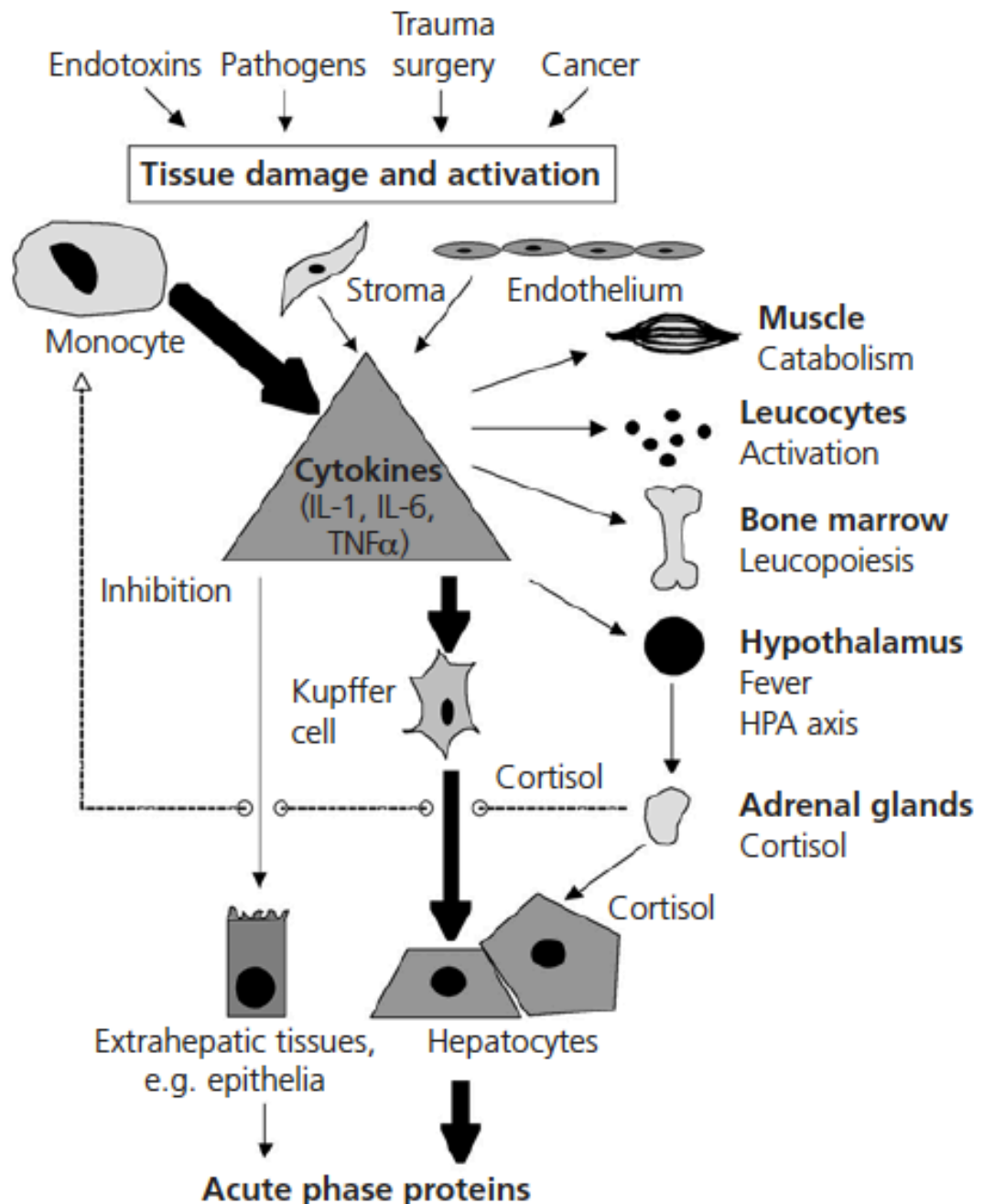
1.2.2 Fibrinogen

Fibrinogen is a complex glycoprotein, composed of three pairs of peptide chains (α , β and γ) joined by disulphide bonds. It is synthesised by hepatic parenchymal cells and has a half-life of approximately 36 hours (Dodds, 1989). Synthesis and turnover rates for fibrinogen are markedly influenced by tissue damage, inflammation, stress and ACTH concentration (Dodds, 1989). The primary function of fibrinogen is in haemostasis and blood clotting, as the substrate for thrombin. In addition to this, fibrinogen is the precursor of fibrin and so plays a key role in the inflammatory process. Fibrin formation, although in many cases beneficial, if excessive may lead to adhesions between serosal surfaces.

Increased utilisation of fibrinogen beyond normal homeostatic functions leads to an increase in synthesis and this is reflected in the serum concentration (Schalm, 1979). Consequently, fibrinogen concentrations are routinely used in equine practice in the diagnosis and evaluation of disease. Fibrinogen concentration in serum gradually increases over a period of five to seven days to attain peak concentration.

Figure 1.1 The Acute Phase Response in Mammals

The acute phase response to injury and disease is common to all mammals, although the specific APPs are often specific to individual species or groups of species. (Diagram from Jacobsen and Andersen, 2007)



While the increase in fibrinogen concentration is non-specific, increased concentrations have been demonstrated following surgery, infection, immunological, tissue injury and other non-specific inflammatory processes (Schalm, 1979; Allen *et al.*, 1988; Auer *et al.*, 1989; Hulten *et al.*, 2002; Pollock *et al.*, 2005).

For reasons that are not fully understood by this author, veterinary practitioners have clung to the use of fibrinogen in the clinical scenario despite its slow response to both the onset of disease and to changes in disease status (Pollock *et al.*, 2005).

1.2.3 Serum amyloid A- History, structure and isoforms

Serum Amyloid A (SAA) was first identified in serum due to its cross reactivity with antisera to the amyloid AA protein isolated from deposits of secondary amyloid (Levin *et al.*, 1973; Husby and Natvig, 1974). Husbebekk *et al.*, 1986 first isolated equine SAA in 1985 and equine SAA was subsequently measured, with an electro-immunoassay, postoperatively and during infectious conditions by Pepys *et al.*, 1989. SAA is an acute phase reactant protein common to humans, cattle, sheep, cats, mice and several other animal species, including the horse. It is an apolipoprotein and is heterogeneous in all of the species in which it has been identified so far. SAA generally consists of a 104-amino acid polypeptide (12kd) in association with a high-density lipoprotein (HDL) class of serum lipoproteins (Sletten *et al.*, 1989). Three isoforms of SAA have been characterised in the horse (Hulten *et al.*, 1997). The SAA group of APPs are so named due to their immunological and biochemical similarity to Amyloid A fibril protein, which is found in reactive systemic amyloidosis. SAA is principally found in serum, however, it has also been isolated from the milk of cattle with mastitis (Eckersall, 2001), from the synovial fluid of horses with arthritis (Jacobsen *et al.*, 2006) and the peritoneal fluid of horses with gastrointestinal disease (Pihl *et al.*, 2015).

1.2.3.1 Synthesis and regulation of serum amyloid A (SAA)

Serum amyloid A is chiefly produced by hepatocytes, however, in the mouse, SAA mRNA is also expressed in extra-hepatic (Meek *et al.*, 1986). It is produced in response to stimulation by a number of inflammatory mediators, including

interleukin-1, interleukin-6 and tumour necrosis factor (Jacobsen and Anderson, 2007). Concentrations of SAA have been shown to increase 1000-fold following tissue injury, cellular necrosis, inflammation and infection and to decline rapidly in the recovery phase. A normal, virtually undetectable concentration of SAA, probably excludes many serious pathological infections, particularly those associated with microbial infection (Pepys *et al.*, 1989; Horadagoda *et al.*, 1994). Indeed several studies indicate that treatment and response to antimicrobials is associated with a rapid fall in SAA concentration (Hulten *et al.*, 2003).

In humans and mice there are a number of isoforms of SAA encoded by multiple genes (Gorevic *et al.*, 1978; Sack, 1983; Yamamoto and Migita, 1985; Raynes *et al.*, 1991). Recent research suggests that this is also the case in equidae (Hulten *et al.*, 1997). However, in the work subsequently presented in this thesis the reagents used do not take account of this diversity.

In the same manner as a prolonged inflammatory response can be detrimental, prolongation of the acute phase response can also have damaging consequences (Jensen and Whitehead, 1998). Currently, the mechanisms of regulation and resolution are not entirely understood, although it seems that a number of pathways are involved. These pathways include repression of transcription (Edbrooke *et al.*, 1991), receptor antagonists and decoys, glucocorticoids, and cytokines which have a direct or indirect effect on down regulation of synthesis (Jensen and Whitehead, 1998). In addition to the mechanisms above, a number of natural mediators, including, reactive oxygen species (ROS) (reactive molecules that contain oxygen, including oxygen ions and peroxides formed as a natural byproduct of the normal oxygen metabolism and that have a role in cell signaling and homeostasis), antioxidants and vitamin E may be involved in stopping the target cells for SAA from responding, thus returning these cells to their normal profiles (Sen and Packer, 1996). Interleukin 1(IL-1), a key mediator in the activation of the production of SAA, is inhibited by the production of Interleukin 1 receptor antagonist protein (IL-1Ra), which has been detected at low concentrations in the serum of normal humans and at increased concentrations in patients with infectious, inflammatory and neoplastic disease (Lennard, 1995), and in horses with gastrointestinal and joint disease (Todhunter

et al., 1996; Lopes *et al.*, 2010). Recently, IL-1Ra has been used therapeutically in the joints of horses in an attempt to reduce the development and progression of degenerative joint disease (Moreira *et al.*, 2014).

1.2.3.2 Function of serum amyloid A (SAA)

The function of SAA is not well understood. However, since SAA is highly conserved throughout all of the species studied, it has been suggested that it is important for survival (Hulten *et al.*, 1997). A number of functions for SAA have been proposed, including a role in cholesterol transport (Meek *et al.*, 1994), platelet function (Syversen *et al.*, 1994) and as a chemoattractant for polymorphonuclear leukocytes and monocytes (Badolato *et al.*, 1994). Preciado-Patt *et al.*, (1994) published results demonstrating the inhibitory effect of SAA on inflammatory processes, mediated by a reduction in cell adhesion to extracellular matrix. A study by Shainkin-Kestenbaum *et al.* (1991) noted that there was a feedback effect between SAA and some inflammatory cytokines in mice, suggesting that SAA also had a role in tissue repair.

1.2.3.3 Clinical use of serum amyloid A (SAA) in the horse

Serum Amyloid A is measured with increasingly frequency in horses: some of the indications for doing so are given in Table 1.1. To date, the majority of workers have focused on changes to SAA concentration following disease or injury, rather than specifically using the kinetics of the concentration change as an aid to the identification of disease. Despite all the research outlined in Table 1.1, routine adoption of measures of SAA in equine clinical practice has not yet taken place and the work to be presented in this thesis will concern the collection and presentation of evidence as to whether more attention should be paid to determination of SAA concentration as a non specific marker for disease or injury.

1.2.4 Haptoglobin structure, synthesis and regulation

Haptoglobin is a glycoprotein composed of two polypeptide subunits (α and β) of 108,000 and 105,000 daltons molecular weight. It is synthesised in the liver and can be found within the α_2 -globulin fraction. The primary function of haptoglobin is to complex free haemoglobin following intravascular haemolysis

(Putnam, 1975). The haptoglobin-haemoglobin complex is removed by the reticuloendothelial system of the liver, thus reducing the risk of haemoglobin-induced nephrotoxicity (Nyman, 1959). Removal of haemoferrum, thus limiting that available for bacterial growth, has led to the term natural bacteriostat being used for haptoglobin (Eaton *et al.*, 1982).

A reduction in serum haptoglobin concentration in cases of haemolytic disease, and an increase following inflammatory, infectious, neoplastic and endocrine disease has been reported in cattle, cats, dogs, rats, mice, horses and humans (Engler *et al.*, 1970; Palmer, 1976; Harvey, 1978; Harvey *et al.*, 1984; Taira *et al.*, 1991; Hofner *et al.*, 1994; McGrotty *et al.*, 2003, Mischke *et al.*, 2007). In addition, haptoglobin has been shown to be an effective measure of health status in production animals (Armory *et al.*, 2007).

1.2.5 Acute phase proteins as prognostic indicators

There are numerous examples in human medicine of the use of APPs as aids to determining prognosis and in the use of APPs to stage, plan, and evaluate the efficacy of treatment regimens. Although never considered in isolation, increased serum concentration of CRP in human beings has been associated with an increased mortality following aortic disease (Schillinger *et al.*, 2002) and is a marker for bacterial infection following surgery (Waleczek *et al.*, 1991). The use of APPs to determine overall survival rates in urogenital (Ito *et al.*, 2011), breast, and head and neck carcinoma patients is also well documented (Dhankhar *et al.*, 2011).

In veterinary medicine, APPs have been evaluated as prognostic markers in small animals (Eckersall and Bell, 2010), including in dogs with immune mediated (Griebsch *et al.*, 2009), neoplastic, infectious, parasitic, (Köster *et al.*, 2009; Andersen *et al.*, 2014) and endocrine disease (Artega *et al.*, 2010) and following chemotherapeutic treatment (Merlo *et al.*, 2007).

Table 1.2 Known functions of the major positive acute phase proteins.

Action	Acute Phase Protein
Coagulation proteins	Fibrinogen
	Prothrombin
	Factor VIII
	Plasminogen
Protease Inhibitors	α_1 -antitrypsin
Complement	C3, C4 and C5
Transport proteins	Haptoglobin
	Haemopexin
	Ceruloplasmin
Miscellaneous actions	Serum Amyloid A
	C reactive protein
	Fibronectin
	α_1 -acid glycoprotein

Known acute phase proteins grouped by their proposed biological function.

Recently, CRP has been measured in dogs with acute gastrointestinal disease (Galezowski *et al.*, 2010), indicating that increased concentrations of the acute phase marker were associated with reduced mortality and were a more sensitive marker of tissue damage than white blood cell count measured at presentation.

1.3 Stress, Inflammation and disease

Stress and inflammation in domestic animals are characterised by a range of physiological, biochemical and behavioural changes (Allen and Kold, 1988; MacKay, 2000). In the horse, such changes are of use in the diagnosis, treatment and prognostication of disease (Patterson *et al.*, 1988; Pollock *et al.*, 2005; Jacobsen and Andersen, 2007). The acute phase response is characterised by an increase in circulating concentrations of a number of proteins. This occurs following tissue injury, inflammation and infection in all mammals that have been investigated (Pepys and Baltz, 1983; Auer *et al.*, 1989). To date, markers of inflammation including fibrinogen and haptoglobin, coupled with changes in a number of haematological and biochemical markers have been used to diagnose, monitor and prognosticate disease and to evaluate response to training in horses (Eurell *et al.*, 1993; Jacobsen and Andersen, 2007; Kristensen *et al.*, 2014). Unfortunately these traditional markers respond fairly slowly to the presence of disease and have wide normal ranges (Sheldrick *et al.*, 1982; Allen *et al.*, 1988; Pepys *et al.*, 1989). As a result, disease states may be well advanced by the time they are identified and, to date, it is widely believed that these traditional markers are of limited value during the identification of subclinical disease.

Thoroughbred horses are principally bred for use in the racing industry and are subject to significant levels of wastage (Rossdale *et al.*, 1985). Horses reared for the purpose of racing are subject to a variety of diseases and stress related conditions that affect their ability to perform, and in some instances that lead to their permanent loss from the industry (Wilsher *et al.*, 2006). Wastage has been defined as any injury or disease which interferes with the training schedule of the horse, resulting in lost days in work, a prolonged spell of rest, or retirement from racing (Rossdale *et al.*, 1985; Bailey *et al.*, 1997; Jackson *et al.*, 2005). Several studies have evaluated losses occurring in the thoroughbred

racing industry. A variety of injuries and conditions are noted as causes of lost days, including lameness, respiratory disease, wounds, colic, and epistaxis (Rossdale *et al.*, 1985; Bailey *et al.*, 1997; Wilsher *et al.*, 2006). The financial consequences of losses in the Thoroughbred industry are significant. Furthermore, there is clearly a welfare issue with regard to the numbers of animals subjected to euthanasia or left with chronic health problems (Jeffcott *et al.*, 1982). In some cases, diagnosis of potentially career threatening injuries or disease may be delayed due to limitations in currently available diagnostic techniques, this in turn may lead to an increased level of wastage. Moreover, it is generally believed that some of these horses may suffer from significant stress during training. This stress may play a part in the development of subclinical and clinical disease but can be difficult to identify (Scoppetta *et al.*, 2012; Cappelli *et al.*, 2013).

The stress response to training has been characterised by haematological changes, in particular, changes in erythrocyte parameters and in the neutrophil/lymphocyte ratio (Snow *et al.*, 1983). Increases in cortisol concentration have been recorded in horses in training and those with clinical evidence of disease, although a study by Irvine and Alexander (1994) found no significant changes in circulating serum cortisol concentration in response to training. Gastric ulceration is prevalent among racehorses in training (Murray *et al.*, 1989; Luthersson *et al.*, 2009a and b) and, in humans, stress has been documented as a cause of peptic ulcer disease (Monning and Prittie, 2011). Similarly, gastric ulceration has been noted in foals with concurrent disease (Furr *et al.*, 1992). Based on these and other reports, the evidence suggests that there are associations between common stressors and disease. However, many of the disease states associated with stress, such as gastric ulceration, have non-specific clinical signs and may be difficult to diagnose without advanced diagnostic equipment. In these circumstances, a new capacity to monitor the response to the stress of daily activity, training, and fulminant and subclinical disease would be of great value to owners, trainers and veterinary surgeons alike, who, using such information, could modify the care patterns of their animals.

1.3.1 Where we are today?

As noted above, a number of APP markers have been identified in horses. Serum Amyloid A (SAA) is one such, and a growing number of articles highlight concentration changes in this and other markers in response to trauma, disease (Copas *et al.*, 2013; Canisso *et al.*, 2014; Pihl *et al.*, 2013), parasitism (Andersen *et al.*, 2014), exercise (Mack *et al.*, 2014) and stress (Paltrinieri, *et al.*, 2008; Belgrave *et al.*, 2013;). Nevertheless, the fact that only a relatively small number of veterinary clinical laboratories offer measurement of SAA concentration, and fewer still as part of a commercial profile, as standard, demonstrates that veterinary medicine has yet to embrace the philosophy of using specific APP markers in the investigation, treatment planning and prognostication of disease.

1.3.2 Defining stress and inflammation, and its consequences in the horse

In 1975, Fraser, Richie and Fraser, defined stress as:

“an abnormal or extreme adjustment in the physiology of an animal to cope with adverse effects of its environment or management”.

Stress is such a broad term and can result in a myriad of different signs in humans and in the veterinary species. The difficulty for the researcher and clinician is that defining, quantifying and subsequently identifying the effects of stressors can be extremely challenging. Stressors include; changes in environment, which may be natural or artificial, handling, disease, treatment, and surgical intervention. Stressors potentially lead to pathophysiological reactions in the host producing changes in behaviour, physiology and disease susceptibility (Capelli *et al.*, 2013). While the stress response may be important for survival initially, long-term exposure to stressors leads to a variety of detrimental changes (Griffin, 1989).

“The indiscriminate manner in which the stress response is activated by afferent nerve stimulation may lead to production of excess hormones which may be life-saving in some patients but life threatening in others” (Bailey *et al.*, 1987).

In domestic animals and humans, the stress response leads to increased oxygen consumption by a number of key tissues and organs (Korthuis, 2011). This may result in the development of a relative hypoxia in a number of body systems and organs non-vital to the stress response, e.g., kidneys and gastrointestinal tract (Berne and Levy, 1998). In humans, increasing blood pressure, heart rate and, therefore, cardiac output may be detrimental in patients with existing cardiovascular pathology. Furthermore, increased concentrations of catecholamines are arrhythmogenic in all species (Bailey *et al.*, 1987) and the development of acidosis may inhibit myocardial function (Berne and Levy, 1998). Finally, animals and humans subjected to chronic stress may enter a catabolic state, resulting in the mobilisation of proteins from muscle. This in turn can delay wound healing (Ellis *et al.*, 1992).

The inflammatory response occurs in all mammals in response to tissue damage and is characterised by a set of clinical signs first described in antiquity by Aulus Cornelius Celsus (ca 30B.C.-38A.D.) and added to by Virchow in the 19th century. These clinical signs of heat, pain, swelling, redness and loss of function can be recognised in some form or another in all inflammatory processes and are associated with both repair and protection of tissue as well as further damage. The different forms of inflammation are determined by the inciting cause, which may be traumatic, infectious or immunological, and the standard inflammatory response is known to consist of four components: inducers of inflammation, the sensors that detect them, inflammatory mediators and target tissues (Medzhitov, 2010). It is generally accepted that acute inflammation plays an important role in tissue protection and repair, and that chronic states may become damaging (Wilmsink *et al.*, 1999). Recognition and identification of the inflammatory process are one of the main bases for the identification and treatment of disease.

1.3.3 Physiology of the inflammatory and stress response

Inflammation is a complex response to trauma, and to the invasion of the organism by foreign substances including infectious particles (Jacobsen *et al.*, 2009). The type of response is almost entirely governed by the nature of the inflammatory trigger, such that bacterial invasion leads to activation of receptors of the innate immune system, which in turn induce inflammatory cells

at the site, i.e, macrophages and neutrophils, to produce an enormous array of mediators, including, inflammatory cytokines, interleukins and prostaglandins (MacKay, 2000). The production of these chemicals is one of the hallmarks of the process, and different inducers lead to the production of different inflammatory mediators. These mediators include, but are not limited to, cytokines (e.g. Tumour necrosis factor (TNF), interleukins (IL1, IL4, IL5, IL6, IL13) and interferons (IFN- α , IFN- β), chemokines (CCL2 and CXCL8) and prostaglandins including, prostaglandin E2 (PGE2). These chemical mediators have a variety of effects both local and systemic; they induce the expression of selectins (carbohydrate-binding membrane proteins) on endothelial cells in the locality of the insult, which promote the recruitment of more inflammatory cells from the circulation. The quantities and quality of the recruited cells are dependent on the type of insult (Jensen and Whitehead, 1998).

Other effects include locally increased tissue permeability and vasodilation of blood vessels, which lead to migration of white blood cells (mainly neutrophils and macrophages) into the damaged tissue to assist in the containment and destruction of invading pathogens and to remove necrotic debris. These processes are assisted by antibody activation and the production of complement (MacKay, 2000). Systemic effects of the inflammatory mediators include the induction of hepatocytes to produce acute phase proteins and the activation of local and central neurones to produce specific behavioural changes (Pecchi *et al.*, 2009). In the case of sterile tissue damage, which may occur during wounding, the inflammatory response acts to prevent colonisation of the damaged tissue by microbes and other pathogens. The mediators produced during this type of tissue damage are not well understood, but are likely to be related to the products and components of dying cells, such as ATP and bradykinin (Basbaum *et al.*, 2009). Activation of nociceptors in the damaged area leads to inflammatory pain: these receptors are activated by some of the clinical signs of inflammation such as swelling and exudation; additionally, prostaglandins increase the sensitivity of nociceptors (Medzhitov, 2010). In order to be effective in the resolution of the infection, or trauma, the acute inflammatory process should normally cease once the insult has been removed or eliminated. This switch from the inflammatory response to the normal homeostatic state is a highly controlled and regulated process that is

characterised by the change from the production of pro-inflammatory prostaglandins to anti-inflammatory chemicals, including resolution-inducing lipotoxins. Recruitment of neutrophils is reduced in favour of phagocytic monocytes to promote clearance of cellular debris and repair of damaged tissue. (Serhan and Savill, 2005). If this resolution of acute infection does not occur, then a persistent state of chronic inflammation follows. Such a state can be caused by chronic infections, foreign bodies, or unrepaired trauma (Kumar *et al.*, 2003).

The inflammatory and neuroendocrine response (Griffin, 1989) to trauma and disease are complex, and the full detrimental and beneficial effects of these physiological responses are yet to be fully understood. Nevertheless, there is widespread acceptance that a better understanding of inflammation holds the key to successful treatment and management of a number of traumatic and other disease states in humans and veterinary species.

1.3.4 Benefits of inflammation and stress

Clearly, the stress response is a normal physiological reaction to a variety of stimuli and, as such, it has a number of benefits to the “stressed” individual. The most notable and immediate effects are increased perfusion to a number of key viscera and therefore increased delivery of oxygen and nutrients to these tissues. This “extra fuel” is maintained through catabolism until the individual is able to resume normal food intake (Griffin, 1989).

Inflammation occurs to restore tissue integrity and re-establish homeostasis, although it should be noted that the response to trauma serves a different purpose than that of the response to infection or immune stimuli. In the case of trauma the primary purpose of the inflammatory response is to remove damaged tissue and promote repair, whereas the response to pathological organisms is primarily protective and important in the induction of adaptive immunity (Medzhitov, 2010)

1.3.5 Methods used to identify stress and inflammation, and associated haematological and biochemical changes

A variety of methods can be used to assess whether an individual animal is stressed. Clearly, clinical examination is of primary importance in assessing inflammation and disease. Changes in behaviour, feeding patterns, weight change, socialisation and performance have all been used. Quantification and identification of stressors can be extremely difficult and this has led previous investigators to examine a variety of haematological, biochemical and other chemical changes that may be associated with stress (Holbrook *et al.*, 2010). The recent interest in pain scores for both humans and animals provides some quantification for stress and inflammation, but these do not provide a direct measure of the inflammatory response (Furr *et al.*, 1995; Hunt *et al.*, 2004).

A number of markers have traditionally been used to identify and quantify the degree of inflammation. Classically, such changes have been used in the diagnosis of clinical and subclinical disease, to measure the stress of certain activities such as exercise and training, and as a guide to the assessment of fitness in racehorses. Significant increases in red blood cell numbers, packed red cell volume and haemoglobin concentrations are well documented in response to exercise and training (Snow and Mackenzie, 1977; Kedzierski and Bergero, 2006). These changes are thought to be due, in part, to splenic contraction (Person, 1967; Person, 1975; Snow *et al.*, 1983). Increases in serum protein concentrations have also been recorded in racehorses and in humans in response to exercise (Astrad *et al.*, 1977; Keenan, 1979). Initially, the plasma protein fibrinogen, but more recently a number of interleukins (IL-6) and the acute phase protein C-reactive protein (CRP) have been shown to increase their concentration in response to exercise (Libardi *et al.*, 2012). It is not understood whether this increase is a response to the development of muscle pathology, however, the fact that increased CRP concentrations can be associated with an increased risk of myocardial infarction in humans (Lindahl *et al.*, 2000) suggests that these acute phase proteins could be used as markers of disease in horses.

In humans, erythrocyte sedimentation rate (ESR) or the Biernacki Reaction, is used as a non-specific measure of inflammation (Westergren, 1957). The ESR is really a measurement of fibrinogen concentration as increased concentrations of

fibrinogen result in the formation of red cell stacks called rouleaux. For that reason, ESR can be considered to be an indirect indicator of the acute phase response (Van Leeuwen and van Rijswijk, 1994). Unfortunately, ESR is of limited use in veterinary species as rouleaux formation can be normal in horses, pigs and cats (Weng *et al.*, 1996).

1.4 Evaluating diagnostic tests

A great variety of tests are used in clinical veterinary medicine, for diagnosis, screening and research. Tests can be evaluated using a number of criteria, including how well they classify the subjects that are tested, their practicality or cost. A diagnostic test is used to determine the presence or absence of disease in the presence of clinical signs, whereas a screening test identifies individuals without clinical signs. Many commonly used tests have a continuous scale and this may complicate the separation of diseased from healthy individuals (Thrusfield, 2007).

The ability of a test to indicate whether or not the individual has the disease is referred to as the validity. Sensitivity describes the ability of the test to correctly identify which individuals have the disease, whereas specificity describes the ability of the test to correctly identify individuals who do not have the disease.

With the introduction of a new test, a gold standard test can be used to evaluate the new test using a 2x2 table to compare the performance of the new test against the currently accepted test. This type of evaluation may also be used to determine the cut points used when the test is applied to potentially diseased individuals. Different cut points will influence the sensitivity and specificity of the test. In daily clinical practice very few tests are used in isolation; instead, multiple tests are performed sequentially or simultaneously and interpreted collectively in order to maximise the chance of the correct diagnosis.

Predictive values can be used to evaluate the performance of a test. The positive predictive value (PPV) is the proportion of individuals that test positive and actually have the disease. The negative predictive value (NPV) is the proportion of individuals who test negative and are actually free of the disease.

The positive predictive value is influenced by the prevalence of the disease in the population tested and on the sensitivity and specificity of the test. The PPV or the NPV can be calculated using a formula or Bayes theorem (Dohoo, 2003).

Finally, all diagnostic tests are evaluated for reproducibility, repeatability and reliability. The ideal test will yield a similar result each time it is conducted. Variations in laboratory practice, testing procedure, and within the subject of the test may affect the ability of the test to consistently yield the same result. For each test the overall percent agreement and percent positive agreement are calculated (Thrusfield, 2007).

Acute phase protein testing is by its very nature non-specific, and such tests are never diagnostic in their own right. In human medicine, acute phase protein tests are interpreted at the bedside alongside clinical findings and the results of other tests. In the same way as the measurement of temperature is universal in medical and veterinary settings, the measurement of acute phase proteins is of great clinical utility (Pepys and Hirschfield, 2003)

1.5 Choosing horses and clinical syndromes in which to evaluate acute phase proteins

A number of disease states and syndromes and their effect on the concentration of acute phase proteins were investigated and are presented in the subsequent chapters. Although opportunistic in nature, the clinical conditions have been chosen because they represent a selection of relatively common ailments / syndromes in relation to which, for various reasons, it would be useful for the attending clinicians and the owners to have more precise indicators of status to inform clinical decision-making.

The horses selected for inclusion in the project were chosen to represent both the professional and leisure horse industry in Northern Europe and with the recognition that in a great many instances in equine practice, clinical cases are available for examination and sampling on a single occasion.

1.6 Measuring the response to surgery and the effect of disease in the horse

Historically, the response to surgery in the horse has been measured based upon surgical success, resolution of the disease and / or various performance indices in competition animals (Cheetham *et al.*, 2008). While many of these measures provide useful data, it could be argued that, to date, none provide information on the general health status of the horse. There are numerous examples of instances in which the presence or degree of subclinical disease and economic output have been used as a general marker of herd health in populations of production animals (Gerardi *et al.*, 2009) but again, no such study has been performed in the horse.

A number of individuals and organisations have attempted to define health (Callahan, 1973; Gunnarsson, 2006) in all species, and there has been a gradual recognition that health is more than just an absence of overt disease or injury. Nevertheless, medical and veterinary professionals alike continue to struggle with the identification of disease states that are not readily apparent or subclinical. In humans, it is widely accepted that measures of disease status alone are insufficient to describe the burden of illness (Muldoon *et al.*, 1998); consequently quality of life measurements have been widely adopted in human clinical practice (Thomas *et al.*, 1994). Many of these definitions of health have been subject to extensive study and validation and are generally divided into measures of objective function and subjective wellbeing (Muldoon *et al.*, 1998). In the veterinary species some of these measures are much more difficult to quantify and assess. Nevertheless it is still quite possible to simply record measures of general health.

1.7 Equine surgical and non-surgical gastrointestinal disease (colic)

There can be few diseases that have a greater emotional effect, or that engender such anxiety amongst horse owners, carers and trainers than gastrointestinal disease or colic. Producing a range of clinical signs from relatively mild and transient, to severe debilitating disease with high rates of morbidity and mortality, horse owner and veterinary led surveys in the UK and the USA suggest that colic is the second most common cause of death in horses after musculoskeletal disease (USDA, 2005; Andrews, 2009). The purpose of this

review is broadly to outline the type of pathology encountered by veterinary surgeons treating horses affected by colic that require surgical intervention, and to demonstrate the need for rapid decision making in such cases.

Rather than a specific disease, the term colic refers to clinical signs associated with dysfunction or derangement of the gastrointestinal tract. These clinical signs vary in severity and include, but are not limited to, sweating, flank watching, rolling or lying down for longer than usual, dog sitting, groaning, pawing, stretching, yawning, alterations in intestinal borborygmi, spontaneous nasogastric reflux, extreme restlessness and violent rolling (Mair, 2002). An array of conditions can lead to the clinical signs described and range from derangements in intestinal motility, impactions, intestinal displacements, structural and functional obstructions, parasitic infestation, neoplastic and inflammatory conditions (Cohen, 2002). Unfortunately, in many instances the cause of colic is not identified and, even when it is, prognostication and determination of the likely outcome of the disease can be extremely difficult (Dukti and White, 2009).

Colic is thought to affect between 4-10 cases per 100 horses presented to veterinary surgeons in first opinion practice (Tinker *et al.*, 1997). Of these horses, the vast majority (80-85%) respond to medical management with an excellent prognosis for resolution and return to normal activity (Proudman, 1992). Strangulating and non-strangulating diseases that lead to rapid development of clinical signs and necessitate surgical intervention are thought to represent 2-10% of colic cases (Hillyer *et al.*, 1997).

1.7.1 Making the decision for intervention and determining outcome in horses affected with colic

A number of workers have developed models in an attempt to better determine the prognosis for horses affected by colic. These models are based on a variety of variables that include, but are not limited to, heart rate, total plasma protein concentration and packed red cell volume, plasma lactate concentration, peritoneal fluid evaluation, capillary refill time and mucous membrane appearance, and the presence of self-inflicted trauma (Orsini *et al.*, 1989; Pascoe *et al.*, 1990; Furr *et al.*, 1995). Although a number of these models

performed well in the hospital environment in which they were developed, with high positive predicative values on the model data sets, they did not perform so well when evaluated using data sets outwith the hospital horse population (Reeves *et al.*, 1989).

Due to the potentially high financial and emotional costs to horse owners of horses affected by, and treated for colic, further work to allow an accurate prognosis to be given at the time of presentation is critical. Human doctors and veterinary surgeons are under increased pressure to practice evidence-based medicine (Freeman and Curtis, 2015). Consequently it is no longer acceptable in terms of animal, and indeed owner welfare to operate on a horse, affected by colic, with a hopeless prognosis (Mair *et al.*, 2007). Objective survival data, mapped onto clinical parameters gathered at the time of presentation are critical to inform policy in equine hospitals (Proudman *et al.*, 2002a, 2002b). The ideal test to determine whether a horse should be subjected to surgery or not, does not exist. Such a test would require to be run quickly, cheaply and if possible “horse-side”. Critically, and in contrast to the myriad of tests and clinical parameters that are currently available to measure, the test would need to be able to take a global view of the state of the horse. In a colicking animal, that would mean the test would assess the degree of gastrointestinal tissue damage, endotoxaemia, sepsis, and provide a global measure of the “inflammatory-state” of the animal, perhaps even the level of stress the animal was experiencing. At its most basic function, the test would determine the likelihood of survival for the patient following successful surgery, but secondary functions might extend to assessing fitness for surgery in the first place.

1.8 The equine athlete-training in horses

It is widely accepted that horses in race training are subject to a certain level of physiological stress (Cayado *et al.*, 2006; Scoppetta *et al.*, 2012; von Lewinski *et al.*, 2013). Strenuous exercise modulates both the autonomic nervous system and the hypothalamic-pituitary-adrenal axis (Galbo, 1986). The activity of these two stress-activated systems can be demonstrated by increases in cortisol, ACTH, adrenaline and noradrenaline concentrations. These endocrine responses to exercise have been extensively studied in horses (Church *et al.*, 1987; Jiminez *et al.*, 1998; Nagata *et al.*, 1999).

Various physiological changes are associated with overtraining in human and equine athletes. Overtraining is a general term for any chronic condition in which there is imbalance between training and recovery resulting in fatigue. A drop in performance is necessary for a diagnosis of overtraining (Hamlin *et al.*, 2002) and the measurement of these reductions in performance is fraught with difficulty. Changes in humans and horses associated with overtraining include; decreased maximal heart rate, changes in muscle composition, modified responses to catecholamines, blunted responses to cortisol, and reduced weight (Hamlin *et al.*, 2002). Perkins *et al.*, (2004) described the effect of duration of training on subsequent performance in racehorses in New Zealand and a number of authors have examined the response of specific body systems and tissues to training (Price *et al.*, 1995). However, to date, very little work has been performed investigating the use of inflammatory markers or acute phase reactants as a measure of general health or “normality” in apparently normal horses in training.

1.9 Gastric ulceration in the horse

Equine peptic disorders are conditions in which hydrochloric acid (HCl) and pepsin, secreted by the cells in the stomach, damage the mucosal lining of the oesophagus, stomach and duodenum. One such condition, which has recently received increased interest amongst clinicians and research workers, is gastric ulceration (Luthersson *et al.*, 2009a, 2009b). Gastric ulceration is common in both adult horses and foals, with a reported prevalence of 25 - 50 % in foals (Wilson, 1986; Murray, 1988; Murray *et al.*, 1990) and 60 - 90 % in adult horses (Hammond *et al.*, 1986; Murray, 1988; McAllister *et al.*, 1992; Vatistas *et al.*, 1994). Indeed two distinct age related syndromes, affecting different anatomical locations within the stomach, have been described. These syndromes may present with different aetiologies and clinical signs, but share a common pathogenesis. Definitive diagnosis relies on visualisation of the lesions with the aid of gastric endoscopy (gastrosocopy). Due to the array of clinical presentations, pathophysiological mechanisms for their development and variety of different types of animals affected, the term equine gastric ulcer syndrome (EGUS) has been coined for this disease (Andrews *et al.*, 1999).

1.9.1 Anatomy of the equine stomach

The equine stomach is divided into two distinct regions anatomically, both grossly and histologically, the non-glandular or oesophageal region and the glandular region. The non-glandular region accounts for one third of the stomach, and like the oesophagus, is white in colour and lined by a thick stratified squamous epithelium (Murray *et al.*, 1989). Separated from the remaining two thirds of the stomach by a raised ridge of tissue, known as the *margo plicatus*, or cuticular ridge, the glandular portion of the stomach is lined by glandular epithelium and is physiologically and histologically similar to the human stomach and that of some of the other domestic animals (Sisson, 1975). The glandular portion contains cells responsible for the production of hydrochloric acid, pepsin, mucus and bicarbonate (Sisson, 1975). The glandular portion of the stomach is protected from damage by hydrochloric acid (HCL) and pepsin by a number of mechanisms including: a mucus/bicarbonate layer which repels HCl and leads to a gradient of increasing pH towards the surface cells, excellent mucosal blood flow, allowing transport of H⁺ ions away from the mucosa and providing a source of oxygen and nutrients. Blood flow is mediated through the production of nitric oxide and prostaglandins E₁ and E₂. Prostaglandins also have a role to play in protection of the mucosa by stimulating the production of surface-active protective phospholipid and mucosal repair, and by preventing cell swelling by stimulating sodium transport (Lees and Higgins, 1985). In stark contrast, the non-glandular squamous epithelium of the equine stomach and oesophagus are poorly protected against the development of peptic ulcers (Orlando, 1991). The major barriers to HCl are the intercellular tight junctions and secretion of bicarbonate by the cells themselves. The oesophagus is protected by the flow of saliva distally, mucus and the integrity of the gastro-oesophageal sphincter.

1.9.2 Ulceration in the equine stomach

Gastric ulcers have been reported in both the glandular and non-glandular regions of the stomach and damage to the surface of the stomach can be classified as inflammation, erosion (superficial mucosa disrupted) or ulceration (penetration of the submucosa) (Rebhun *et al.*, 1982; Roberts, 1990; Andrews *et al.*, 1999; Lutherson *et al.*, 2009a,b)

1.9.3 Prevalence and aetiology of gastric ulceration

The prevalence of gastric ulceration has been reported to range from 10.3% at necropsy to as much as 100% when limited to animals in full race training (Murray *et al.*, 1989; Sandin *et al.*, 2000). Gastric ulceration is a condition of multi-factorial aetiology and, as a result, a variety of factors have been implicated in its development. Mucosal protective factors are more developed in the glandular portion of the equine stomach, compared to the squamous region, and different mechanisms are probably responsible for the development of ulcerative lesions in different regions (Sanchez, 2004). Ulcers in the non-glandular, squamous region are primarily the result of increased exposure to hydrochloric acid and, as such, are similar to gastro-oesophageal reflux disease syndrome (GERDS) noted in humans (Collier and Stoneham, 1997). In contrast, ulcers in the glandular mucosa result from interruptions to blood flow and decreased mucus and bicarbonate secretion. This leads to back diffusion of hydrogen ions and resultant damage to the submucosa (Andrews *et al.*, 1999). Hydrochloric acid is secreted continuously by equine parietal cells within the gastric glands and, as a result, equine gastric pH can range from 2 to 6 depending on the fasted state of the horse (Campbell-Thompson and Merritt, 1990; Murray *et al.*, 1993). Anything interfering with HCl concentration or volume, within the stomach, or that prolongs HCl contact with the mucosa, or that interferes with mucosal blood flow and mucus production, may lead to gastric ulceration.

In humans and horses, stress has been implicated as a potential cause of ulceration. The mechanism is not well understood but may be related to increased production of endogenous corticosteroid, which can inhibit prostaglandin synthesis. In foals, parturition and hospitalisation, and in adults, confinement and training, have been suggested as possible stressors (Andrews *et al.*, 1999).

1.9.4 Diagnosis of gastric ulceration

In yearlings and adult horses, diagnosis of EGUS is based upon clinical signs and the response to treatment. Definitive diagnosis of gastric ulceration requires endoscopic evaluation of the stomach in fasted horses. Gastroscopy is simple to perform in the standing horse and is the current gold standard for identification

of pathology (Murray *et al.*, 1990; MacAllister *et al.*, 1997). Clinical signs are variable, and erosions and ulcers are usually categorised as clinical or subclinical and may be associated with poor appetite, reduced body condition, recurrent colic, diarrhoea, poor performance and changes in demeanour (Sanchez, 2004). The presence of gastric ulceration is often overlooked in adult horses, although the syndrome may, in fact, be of greater clinical and economic significance in mature rather than young animals (Sanchez, 2004).

A number of scoring systems have been used to describe lesions in the glandular and squamous regions of the equine stomach (Murray *et al.*, 1996; MacAllister *et al.*, 1997; Andrews *et al.*, 1999; Bell *et al.*, 2007). The scoring system used in the studies presented in this thesis was the accepted system during the study period (Murray *et al.*, 1996).

1.9.5 Treatment of gastric ulceration

A number of treatment options are available, including histamine H₂ receptor antagonists (cimetidine and ranitidine), mucosal protectants, such as sucralfate, and, more recently, and so far most effectively, the proton pump inhibitor, omeprazole. Omeprazole works by irreversibly binding to the H⁺,K⁺-ATPase proton pump of the parietal cells. The drug has shown excellent efficacy against gastric ulceration (MacAllister, 1999) and, additionally, continued low dose therapy is protective against the re-development of erosions and ulceration (Murray *et al.*, 1997). Treatment with omeprazole has also been shown to reduce the severity, and even eliminate, ulceration in horses in full race training (Vatistas *et al.*, 1999). Nevertheless, treatment is expensive and can be protracted.

1.9.6 Acute phase proteins and gastric ulceration

If the concentration of an acute phase protein or proteins were noted to change in response to the presence of clinically significant gastric ulceration, testing for this marker or markers could be added to the panel of biochemical analytes commonly examined during investigations for poor performance or as part of a routine screening regimen for performance horses.

1.10 *Rhodococcus equi* in Thoroughbred foals

Rhodococcus equi, formerly known as *Corynebacterium equi*, was first described as a cause of purulent bronchopneumonia in foals in Sweden by Magnusson (1923). This gram-positive pleomorphic coccobacillus is frequently found in V and L shaped groups that are referred to as “Chinese character formations” (Wilkins 2004). Since its isolation, it has become a well-known pathogen in horses and, more recently, it has been identified as an opportunistic pathogen in immunocompromised humans (Drancourt *et al.*, 1992). A facultative intracellular organism, many species of *Rhodococcus*, including *R. equi*, can be isolated from the soil and the air in accommodation used to house horses (Woolcock *et al.*, 1980; Takai *et al.*, 1987). The organism has also been isolated from the faeces of foals, from the first week of life onwards, and adult horses (Takai *et al.*, 1986). The welfare and financial consequences for farms producing foals affected by *R. equi* are significant and, as such, a great deal of research has been conducted into the epidemiology, diagnosis and treatment of this disease. The result of infection with *R. equi* is a severe bronchopneumonia with granulomatous abscess formation and, in approximately half of all infected animals, the disease progresses to involve other body systems (Zink *et al.*, 1986).

1.10.1 Pathogenesis and virulence of *Rhodococcus equi*

Rhodococcus equi inhabits, and replicates within, alveolar macrophages, where it can resist the innate immune system and can prevent phagosome lysosome function in infected animals and humans (Hietala and Ardans, 1987). Within the macrophage, the bacterium is also protected from neutrophil mediated killing. Ability to replicate within a macrophage is dependent on virulence and this, in turn, is dependent upon the capsular polysaccharide, the exoenzyme cholesterol oxidase, cell wall mycolic acids and, most importantly, the possession of a large plasmid, which encodes for a surface expressed lipoprotein, VapA. All strains of *R. equi* isolated from horses with clinical disease possess the large plasmid and express VapA antigens (Hondulas, 1997). Plasmid-cured *Rhodococcus equi* are unable to produce infection and are cleared from the lung of foals within two weeks (Giguere *et al.*, 1999).

The consequences of infection with *R. equi* in all species is the development of a pyogranulomatous pneumonia, which is due to the presence of mycolic acid in

the *R. equi* cell wall which promotes granuloma formation (Wilkins, 2004). Early lesions are characterised by a cellular influx of macrophages and multinucleate giant cells into the alveolar spaces.

1.10.2 Development of disease and clinical signs

Although infection with *R. equi* is reported in adult horses (Lavoie *et al.*, 1994), the majority of clinical cases are reported in foals less than three months of age. Foals are exposed to infection by inhalation of the pathogen, although the alimentary and genital systems are other potential routes of infection. Characteristically, clinical disease is insidious in onset and clinical signs may be dependent upon the route of infection (Yager, 1987). Respiratory infection leads to an extensive bronchopneumonia and the development of large abscesses, which may coalesce (Hondalus, 1997). A proportion of infected individuals develop ulcerative colitis and / or mesenteric lymphadenitis (Zink *et al.*, 1986). The intestinal form originates in the Peyer's patches, which become ulcerated; with time, infection extends to involve the local mesenteric lymph nodes. Dissemination of infection from the lung to other sites leading to a variety of clinical syndromes has been described, including: septic arthritis, septic pharyngitis, discospondylitis, serositis, and cutaneous ulcerative lymphangitis (Hondalus, 1997; Wilkins, 2004).

1.10.3 Susceptibility of the foal compared to other species

Although infection with *R. equi* has been reported in cattle, pigs, sheep, goats, deer, buffalo, cats, and dogs (Soedarmanto *et al.*, 1997; Passamonti *et al.*, 2011), disease in species other than the horse, and more recently immunocompromised humans (Drancourt *et al.*, 1992), is rare and generally confined to local lymph node abscessation or wound infections. The clinical syndrome of suppurative bronchopneumonia is almost entirely confined to young horses (Wilkins 2004). Furthermore, while experimental studies have readily induced the condition in foals, similar results have not been demonstrated in other animal species following inoculation with *R. equi*. Breed predispositions have been reported, but these differ according to geographical location (Taki *et al.*, 1987); for example, despite an extensive study, no virulent strains of *R. equi* have ever been isolated from Prezwalski's horses in Mongolia (Taki *et al.*, 2005).

Provided foals are exposed to a sufficient challenge dose, experimental studies have demonstrated that all foals are similarly susceptible (Yager, 1987).

1.10.4 Diagnosis and treatment of *Rhodococcus equi*

Diagnosis of *R. equi* is based upon the identification of the organism in direct smears or culture of tracheobronchal washes or faecal samples (Wilkins, 2004). Radiographs of the thorax may demonstrate the presence of multiple abscesses (Bertone, 1998) and thoracic ultrasound is very sensitive, particularly in foals with disease involving the peripheral sections of the lungs (Wilkins, 2004). Haematological examination may indicate the presence of a neutrophilia, increased fibrinogen concentration and thrombocytophilia. A number of serological tests have been described, although results can be difficult to interpret as young foals that become exposed to the organism may produce antibodies in the absence of clinical disease. Recently, a polymerase chain reaction (PCR) test has been validated for the identification of *R. equi* and results of two clinical trials suggest that this method is likely to be effective in the diagnosis of the disease (Sellon *et al.*, 2001), although, again, this technique may only confirm the presence of the organism, and not necessarily clinical disease. Treatment can be difficult, and protracted, due to the intracellular nature of the pathogen and the fact that the disease may be extensive prior to the onset of clinical signs. The most common treatment is the combined oral use of erythromycin (25mg/kg tid) and rifampicin (5mg/kg bid). However, recently there has been a move to the use of azithromycin (10mg/kg sid PO or clarithromycin (7.5mg/kg bid PO) in combination with rifampicin (Wilkins 2004).

1.10.5 Why do we need to do better with early identification of infected foals?

The prognosis for survival of foals, following successful treatment, is particularly good for those individuals that are mildly or subclinically affected. Additionally, these animals are more likely to have an athletic career. However, despite this, diagnosis continues to rely heavily on the recognition of clinical signs by which time significant tissue damage may have occurred (Bertone, 1998; Johns, 2013). To this end, the development of a cheap, reliable test, which could form part of the routine biochemical screening panel utilised by many equine commercial breeding operations, is much needed.

1.11 Patient side acute phase protein testing

Since the work described in this thesis was undertaken, patient side SAA testing has become available to equine veterinary surgeons. Although not yet widely used, the tests, performed on whole blood, provide two options for results; either, a binary result which simply tells the operator whether the SAA concentration is within the normal range or not; or a numerical value. The emphasis is on the identification of individuals who may be abnormal before the onset of overt clinical signs. The manufacturers advocate serial testing of individuals to develop an acute phase profile for each horse, as well as one off testing if disease is suspected. To date the tests have been used to successfully identify individuals, which with further screening have been diagnosed, with infectious airway disease, exercise induced pulmonary haemorrhage, gastrointestinal disease and surgical site infection.

1.12 Summary, objectives and hypothesis

The various traumatic interventions, inflammatory stimuli, acute and chronic disease states described in this introduction and literature review represent some of the conditions, affecting horses, in which the use of APP markers to diagnose, monitor response to interventions and treatment and prognosticate disease and injury, might be more generally adopted. The purpose of the work presented in the chapters that follow was to attempt to bridge the present gap between demonstrating that these markers are present following injury or disease, and their effective clinical use in everyday equine veterinary medicine.

The specific objectives of this project were to answer the following questions;

- (1) to define differences, if any, in the serum concentration of the acute phase proteins, serum amyloid A and haptoglobin in clinically normal horses.
- (2) to determine if surgical stress is a significant factor in the serum concentration of serum amyloid A and haptoglobin in the horse.

- (3) to determine if the serum concentrations of the acute phase proteins serum amyloid A and haptoglobin are associated with the severity and eventual outcome in horses with acute gastrointestinal compromise.
- (4) to determine if differences in training regimens are a significant factor in the serum concentration of serum amyloid A and haptoglobin within a group of Thoroughbred racehorses.
- (5) to determine if gastric ulceration has a significant association with the serum concentration of serum amyloid A and haptoglobin, and if these acute phase proteins are useful markers for identification of different grades of ulceration within a group of Thoroughbred racehorses in training.
- (6) to determine if the concentrations of serum amyloid A and haptoglobin are significantly increased in foals subclinically infected with *Rodococcus equi*, but lacking clinical signs.
- (7) to determine if serum amyloid A and other acute phase proteins have a role to play as indicators of normal health in horses.

Overall, the work explored the extent to which the acute phase proteins, serum amyloid A and haptoglobin, can be used as non-specific tests in horses for:

- the presence of inflammation,
- tissue injury,
- training stress and
- the presence of subclinical pathology,
- the diagnosis and prognostication of disease.
- as a general measure of health.

Chapter 2

General Materials and Methods

2.1 Introduction

The clinical studies presented in this thesis were undertaken between 2003 and 2007. Full details of the animals used, specific testing regimens and statistical methods are given in the subsequent relevant chapters. The general section details procedures common to all groups and provides an overview of the investigations outlined in the subsequent chapters.

2.2 Animals

Two hundred and forty six horses were used in this project. The majority of these horses (221) were Thoroughbreds. These horses could be separated into a number of groups. All were examined and sampled with permission from the owners, keepers or trainers. In the groups presented with evidence of disease, all of the findings from the project were made available to the owners/keepers or trainers during treatment planning. Sample size calculations to evaluate the power of the studies to detect biologically meaningful effects were performed retrospectively and are included in the subsequent chapters where relevant.

2.2.1 Group One - 50 Clinically normal, Thoroughbred horses

Fifty horses from a stud farm were blood sampled in August 2003. These horses were selected to be representative of the population of animals available. Each horse was subjected to a full clinical examination, including temperature, pulse, respiration and palpation of superficial lymph nodes. In addition, a thorough history was taken in an attempt to ensure that there was no evidence of disease. Clinical examination was carried out following blood sampling to reduce the effects of the stress of handling on the various haematological and biochemical parameters. This group of apparently normal horses was further subdivided into foals (n=16), young adults (n=28), and adults (6). For the purpose of this study an animal was considered to be a foal between birth and eight months of age, a young adult between 9 months and 4 years and an adult if it was 4 years or older. These subdivisions were created for the purpose of this study as there is no generally accepted definition in the literature. The young horses sampled, bred for sale, were all weaned, housed individually at night and mixed at pasture during the day. All brood mares had foals at foot, were housed individually during the night and mixed with other brood mares and foals throughout the day. All of the “in training” group had begun initial (pre) training

and were managed by full time stall confinement when not exercising. Full details of the horses in Group 1 can be found in Appendix 1.

2.2.2 Group 2 - 25 horses presented for a variety of surgical procedures

Twenty-five horses admitted to the University Veterinary Hospital, University College Dublin, Ireland were sampled in one month in 2003. These horses were further subdivided into two groups. The first group (n=18) (the elective group) included horses presented for elective surgical procedures, that is animals with no active inflammation or concurrent disease. The second group included 7 horses with on-going disease presented for non-elective surgery.

2.2.3 Group 3 - 59 horses presented for the investigation and treatment of colic

Fifty nine horses were presented to one of three university veterinary hospitals, including University College Dublin, Ireland, The University of Copenhagen, and The University of Glasgow for the investigation and treatment of colic. The selection for this group was as follows; any horse presented to the equine emergency service at any one of the above three clinics between 2004 and 2006 which was determined to have a lesion which required surgical intervention and was thereafter subjected to surgery no matter the surgical outcome.

This group was further subdivided into one of two categories: (1) Horses recovered from surgery and subsequently discharged from the hospital, (2) Horses that were either euthanised or died were consequently not discharged from the hospital.

Further details of these animals including reason for admission and details of treatment and outcome are given in Chapter 4.

2.2.4 Group 4 - 100 racehorses in training

One hundred Thoroughbred racehorses from nine separate training premises were blood sampled on three separate occasions between May 2003 and September 2003. This number was chosen due to numbers available, and based on geographical location of the training premises and the numbers that could be reasonably sampled on individual occasions. These horses were selected

randomly using a random number generator system based on stable door number, from eight national hunt and one flat training yard. History, including concurrent illness, past and present performance and current level of exercise, if any, was recorded for all individuals included in this group. Each horse was subjected to a full clinical examination, including temperature, pulse, respiration and palpation of superficial lymph nodes. Clinical examination was carried out following blood sampling to reduce the effects of the stress of handling on the results.

Following sampling, these horses were subjected to gastroscopic examination. The degree of gastric ulceration, if present was scaled using the grading system described in 2.5 below. At the time of the research, it was accepted practice to use the grading system described to give a global score for ulceration across both regions of the stomach, rather than a separate score for squamous and mucosal ulceration as is common practice today (Murray *et al.*, 1996) Full details of the horses in Group 4 can be found in Appendix 2.

2.2.5 Group 5 - Thoroughbred foals exposed to, and subsequently naturally infected with *Rodococcus equi*

Twelve Thoroughbred foals from the same commercial stud operation from which normal horses were collected in 2.2.1, but from another premises, were blood sampled on one occasion. These foals were part of a larger group of foals sampled as part of an on-going disease surveillance regimen on this part of the farm. The selected foals were between the ages of 3 and 6 months and clinically normal at the time of blood sampling, but subsequently developed clinical signs of infection with *R. equi*, confirmed by bronchoalveolar lavage and culture of the organism, within 4 weeks of the sampling date. All 12 foals were subjected to a clinical examination, including temperature, pulse, respiration and palpation of superficial lymph nodes at the time of sampling (i.e. before they became infected).

2.3 Anaesthetic Protocol

The horses in Groups 2 and 3 were subjected to general anaesthesia. All of the horses were anaesthetised using a standard protocol which consisted of sedation with 0.05mg/kg acepromazine maleate (ACP-Novartis), 1.1mg/kg xylazine HCL (Chanazine; Chanelle) and 0.05mg/kg diazepam (Valium; Roche). Anaesthesia

was induced with 2.2mg/kg ketamine HCL (Narketan 10; Vetoquinol) and maintained with halothane or Isoflurane (Halothane-Vet; Merial or Isothane-Novartis) in oxygen delivered in a circle system. All the horses received 22,000 iu/kg procaine benzyl penicillin (Depocillin; Intervet) tetanus antitoxin (Intervet) and 4.4 mg/kg phenylbutazone (Phenylarthrite; Vetoquinol) before anaesthesia was induced. Recovery from anaesthesia was not assisted for Group 2 but was assisted for Group 3.

2.4 Techniques

2.4.1 Blood sampling

Blood was obtained from each horse by jugular venopuncture using a vacutainer system and a 21g 1.5inch needle. Samples were obtained to determine full haematology, selected biochemical analysis and concentration of the acute phase proteins, fibrinogen, haptoglobin and SAA. Blood samples were collected into a plain tube, a tube containing lithium heparin, and a tube containing ethylenediaminetetracetic acid (EDTA). Clinical examination was carried out following blood sampling and gastroscopy (in Group 4) to reduce any effect of the stress of examination and handling on the concentration of the analytes. All of the horses were restrained by their regular handler where appropriate.

Blood samples preserved in EDTA and lithium heparin were analysed immediately for routine haematological and biochemical analytes and fibrinogen, at the diagnostic laboratory of the School of Veterinary Medicine, University College Dublin (Groups 1, 2, 4 and 5) and the diagnostic laboratories of the School of Veterinary Medicine, in Dublin, Copenhagen or Glasgow (Group 3). Quality assurance testing was in place for each of the clinical laboratories and identical analysers were used for automated analysis.

Blood in the plain tube was centrifuged at 5000rpm for five minutes, and the serum separated into three, 1.5mL aliquots. These samples were stored at -80°C until they were analysed for concentration of SAA and Haptoglobin.

While it would certainly have been desirable to run all of these samples in one laboratory over a short time frame, due to the nature of the project this was simply not possible and, had this been attempted, the effects of transport and storage on samples could well have introduced discrepancies in any comparisons that were subsequently made. All of the laboratories conformed to ISO standard 15189 and had been approved by the ECVCP laboratory standards committee.

2.4.2 Sampling Details

Information regarding timing of samples and details of testing are given in the subsequent chapters where relevant.

2.4.3 Routine haematology and biochemical analysis

Haemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte count (RBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), were determined using an Abbot Cell-dyn 3500 analyser. The differential white blood cell count and platelet counts were determined using a manual blood smear technique, using a Giemsa stain.

Clinical biochemistry analytes were measured using a Bayer Opera Automated Analyser. Analysis of groups 1, 2, 4 and 5 were all carried out in the same laboratory at the School of Veterinary Medicine, University College Dublin. Analysis of Group 3 bloods were performed at the Schools of Veterinary Medicine, in Dublin, Glasgow and Copenhagen. The same analysers were used, operated by similarly qualified individuals and while there were minor differences in reference ranges, these were small. Each of the laboratories in which the samples were run was subjected to national/international accreditation programmes as discussed.

2.4.4 Acute phase protein assays

All of the SAA and haptoglobin tests were performed in the biochemistry laboratory at the Irish Equine Centre. Where possible sample groups were mixed for analysis. Mixing of sample groups was performed in order to reduce any effects of changes in reagents, sample kits and operator on the results.

2.4.4.1 Serum Amyloid A Assay

Serum amyloid A concentration was determined using a commercial, enzyme-linked immunosorbent assay (ELISA) (Tridelta Phase series) and a Multiscan Multisoft photometer plate reader. The detection limit for SAA was 0.5 µg/mL. Six standards and 90 samples were added to 96 well plates coated with monoclonal antibody specific for SAA. Biotinylated anti-SAA monoclonal antibody was then added. Any SAA present in the well was both captured on the plate by the immobilised antibody and labelled with conjugate antibody in a one-step

procedure. After washing off any unbound material, streptavidin-horse radish peroxidase conjugate was added to the well and incubated. Following a second incubation, Tetramethylbenzidine (TMB) substrate solution was added. The intensity of the colour produced was proportional to the concentration of SAA present in the original sample.

The intra-assay coefficient of variation was 7.7% and the inter-assay coefficient of variation was 10.8% at mean concentrations of 74 µg/ml and 81 µg/ml, respectively. The coefficients of variation were determined by screening three samples 16 times in three consecutive batches.

Detailed Procedure

- (1). Each serum sample was diluted 1: 500 in diluent buffer prior to analysis. This amounted to 5ul of sample in 2.5ml of diluent buffer. Following dilution samples were vortexed vigorously to ensure mixing.
- (2). SAA standards were prepared using the standard provided and diluent buffer. This provided the working standards shown in the table 1 below along with the values for the horse.
- (3). 50ul of diluted (50ul in 5mls of diluent buffer) biotinylated anti-SAA was added to each well
- (4). Vortexed diluted samples were then added to each well and the plate was tapped gently to mix the well contents. The plate was then covered and incubated at 37°C for one hour.
- (5). Following incubation the plate was washed four times with diluted wash buffer, before drying the plate by tapping on absorbent paper.
- (6). 100ul of streptavidin peroxidase was then added to each well. The plate was then recovered and incubated at room temperature for 30 minutes in the dark.
- (7). The plate was then re-washed as described in step 5.
- (8). 100ul of TMB substrate was then added to each well. The plate was then recovered and incubated at room temperature for 13minutes in the dark.
- (9). Finally 50ul of stop solution was added to each well.
- (10). Absorbances were measured and results calculated using a Multiscan Multisoft photometer plate reader. Absorbance of each well was read at 450nm.

2.4.4.2 Haptoglobin assay

Tridelta Phase range kits were used to determine haptoglobin concentrations in each sample. The principle of the assay uses the peroxidase-like activity exhibited by free haemoglobin, this activity is inhibited at low pH. Haptoglobin present in the sample combines with haemoglobin and at low pH preserves the peroxidase activity of the bound haemoglobin. Preservation of the peroxidase activity of haemoglobin is directly proportional to the amount of haptoglobin present in the sample. Although the assay can be performed manually, all samples were processed using an automated method on a Cobas Mira analyser. Reagent one (premixed haemoglobin), chromogen and substrate (stabilised hydrogen peroxide) were placed in the required storage vessels on the instrument. Single point calibration was carried out using the 2.0mg/ml standard, provided in the kit, diluted 1:1 to give a 1mg/ml calibrator. The standard was then run on the instrument as a quality control. Samples were placed on the instrument and results produced. The Cobas Mira instrument was set to the protocols provided by the Tridelta company for haptoglobin measurement. The intraassay coefficient of variation was 1.36%, and the interassay coefficient of variation was 11%. The analytical sensitivity was determined as 0.05mg/mL.

Table 2.1 Working Standards and Volume of Diluent used in the SAA Assay

Tube Number	Volume of Callibrator (ul)	Volume of Diluent (ul)	Serial Dilution	Equine Standards (ng/ml)
T1	60	240	-	20
T2	-	150	150 T1	10
T3	-	150	150 T2	5
T4	-	150	150 T3	2.5
T5	-	150	150 T4	1.25
T6	-	150	-	0.625

2.4.4.3 Fibrinogen Assay

The concentration of fibrinogen was determined using the heat precipitation method (Millar *et al.*, 1971) and was carried out in the diagnostic laboratory of the School of Veterinary Medicine, University College Dublin (Groups 1, 2, 4 and 5) and the diagnostic laboratories of the School of Veterinary Medicine, in Dublin, Copenhagen or Glasgow (Groups 3).

A potential source of error with the heat precipitation method for fibrinogen concentration determination is localisation of the interfaces of the pale brown buffy coat and the white layer of packed fibrinogen. Occasionally these are not at right angles to each other in which case the test should be repeated. Precise vernier reading is essential. Using 20 randomly selected blood samples at each laboratory, two separate aliquots were taken and estimations of fibrinogen concentration were made on each of these. The mean difference was 0.124mL/100mL (SD \pm 0.114). Ninety-three per cent of the estimations had a test/retest difference of less than 0.3mL/100mL. Any test showing a difference of more than 0.3mL/100mL was repeated: this difference was equivalent to 30 mg/100 ml, i.e., about 10% in the normal plasma fibrinogen range. The intraassay coefficient of variation was 3 -7%, and the interassay coefficient of variation was 6-9%. The analytical detection limit was set at 0.5g/dL.

Procedure

- (1). Two microhaematocrit tubes with freshly drawn and anticoagulated (EDTA) whole blood were centrifuged as for measurement of the standard microhematocrit.
- (2). The first tube was broken just above the RBC/plasma interface. The plasma was dropped onto the prism of the refractometer and the total protein concentration (g/L) was read and recorded.
- (3). The second tube was incubated for 3 minutes in a 58° C water bath. The fibrinogen precipitates as a white ring above the RBC.
- (4). At the completion of the 3-minute incubation, the second tube was re-centrifuged and then broken above the plasma/fibrinogen interface. Total protein of this remaining plasma was measured as previously described.

(5). The fibrinogen concentration was determined to be the difference in the total protein measurement of the first microhematocrit tube minus the incubated and recentrifuged second microhematocrit tube.

2.5 Gastric endoscopy

The trainers were instructed to starve each horse for at least 24 hours prior to examination, muzzles were provided for this purpose.

Using a three metre, 12.8mm diameter video endoscope (VES, UK) passed naso-oesophageally, each horses stomach was examined on three separate occasions. The entire stomach was examined and the scope was then retroflexed to allow examination of the cardia and fundus (Figure 2.3a and 2.3b). Each examination was video recorded.

Figure 2.1a Schematic showing the technique for examination of the stomach

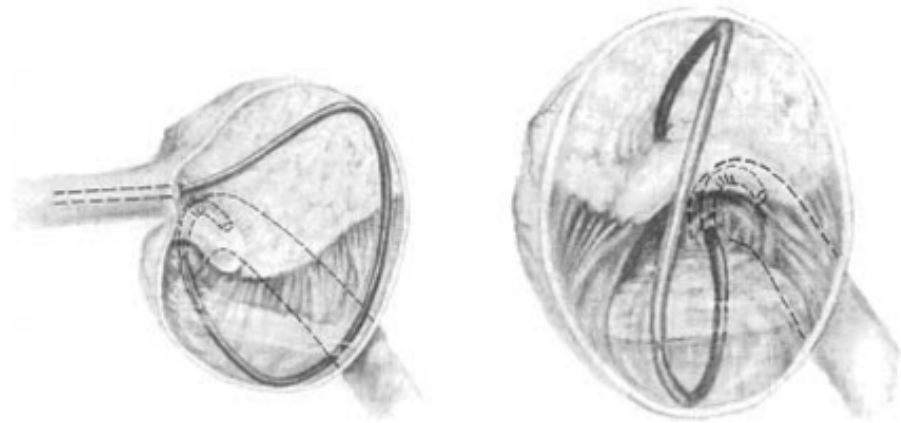


Figure 2.1b Gastroscopic image showing the endoscope retroflexed in the stomach in order to image the cardia region

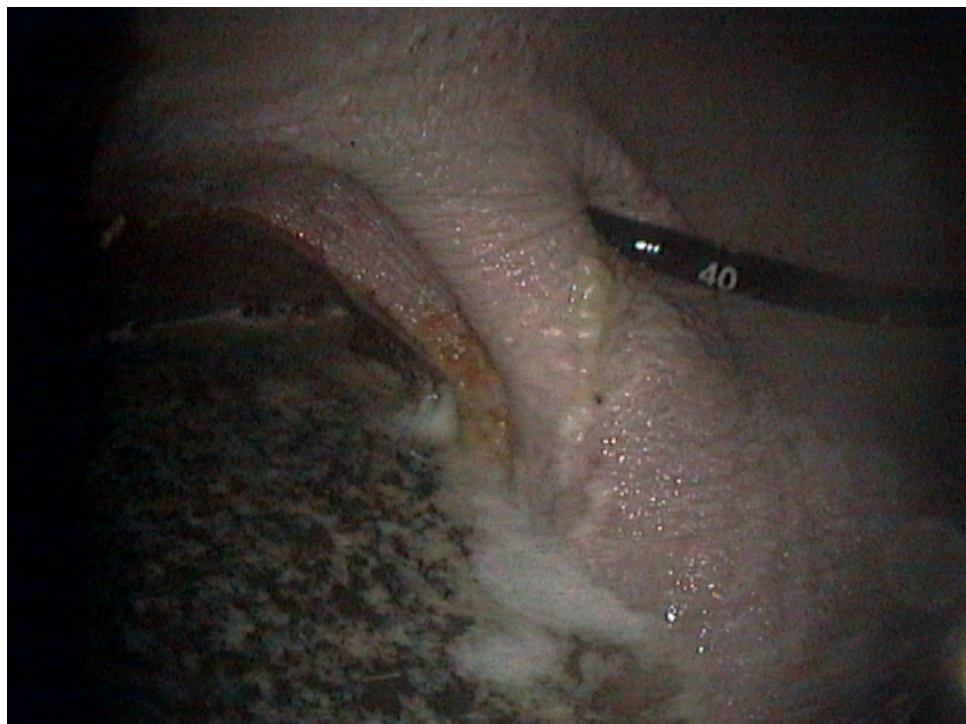


Table 2.2 Scoring System for Gastric Ulceration Used in the 100 Thoroughbred Horses in Training (after Murray *et al.*, 1988)

Score	Squamous mucosa	Glandular mucosa
0	Normal	Normal
1	Mild hyperkeratosis, hyperaemia	Focal areas of hyperaemia
2	Moderate hyperkeratosis, hyperaemia, 1-2 small erosions	Multifocal hyperaemia, up to 3 small lesions
3	Multifocal small erosions, with hyperaemia +/- hyperkeratosis	Multifocal hyperaemia, up to three small lesions
4	1-4 small ulcers, minimal thickening at margin, +/- small erosions	> 3 small lesions, multifocal hyperaemia
5	Deeper appearing ulceration, mild to moderate thickening of margins, +/- bleeding	1-2 moderate sized lesions, hyperaemia
6	Multifocal ulceration, mild to moderate thickening of margins, +/- bleeding	1-2 large lesions, hyperaemia
7	More extensive, deep-appearing ulceration, +/- bleeding	1-2 large lesions, + smaller lesions, hyperaemia
8	Focal large, deep-appearing ulceration, +/- multifocal erosions/ulcers, +/- bleeding. More extensive and with more changes in surrounding tissue than for 7	1-2 large, deep-appearing lesions, +/- smaller lesions, hyperaemia
9	Extensive, deep ulceration with bleeding, covering larger area than for 8	3-4 large, deep-appearing lesions, +/- smaller lesions, hyperaemia
10	Most severe, most extensive, deepest-appearing ulceration. Active bleeding or evidence of recent bleeding. Majority of mucosa ulcerated	5 or more large, deep-appearing lesions, +/- small lesions, hyperaemia

Figure 2.2 Gastroscopic image of a normal stomach showing the margo plicatus, glandular and squamous regions



Figure 2.3a and b. Gastroscopic images of a stomach with Grade 4 ulceration



Figure 2.4 Gastroscopic image showing moderate ulceration (Grade 6)

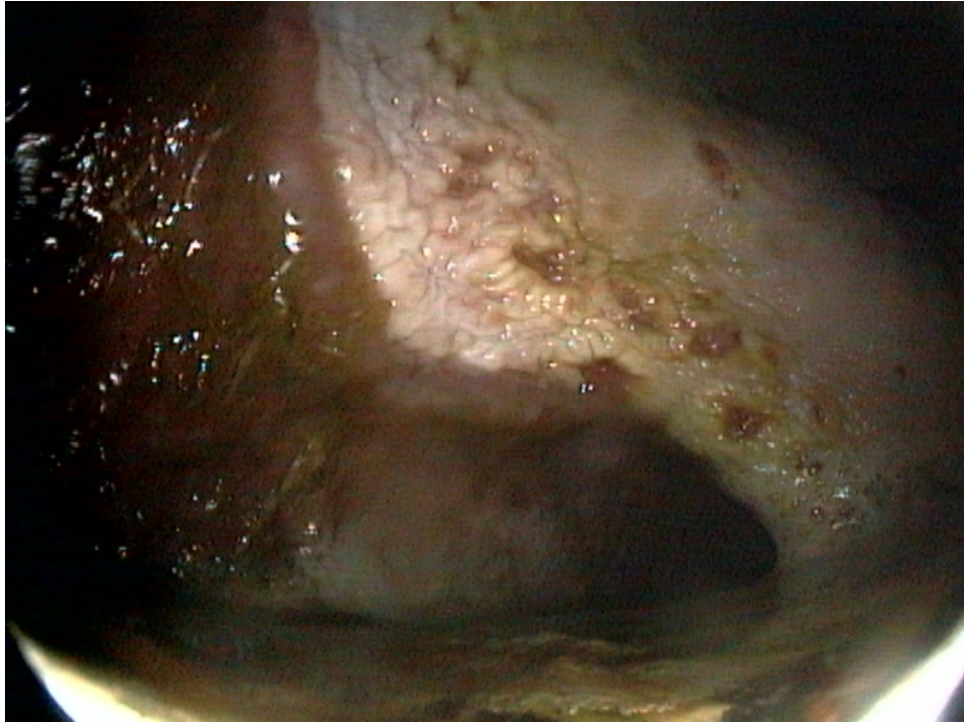
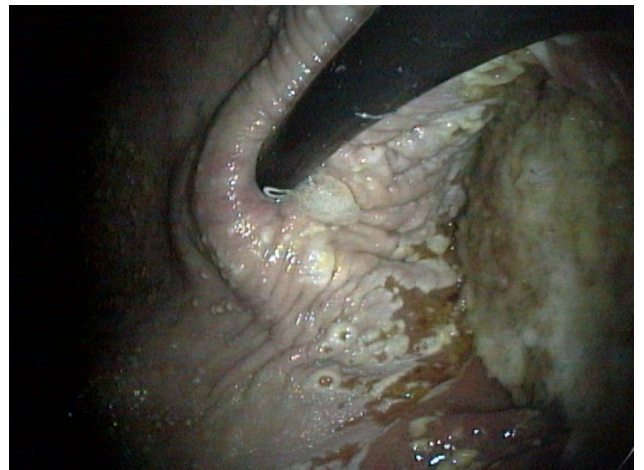


Figure 2.5a and b. Gastroscopic images showing severe ulceration around the cardia (Grade 8)



2.6 *Rhodococcus equi* Diagnostics

2.6.1 Bronchoalveolar lavage technique

1. All foals were manually restrained and a nose or ear twitch applied for restraint. A number of fractious individuals were sedated with a combination of xylazine hydrochloride (0.3-1mg/Kg) and butorphanol tartrate (0.1mg/Kg).
2. A standard coiled, guarded, bronchoalveolar (BAL Cook) lavage tube, with a sterile sample tube, was lubricated with KY-jelly and passed into the ventral meatus of the right or left nostril. As the tube reached the pharynx the neck was extended to facilitate passage of the tube through the rima glottis (a combination of lack of resistance, and coughing confirmed correct placement). The tube through which the sample is obtained is contained within an outer tube to prevent contamination from the upper portion of the respiratory tract. A new sterile tube was used for each foal.
3. The BAL tube was advanced until resistance was encountered; some coughing was usually encountered at the level of the carina. 5-10mL of sterile fluid was used to inflate the BAL tube cuff in order to maintain the tube in the correct position and 10mL of lidocaine hydrochloride was instilled into the tube at this point.
4. 40mL of sterile saline was instilled through the BAL tube into the lungs and immediately aspirated. The aspiration was repeated 2-3 times until at least 20mL was retrieved. The fluid was split between sterile plain and EDTA filled containers. Finally samples were submitted for culture and sensitivity, differential cell counts and qualitative cell morphology.

2.6.2 Cytology and culture of *Rhodococcus equi*

Samples were prepared for cytological examination using the following technique.

1. Samples preserved in EDTA were centrifuged in order to concentrate cellular material and smears were made and allowed to air dry.
2. Air dried smears were stained with H&E stain and allowed to air dry.
3. The slides were examined at high power and >500 cells were typically examined.
4. A diagnosis of *R. equi* was confirmed based on the presence of multiple gram positive coccobacilli during cytological examination of the BAL fluid (Figure 2.8).

The fluid contained in the plain BAL tube was subjected to microbial culture. *R. equi* colonies form at 30°C on sheep blood agar, they are characteristically small smooth and shiny at 24 hours becoming salmon pink in colour and mucoid by 72 hours (Figure 2.9). The organism is catalase and urease positive and CAMP + with a characteristic shovel shaped zone of hemolysis

Figure 2.6 BAL cytology showing macrophages containing multiple cocci-shaped bacteria. (H&E x 400)

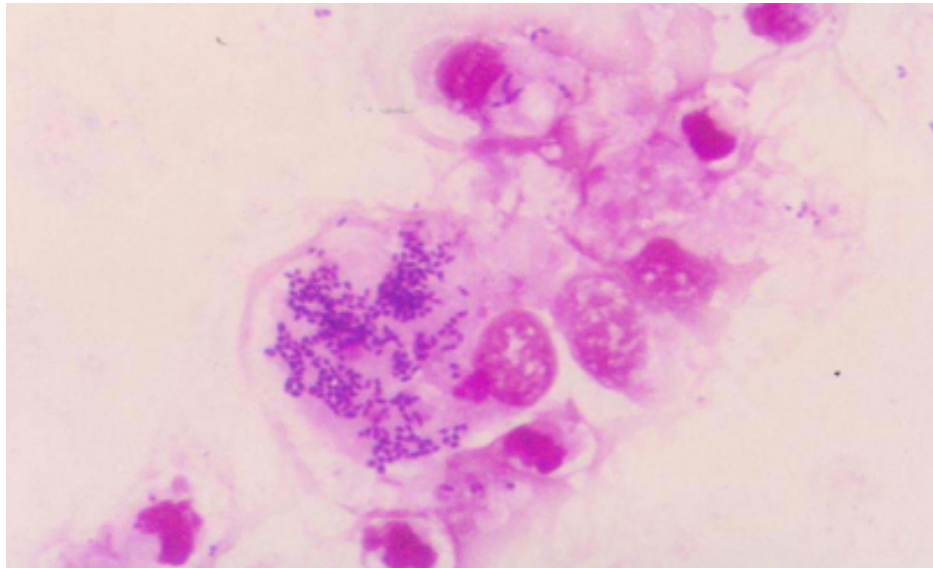
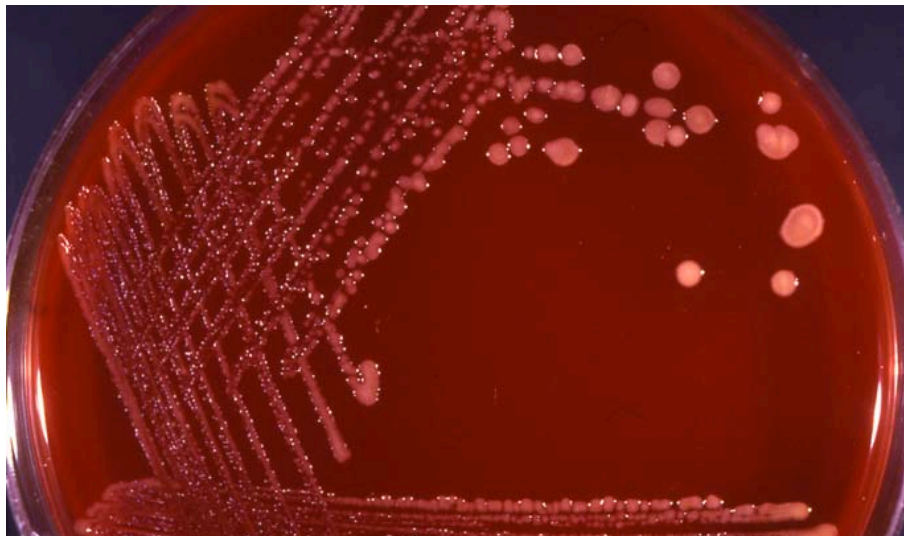


Figure 2.7 Agar plate showing growth on sheep blood agar at 72 hours
R. equi colonies are typically smooth, mucoid and salmon pink.



2.7 Statistical Analysis

These data were manipulated using Excel (Microsoft Office for Mac, Versions 2007 -2011). Statistical analysis was performed using STATA Version 8, Minitab Version 16 and SPSS version 12. Graphs and tables were produced using Excel and Minitab. The following tests were used to evaluate statistical significance in the studies presented in the Chapters that follow.

2.7.1 One-way analysis of variance (ANOVA) (Chapter 3)

Analysis of variance is a technique to determine whether the mean of a variable is influenced by different types and combinations of factors. One way analysis of variance is an extension of the independent t-test and was used to compare any number of groups or treatments. The reason ANOVA is used instead of multiple t-tests is to reduce the chance of a type 1 error. That is incorrectly rejecting the null hypothesis and incorrectly concluding that a difference did not occur by chance. In ANOVA calculations variances are used rather than standard deviations to measure variability (Bland 2001; Bewick, Cheek, Ball 2004). ANOVA testing was completed using the statistical package STATA V8.

2.7.2 Logistic regression (Chapters 5 and 6)

During analysis of much of the data presented there were multiple explanatory variables examined; consequently, methods to facilitate multivariable analysis and account for potential confounding were used. In most cases, the outcomes of interest were binary in nature, e.g. presence or absence of gastric ulceration, survival or death following colic surgery; as a result, logistic regression modelling was utilised. Logistic regression models the natural logarithm (ln) of the odds of an outcome for a given value of explanatory variable(s). The odds are defined as the probability of having an outcome (e.g. ulceration) divided by the probability of not having the outcome (e.g. no ulceration). A major advantage of performing multivariable logistic regression is that multiple explanatory variables can be considered at once.

A logistic regression model can be represented using the following equation:

$$\ln \left[\frac{p}{1-p} \right] = \alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_i x_i$$

In the above equation: $\ln \left[\frac{p}{1-p} \right]$ the log transformation of the odds of outcome, where p = probability of outcome

α = the intercept term, which represents the value of y when $x=0$

B_i = the regression coefficients, which represent the change in y for a unit change in x_i , whilst the values of the other explanatory variables remain constant

x_i = the explanatory variables.

(a) Univariable logistic regression

Logistic regression was used to develop models to determine whether any of the variables measured during the studies presented here could be used to predict the outcome. In logistic regression the relationship between the variables is asymmetric, in that the value of one variable is caused or can be predicted by the value or state of another variable or variables (Dohoo *et al.*, 2003). Each potential predictor variable was examined with respect to its association with the relevant outcome variable one at a time. Predictor variables were considered for inclusion in multivariable models where the likelihood ratio p -value during univariable analysis was less than 0.25. Continuous explanatory variables were examined in their original continuous form (assuming a linear relationship between explanatory variable and outcome) and in their quartiles in order to identify any deviations from linearity. Where continuous variables fitted the model best in a categorical form and two or more levels of that variable were not significantly different from each other, category levels were collapsed together in order to include categorical variables with the fewest possible levels, without compromising model fit. Model fit was assessed by examination of likelihood ratio statistic and models were compared using the *LRTEST* command in STATA.

(b) Multivariable logistic regression

A forward stepwise process was used to build multivariable models. Variables were selected in turn, based on log likelihood values, biological relevance/plausibility and P values. In the models developed for this work, which generally had relatively few potential explanatory variables available, all variables not otherwise included in final multivariable models, were forced into models to examine potential confounding. Confounding was regarded as

significant if the values of coefficients altered by more than 30% (Dohoo *et al.*, 2002). Final multivariable models were compared using the *LRTEST* command in STATA and decisions over the best fit model were made by examination of Akaike Information Criterion (AIC). Hosmer-Lemeshow tests were performed to identify any lack of fit of the final multivariable models.

2.7.2.1 Clustering of data

The structure of data in a number of the studies presented here represents populations in which there is a hierarchical relationship between some of the variables, that is to say that one variable affects and may be dependent on another (Goldstein, 2003; Rahaman *et al.*, 2011). In addition, in a number of the studies presented, repeated measurements were taken from individual horses and these horses may have had further relationships (managed in the same way, on the same yard etc.). Repetition of analysis following these types of relationships between horses violates some of the assumptions made regarding the independence of the logistic regression model. The consequence of this is that failure to account for clustering may lead to artificially small standard errors, resulting in narrower confidence intervals, P-values that are too small and therefore incorrect inferences (Dohoo *et al.*, 2010) i.e. identifying associations that do not exist. To account for this, multi-level models were also developed where appropriate to allow for these relationships which particularly related to clustering of data due to animals being kept together or managed in a similar way.

2.7.3 ROC curves and predictive ability (Chapters 5 and 6)

A receiver operating characteristic (ROC) curve is a plot of the sensitivity of a test against the false positive rate of the test. The purpose of the ROC curve is to determine the optimal cut point for distinguishing between affected and non-affected individuals. The shape/angle of the line provides information on the discriminating ability of the test with a 45 degree angled line describing a test that is no better at predicating than chance alone. As the ROC curve moves towards the top left hand corner, the better the ability of the test, described by the plot, to predict outcome the outcome in question it becomes.

The area under the curve can be used to predict the percentage of animals which will be correctly classified by the test. The advantage of using ROC curves

to determine a cut point for sensitivity and specificity is that it describes the overall ability of the test to discriminate affected from non-affected animals over a range of cut points (Dohoo *et al.*, 2003).

2.7.4 Mann Whitney u Test (Chapters 4, 6 and 7)

The Mann Whitney u Test is a test used to compare groups when the dependent variable is either ordinal or continuous but not normally distributed (Dohoo *et al.*, 2010).

The Mann-Whitney U test is a non-parametric test, hence it does not make any assumptions related to the distribution, although the following assumptions are made;

1. The sample drawn from the population is random.
2. Independence within the samples and mutual independence is assumed.
3. Ordinal measurement scale is assumed.

The calculation is as follows, where:

U=Mann-Whitney U test

N1 = sample size one

N2= Sample size two

Ri = Rank of the sample size

$$U = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - \sum_{i=n_1+1}^{n_2} R_i$$

2.7.5 Post Hoc Power Calculations - 2 Sample t test

There are two approaches to power analysis, the first is to calculate the necessary sample size for a specific power, the second is to calculate the power for a given sample size. Sample sizes were based on available data and *post hoc* power calculations were conducted for all of these studies.

STATA version 8.0 was used to conduct the power calculations and perform the two-sample t tests.

2.7.6 Kruskal wallis (Chapter 3)

The Kruskal-Wallis test is non-parametric, rank-based test used to determine whether there are statistically significant differences between two or more groups of an independent variable on a continuous or ordinal dependent variable.

The calculation is as follows, where:

$$H = (N - 1) \frac{\sum_{i=1}^g n_i (\bar{r}_{i\cdot} - \bar{r})^2}{\sum_{i=1}^g \sum_{j=1}^{n_i} (r_{ij} - \bar{r})^2},$$

N_i is the number of observations in group i

R_{ij} is the rank (among all observations) of observation j from group i

N = is the total number of observations across all groups

$\bar{r}_i = \frac{\sum_{j=1}^{n_i} r_{ij}}{n_i}$ is the average rank of all observations in group i

$\bar{r} = 1/2(n_i/N+1)$ is the average of all the R_{ij}

2.7.7 Wilcoxon signed-rank test (Chapter 4, 6)

A Wilcoxon signed-rank test was used to compare the pre-surgery concentrations of SAA, haptoglobin and fibrinogen between the horses undergoing elective and non-elective surgery because these data were not normally distributed. The test is a non-parametric statistical hypothesis test which can be used to compare to related samples, matched samples or repeated measurements from a single individual. The test is used as an alternative to the paired t test for data that is not normally distributed.

Chapter 3

Acute Phase Proteins, Haematological and Clinical Biochemistry in Thoroughbred Horses, a “Snapshot” of the Normal Horse

3.1 Introduction

The aims of this chapter were as follows:

1. To determine the serum concentrations of Serum Amyloid A (SAA), haptoglobin and fibrinogen amongst a representative group of horses, judged to be clinically normal, from a well-maintained equine breeding and training establishment.
2. To test the hypothesis that the chosen acute phase proteins are simple to measure in the Thoroughbred and that normal values of SAA are associated with currently accepted indices of clinical normality in both sexes and across a range of ages.

In chapters 1 and 2 acute phase proteins were introduced and the case made for their quantification in the horse in a variety of circumstances some with known overt pathology and others more concerned with particular circumstances, often associated with competition, in which the animals involved are in various ways subject to stress. It was important that before embarking on this study experience was gleaned of the values and range of values of the chosen acute phase proteins in normal healthy animals. The results obtained were intended to serve as an independent set of base line values and to explore in outline the extent to which gender and age were associated with the average values observed. Initially 50 horses were chosen at random, as indicated in chapter 2, in a premises with approximately 250 available horses. It was considered necessary to perform this investigation because, at the time the study was performed, the measurement of SAA was not routine in equine clinical practice. Reference ranges (0-200µg/mL) had been described based on serial examination of small groups of horses (Lumsden and Mullen, 1978; Husebekk *et al.*, 1986; Pepys *et al.*, 1989; Nunokawa *et al.*, 1993; Hulten *et al.*, 1999), and it was generally accepted that serum concentration of SAA could be expected to increase up to 1000-fold following inflammation, infection, tissue injury and cell necrosis and decline rapidly following recovery in horses (Pepys *et al.*, 1989; Hulten *et al.* 1999). However this had never been tested in a group of thoroughbreds and never in the geographical location in which the study was performed.

The acute phase protein concentrations were determined by the methods already given. In addition, to serve as more usual indicators of health, standard haematological and clinical biochemical measurements, again determined as outlined previously, were derived. These measurements are given in Appendix 1. Initially no power calculations were attempted with the intention being to see what was found and from the results to determine whether greater numbers of animals needed to be recruited. Post hoc power calculations were then performed and are presented. The animals available were of three age groups, foals, young adults and adults. As indicated in Chapter 2 horses were defined as foals if they were aged between birth and eight months of age, young adults if they were between nine months and four years, and adults if they were aged from four years or more. It should be noted that these were based on actual time since birth and not the conventional ageing system used for all Thoroughbreds that assumes the 1st January as day one of life. The young horses sampled, bred for sale, were all weaned, housed individually at night and mixed with other individuals of a similar age at pasture during the day. All brood mares had foals at foot, were housed individually during the night and mixed with other brood mares and foals throughout the day. All of the “in training” group had begun pre-training, which involved lunging exercise, with full tack, and were managed, from a housing point of view, in the same way as the other horses. The details of the horses sampled are given in Appendix 1. This group of horses, from an internationally recognised breeding and training establishment, was considered to be representative of the typical thoroughbred common to Europe, the Americas and Australasia.

3.1.1 Variation in SAA measurement

There are a number of potential sources of variation in APP concentration in normal horses. These include, but are not limited to, variation between individuals, different age groups, sex, breed, and variation based on management system, use of the horse, storage and handling of samples, assay used and geography. Some of these potential variables are difficult, if not impossible to test, and are not tested by the investigation described here. The effect of age, sex (Hulten *et al.*, 1999), storage (Hillström *et al.*, 2010), and to some extent breed (Pepys *et al.*, 1989) have been described by previous studies, albeit based on small numbers of horses, and only age has been consistently

shown to result in a small variation in SAA concentration (Hulten *et al.*, 1999). Critically all of the previous work investigating SAA concentration in horses suggests that an increase in serum concentration of 1000 times or more can be expected in response to inflammation, infection, necrosis and trauma (Pepys *et al.*, 1989; Hulten *et al.*, 1999; Pollock *et al.*, 2005; Jacobsen and Andersen, 2007) and therefore in the clinical setting, a small variability between groups of horses based on the variables described are likely to be of little biological significance in the detection of abnormal horses.

3.2 Materials and methods

3.2.1 Ethics

Ethical approval for this study was granted by the Ethics and Welfare committee, School of Veterinary Medicine, University College Dublin. This particular equine premises was chosen due to the numbers of horses available and the fact that a routine sampling regimen was already in place, allowing surplus samples to be collected for the purpose of this study with minimal disruption to the animals and to the routine. The numbers of animals were selected based roughly on the proportions of each age group in the general population. Within these age groups individual animals were selected randomly by placing their stable door number on a card in a box and selecting these by yard (i.e mare and foal yard, brood mare yard, young adult yards).

3.2.2 Experimental design

This was a prospective analytic cross sectional study, 50 horses of mixed age and sex were selected randomly from a population of approximately 250 horses. This number was chosen initially as it was the number of horses that could reasonably be sampled by one person in one morning while causing minimal disruption to the monthly sampling regimen in place on the premises.

3.2.3 Sample collection

Full details of the sampling methods, haematology, biochemistry and acute phase protein assays are given in Chapter 2-General Materials and Methods. Samples were obtained on one occasion in August 2003.

3.2.3.1 Acute phase proteins

The acute phase proteins SAA, haptoglobin and fibrinogen were measured using the assays outlined in Chapter 2.

3.2.3.2 Haematological and clinical biochemistry examination

Haematological examination included determination of haemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte count (RBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and a differential white blood cell count.

The following biochemical analytes were also measured: gamma glutamyl transferase, alkaline phosphatase, creatinine, urea, total protein, albumin, and globulin. These biochemical and haematological analytes were chosen in an effort to determine whether any of the horses sampled had evidence of infectious or inflammatory disease.

3.2.4 Clinical examination

Each horse was observed from a distance (usually over the stable door) for a period of not less than one minute; thereafter the animals were caught and restrained by the regular handler. A full clinical examination including measurement of heart and respiratory rate, rectal temperature, and palpation of superficial lymph nodes was performed. In addition, a history was obtained to determine whether there had been any evidence of disease in the previous month or if veterinary intervention had been sought.

3.2.5 Data processing and statistical methods

3.2.5.1 Data processing

All data were entered into a Microsoft Excel spread sheet (Microsoft Excel for mac). Data were checked for errors, consistency and validity using Excel's standard functions. The data were separated into groups based on age, and sex and, once a final database was produced, it was imported into SPSS, version 8.0, for analysis. Minitab, version 16, and Microsoft Excel for mac, were used to produce graphical summaries of the data.

3.2.5.2 Statistical methods

The results were analysed using the statistical package SPSS version 8.0. The results from all of the horses sampled were analysed using one-way analysis of variance tests.

3.2.5.2.1 Analysis of variance (ANOVA)

Analysis of variance (ANOVA) was used to analyse the differences between group means and variation among and between groups for SAA and haptoglobin. The variance observed in a group of data is partitioned into components attributable to different sources of variation. In this study ANOVA was used to assess whether or not the means of several groups were equal, and therefore generalizes the t-test to more than two groups. This is important as performing multiple two sample t-tests can lead to an increased likelihood a type 1 error. A t-test was also used to analyse the differences between age and sex.

3.2.5.2.2 Kruskal-Wallis analysis

The Kruskal-Wallis test (or One-way ANOVA by ranks) is a non-parametric equivalent of the ANOVA. The test does not assume normal distribution, unlike the one-way analysis of variance. In this test, the null hypothesis is that the medians of all groups are equal, and the alternative hypothesis is that at least one population median of one group is different from the population median of at least one other group. This test was used to analyse the differences between age and sex for fibrinogen concentration.

3.2.5.2.3 Normality testing

These data were tested for normal distribution using Minitab, version 16 standard functions. This software uses an Anderson-Darling test of normality to assess whether the data are normally distributed. A p-value of greater than 0.05 suggests that the data are normally distributed.

3.2.5.2.4 Power calculations

Post hoc power calculations were performed following data collection to determine what change in the concentration of SAA could be detected by the test. Minitab, version 16, standard power calculation functions, were used to

perform this analysis. This uses a one sided t-test with a statistical power of 80%.

3.3 Results

Of the 50 animals identified, there were 16 foals (32% of group), 28 young adults (56% of group) and 6 adults (12% of group). The foals ranged in age from 5 to 8 months, the young adults from 2 to 3 years, and the adults from 7 to 16 years. Of the foals, there were 8 males and 8 females. Amongst the young adults there were 17 males and 11 females. All of the adults were female. Six of the foals sampled were with the adult mares sampled. Signalment details for the 50 horses are given in Appendix 1.

The results of the analysis for the APPs are presented in table 3.1, results for haematology and clinical biochemistry are given in Appendix 1.

3.3.1 Initial Review of Data

As a first step in examining the data, simple graphical representations of selected variables of interest, were produced (Figure 3.1). This was done for a number of reasons: firstly examining the data in this way facilitated identification of errors or missing values in the data; secondly it helped to guide further examination of the data. Of the 50 horses included in the study, a full set of data was available for all of them.

Box plots were produced showing each of the APPs by age group and by sex; the concentration of the acute phase proteins, SAA, haptoglobin and fibrinogen are shown (Figures 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7).

3.3.1.1 Method

Box plots were produced using Minitab, version 16 for each of the variables outlined above (Figures 3.2-3.7). A histogram (Figure 3.1), of mean results for the 3 acute phase proteins was produced using Excel for mac. These graphical representations suggested that the results were similar between the 3 age groups for all of the analytes examined except for haptoglobin and fibrinogen concentration for which the foals were noted to have a higher concentration than the young adult or adult groups (Figures 3.3 and 3.4).

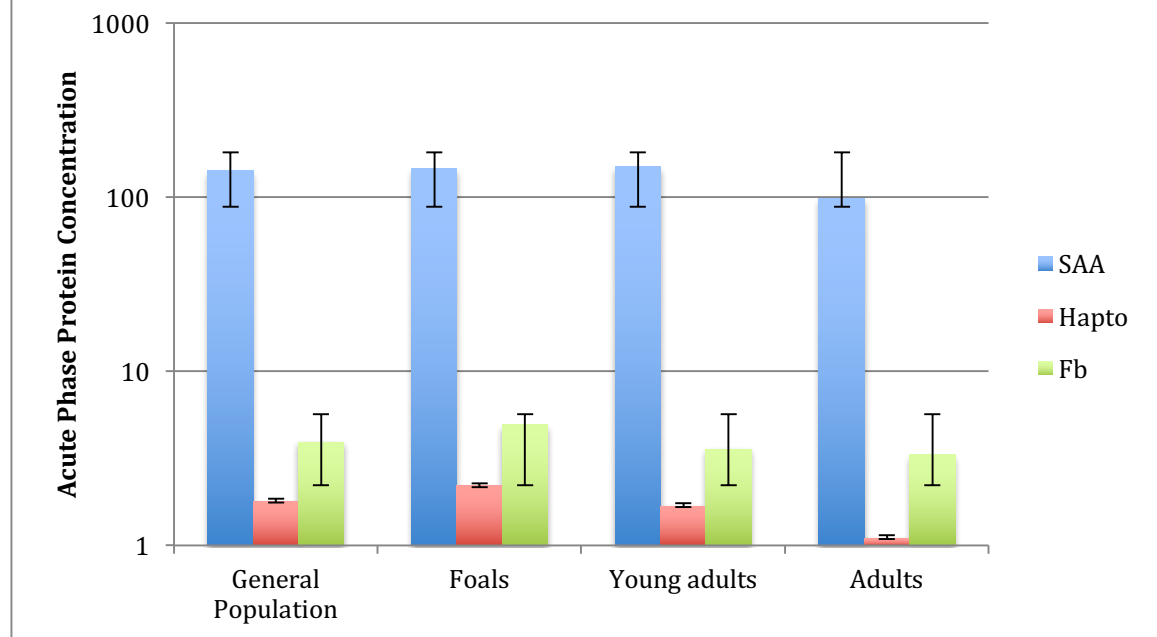
Table 3.1. Acute Phase Protein Profile for the General Population, Foals, Young Adults and Adults in Group 1

	SAA	Haptoglobin	Fibrinogen
	(µg/mL)	mg/mL	(g/L)
	Mean (sd)	Mean (sd)	Median
	Range	Range	Range
General Population n=50	141.9 (65.22)	1.8(0.58)	3.96
	16-299.5	0.5-2.7	2.0-6.0
Foals n=16	144.92 (45.27)	2.21* (0.35)	4.0
	53.5-196.1	1.0-2.7	4.0-6.0
Young adults n=28	149.52 (73.63)	1.7 (0.50)	3.6
	16-299.5	0.8-2.6	2.0-6.0
Older adults n=6	98.68 (45.27)	1.11 (0.70)	4.0
	42.5-194.8	0.5-2.4	2.0-4.0

* Significantly greater concentration than the two older groups, (P=0.01)

The results for fibrinogen were not normally distributed and thus are presented as medians with a range.

Figure 3.1. Mean Concentration for the Acute Phase Proteins



Histogram showing the concentrations of the three APPs for the general population, and for the three age groups. Error bars show 95% C.I. for SAA and haptoglobin and 2.5% and 97.5% percentiles for fibrinogen.

3.3.2 Normality testing

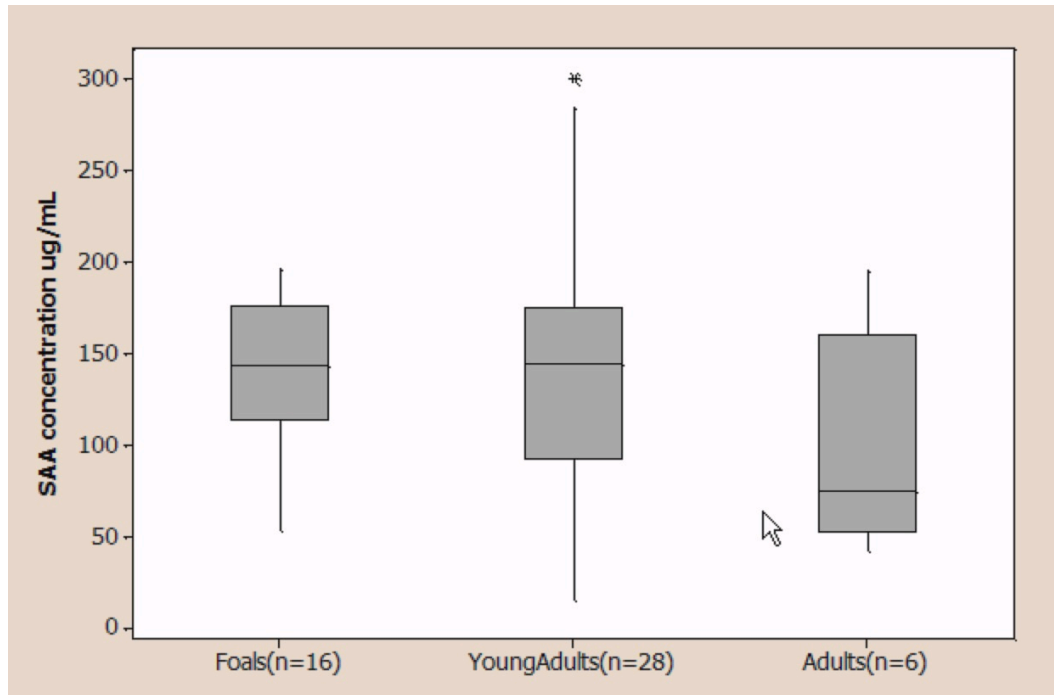
The data obtained for each of the acute phase proteins were assessed for whether they satisfied the conditions for normal distribution. Minitab, version 16 was used to produce scatterplots comparing the data to values that would be attained by numbers drawn independently from a standard Normal distribution. When the points plotted are close to the line along the diagonal, they are close to normal; horizontal departures (along the data axis) indicate departures from normality. The scatter plots produced demonstrated that the results for SAA and haptoglobin were normally distributed; however, the results for Fibrinogen concentration were not.

3.3.3 Statistical analysis and summary

Haematology and clinical biochemistry results, including the acute phase proteins, haptoglobin and fibrinogen, were all within previously reported reference ranges described for the domestic equid, *Equus caballus*. All of the results for SAA were within the reference ranges described except for three of the young adults. (Lumsden and Mullen, 1978; Taylor-MacAllister *et al.*, 1997; Tyler-McGowan *et al.*, 1999). Close inspection of the results including haematological, clinical biochemical and clinical examination findings from all of the animals included, revealed no analyte or clinical finding, or combination of findings, that would lead an experienced clinician to suspect any of these animals to be anything other than healthy and normal. The yard manager reported that the three young adults with mild increases in SAA concentration developed evidence of an upper respiratory tract infection between 5 and 10 days after sampling.

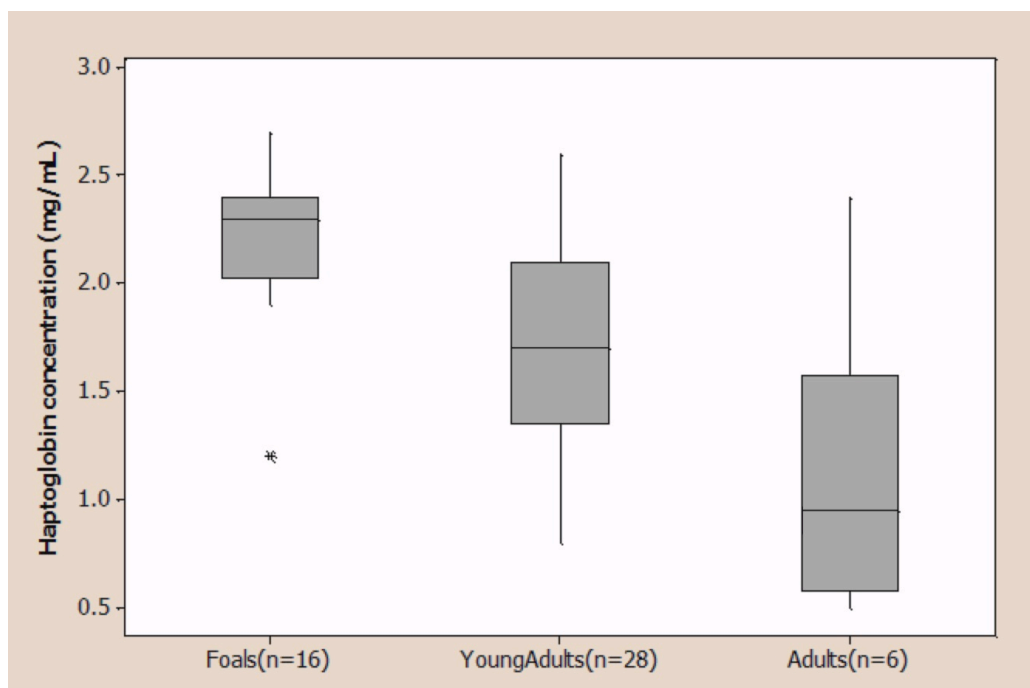
There were no significant differences in the concentration of serum amyloid A between horses of different ages and sexes (compared using ANOVA), but the concentration of haptoglobin was significantly higher in the foals than in the two adult groups ($P < 0.001$). There were no significant differences in the concentration of fibrinogen between the groups ($P = 0.435$) (these were compared using a Kruskal-Wallis analysis as the fibrinogen results were not normally distributed). There was no significant difference between the concentrations of haptoglobin or fibrinogen in the two adult groups.

Figure 3.2 Box plot of SAA concentration for the three groups of normal horses



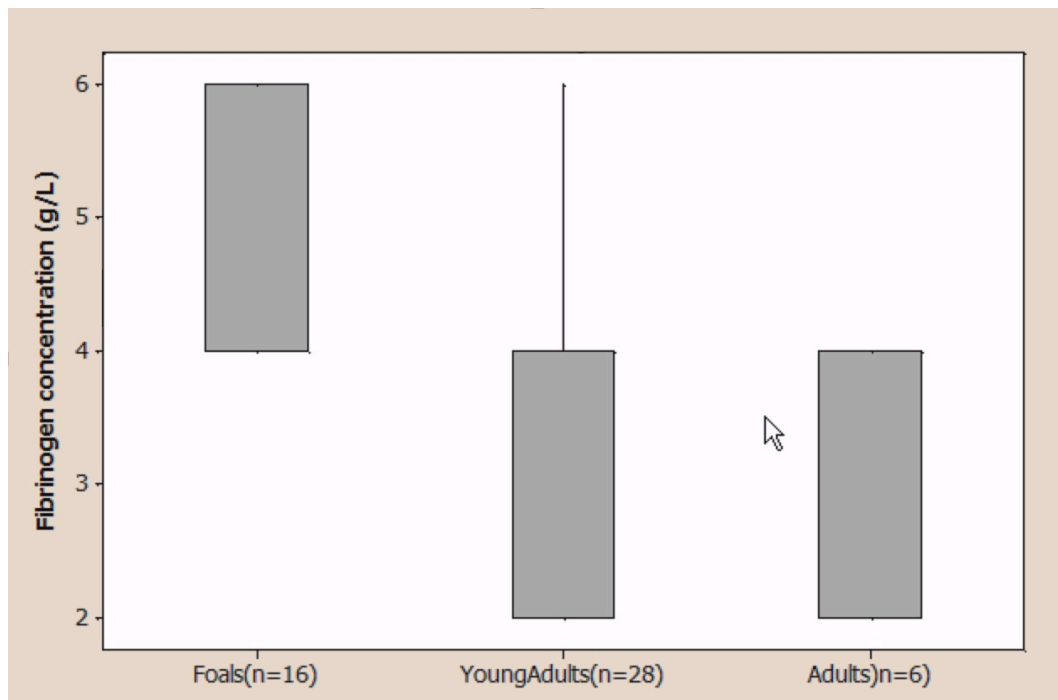
The box and whisker plot allows comparison of SAA concentration across the three age groups of horses and further shows the maximum, minimum, median and any outliers for each group. The horses that later developed an upper respiratory tract infection can be clearly seen in the young adult group.

Figure 3.3 Box plot of Haptoglobin concentration for the three groups of normal horses



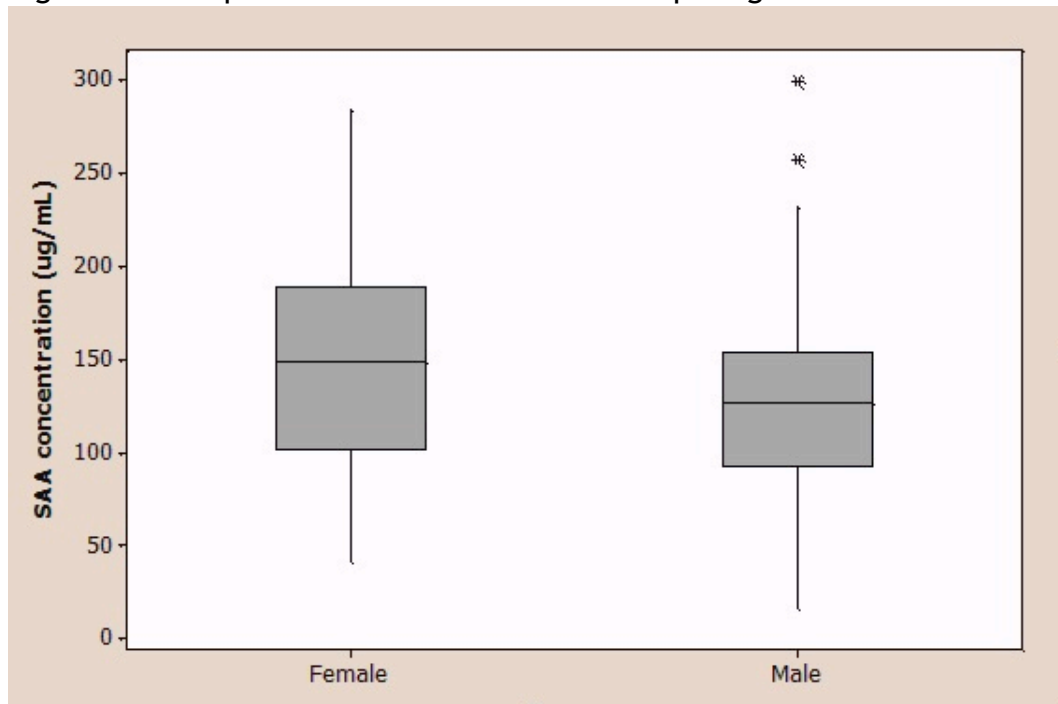
The box and whisker plot allows comparison of Haptoglobin concentration across the three age groups of horses and further shows the maximum, minimum, median and any outliers for each group.

Figure 3.4 Box plot of Fibrinogen concentration for the three groups of normal horses



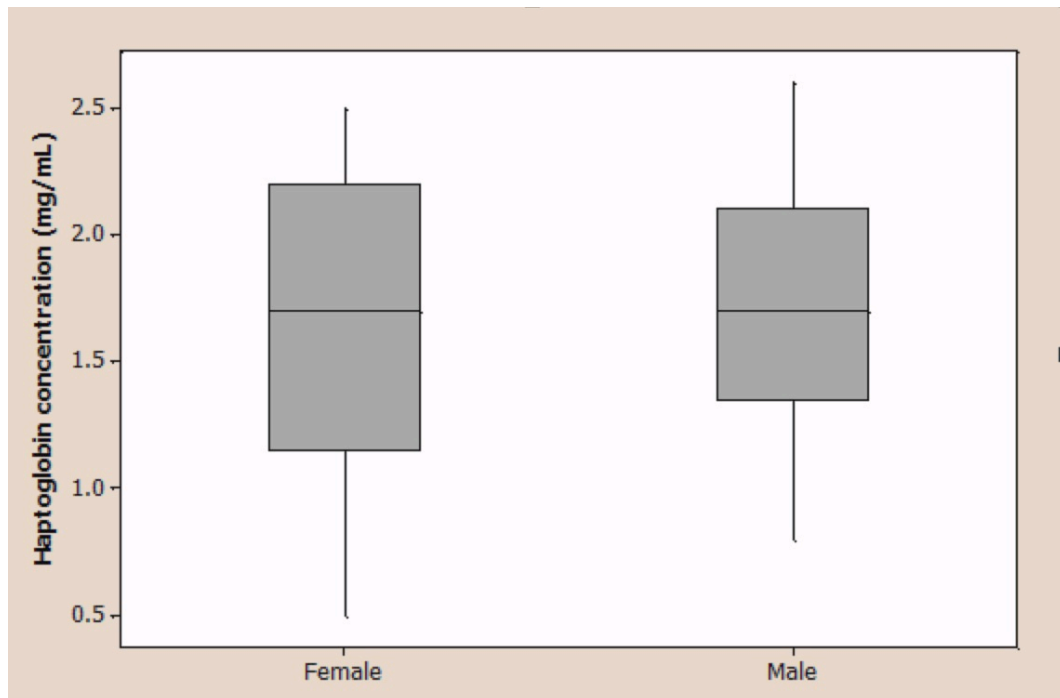
The box and whisker plot allows comparison of SAA concentration across the three age groups of horses and further shows the maximum, minimum, median and any outliers for each group.

Figure 3.5 Box plot for SAA concentration comparing females and males



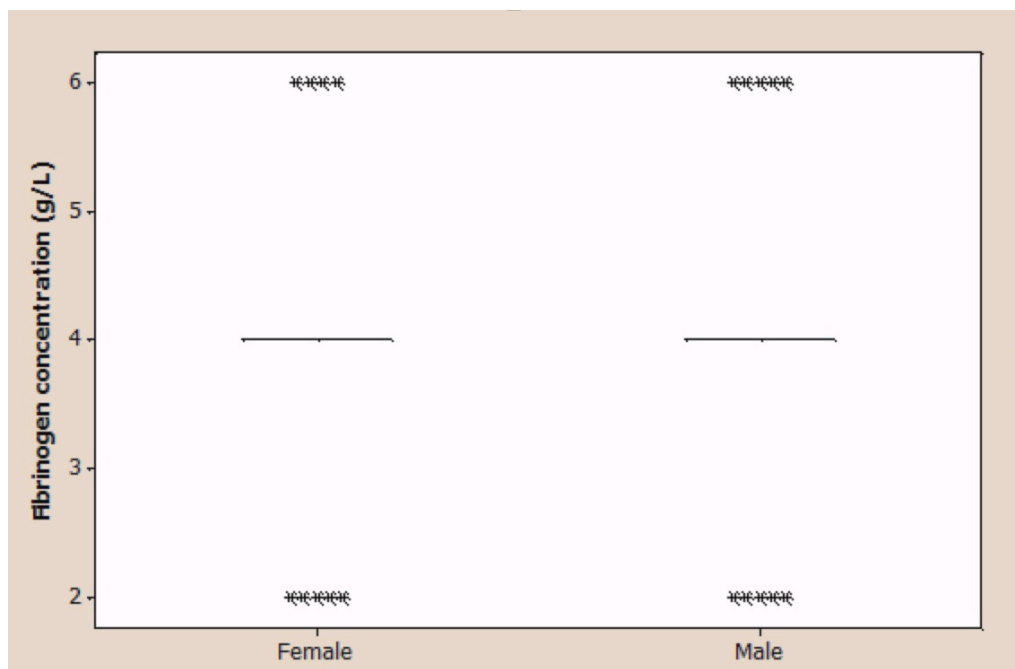
The box and whisker plot allows comparison of SAA concentration by sex and further shows the maximum, minimum, median and any outliers for each group.

Figure 3.6 Box plot for haptoglobin concentration comparing females and males



The box and whisker plot allows comparison of haptoglobin concentration by sex and further shows the maximum, minimum, median and any outliers for each group.

Figure 3.7 Box plot for fibrinogen concentration comparing females and males



The box and whisker plot allows comparison of fibrinogen concentration by sex and further shows the maximum, minimum, median and any outliers for each group.

Post hoc power calculations performed using a one sided t-test suggested that with an 80% power and with the sample size of 50 animals, a difference in SAA concentration of greater than 26.36µg/mL would be identified as a statistically significant difference. Based on clinical use of SAA in horses, 1000 fold changes in concentration can be expected in abnormal individuals and therefore it was considered that from a biological perspective there was sufficient power in the study.

3.4 Discussion

A composite “snapshot” of the haematology and clinical biochemistry including acute phase proteins was carried out in a randomly selected group of clinically normal Thoroughbred horses. Haematology and clinical biochemistry results were all within previous references ranges described for the domestic equid, *Equus caballus*. The results for the acute phase proteins SAA, haptoglobin and fibrinogen were all within the normal range except for the SAA results in three horses, which were subsequently reported to have developed a respiratory tract infection (Lumsden and Mullen, 1978; Taylor-MacAllister *et al.*, 1997; Tyler-McGowan *et al.*, 1999).

There were no significant associations with age or sex for any of the parameters that were measured except for haptoglobin, for which there was a significantly higher mean concentration in the sub group of foals. However, as there were only six horses over the age of 4, these results need to be treated with caution. The results indicate that SAA concentration is normally distributed in horses and that there are no significant effects of age or sex based on this group of Thoroughbreds. This marker of tissue damage has a narrow normal range and previous work suggests that it is unaffected by normal handling, season, time of day and normal management practice (Pepys and Hirschfield, 2003). Investigations in other species support the fact that clinically normal animals can be expected to have SAA concentrations within the reference range no matter what time of day, season, sex or age they may be (Nunokawa *et al.*, 1993; Satoh *et al.*, 1999; Stoneham *et al.*, 2001; Pollock *et al.*, 2005; Hillstrom, 2010). To date, the majority of studies have been limited to the measurement of SAA in the diagnosis of specific infectious diseases in horses (Pepys *et al.*, 1989; Hulten *et al.*, 1999; Stoneham *et al.*, 2001; Hulten 2002; Cohen *et al.*, 2005; Copas *et*

al., 2013), with responsibility for defining the normal range left to individual commercial laboratories offering these assays. Definition of normal ranges can be complex and requires large groups of animals. They must be defined using only healthy individuals. However, in practice, exclusion of abnormal animals or those with subclinical disease can be difficult. Various techniques can be used to determine a normal range but all require removal of outliers, and determination of the distribution of the data and appropriate normalising transformations (Shine, 2008). For the purpose of this study, animals were considered to be normal if their behaviour upon observation was as expected by their usual handler, no abnormalities were identified during routine clinical examination and haematological and biochemical parameters were within the normal range. It is reasonable to question how representative this group of 50 Thoroughbred horses was in comparison to the general population. Every effort was made to identify individuals with evidence of disease through clinical examination and an extensive history; furthermore, the horses were selected from a management system typical of the standard breeding and training establishments across the UK and Ireland.

The ELISA method for determining the concentration of SAA is relatively simple and allows for large numbers of samples to be analysed concurrently making the test potentially attractive for a commercial laboratory from a financial perspective, and to equine clinicians. In order to be an effective test in animals with acute disease, it is important that accurate results can be returned quickly. Since the work described here was carried out, a number of horse-side SAA tests have been developed, some of which provide a numerical result, others which simply indicate whether the SAA concentration is within the normal range or not (Stablelab, 2015) and these are becoming increasingly popular and useful in the identification of abnormal individuals.

Post-hoc power analysis was performed on the results presented in this chapter. *Post-hoc* power calculations are conducted after a study has been completed, and utilise the obtained sample size and effect size to determine what the power was in the study, assuming the effect size in the sample is equal to the effect size in the population. Whereas the utility of prospective power analysis in experimental design is universally accepted, the usefulness of retrospective techniques can be controversial (Thomas, 1997). Falling for the temptation to

use the statistical analysis of the collected data to estimate the power could result in uninformative and misleading values. In particular, it has been shown (Hoenig and Heisey, 2001) that *post-hoc* power in its simplest form is a one-to-one function of the p-value. In this work, Hoenig and Heisey suggest that that all *post-hoc* power analyses suffer from what is called the "power approach paradox" (PAP), in which a study with a null result is thought to show more evidence that the null hypothesis is actually true when the p-value is smaller, since the apparent power to detect an actual effect would be higher. Nevertheless the power calculations performed, interpreted in light of the biological relevance of changes in SAA concentration presented in the subsequent chapters, suggest that large changes in SAA concentration are to be expected in abnormal animals and therefore there is sufficient power in this study.

There are a number of limiting factors when dealing with a group of horses, which are by definition non-experimental animals. Nevertheless, with the reservation that the relatively small number of horses sampled in this study means it is difficult to confirm that the results are normally distributed (despite normality testing), all of the results for the acute phase proteins fell within previously published reference ranges, except for three individuals with relatively small increases in SAA concentration which subsequently developed low grade respiratory tract disease (Nunokawa *et al.*, 1993). The low number of older adults and adult males in the study group means that larger differences in the results from this group might not be statistically significant. This fact would be worth considering in any future study. It is also possible that the results were subject to an effect of environment common only to this establishment at this geographical location. However, it should be noted that the effect of environment, location, season and time of day on the concentration of APPs has been studied in humans and shown to be of no consequence (Pepys and Hirschfield, 2003). In respect of this, these premises were chosen as they contained a large population of horses kept extensively, that is to say that the majority of the horses sampled were not kept in close proximity to one another, nor were all of the horses sampled managed in the same manner, i.e. foals were at foot and kept at pasture during the day and housed at night, the young stock were housed and in pre-race training. The fact that the animals on the premises that were used were already subject to a regular blood-sampling regimen

allowed for surplus samples to be collected with minimal effects on animal welfare. As in all clinical studies, it would have been of interest to sample animals from other sources.

Access to “real”, that is clinically normal, horses in a standard environment can be difficult; equine breeding premises are, by definition, commercial operations which rightly protect the welfare of their stock and are often keen to maintain a degree of confidentiality. Although the numbers used in this part of the thesis are small, they represent a group of “typical” Thoroughbreds bred for the racing industry from a well established and respected international operation.

While it would be dangerous to draw too many conclusions based on the results presented in this initial “snapshot” of the serum concentration of SAA in the normal Thoroughbred, this pilot work indicates that SAA concentrations appear to be low in horses with no clinical, haematological or biochemical evidence of disease. SAA is simple to measure and if, as was noted in this group of horses, normal horses can be expected to have a low concentration of SAA, SAA could be used as a sensitive, although non specific indicator of health. As has been described during previous published work, and as will be shown in the subsequent chapters, the magnitude of increase in SAA concentration in clinically abnormal horses is greater than the observed differences between the groups presented here and therefore the measurement of SAA is potentially biologically and clinically useful. These findings form the basis of the in depth studies presented subsequently in this thesis.

Chapter 4

The Effect of Surgery on the Acute Phase Response in Horses

4.1 Introduction

Operative surgery can be considered to be goal orientated violence towards tissue and a number of authors have demonstrated that surgical intervention leads to an acute phase response in horses which can be measured and which then reduces during the post operative period (Pollock *et al.*, 2005; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2009; Hillstrom *et al.*, 2010). Surgical trauma leads to inflammation that creates a wide range of physiological, biochemical, and behavioural changes in animals and in humans. Changes in haematological and biochemical parameters have been used to diagnose, monitor, and prognosticate the course of disease in horses. However, these analytes are relatively insensitive indicators of inflammation (Hillstrom *et al.*, 2010). Changes in serum concentration of traditional markers of inflammation, such as fibrinogen and haptoglobin, unfortunately, occur relatively slowly in the presence of disease and these analytes have a wide normal range (Allen *et al.*, 1988, Pepys *et al.*, 1989). For this reason, these analytes, although fairly commonly used in veterinary clinical practice to diagnose, monitor and determine prognosis, have major limitations.

4.1.1 Searching for the optimum biomarker

Biological analytes used to indicate the presence of a disease or injury, or to prognosticate such pathology are referred to as biomarkers. Clearly the ideal biomarker has both high sensitivity and specificity for the disease or traumatic damage and for detecting a change in the status of the affected organism and for the outcome of the disease or injury (Hsu *et al.*, 2014). However, the widespread use of CRP in human medicine as a highly sensitive, but non-specific, marker of most forms of inflammation, infection and tissue damage suggests that acute phase proteins, although rarely diagnostic in their own right, have a major role in the management of disease (Pepys and Hirschfield, 2003). Typically a panel of biomarkers is used, or individual biomarkers are used in conjunction with haematological and clinical biochemical analytes and / or clinical findings. In humans the use of a panel of biomarkers, including CRP, fibrinogen and matrix metalloproteinase 7, among others (La Framboise *et al.*, 2012), is widespread during diagnosis and prognostication of disease. When

choosing treatment modalities multiple logistic regression models and ROC curves have been used to evaluate the biomarker set (Hsu *et al.*, 2014).

4.1.2 Acute phase proteins as biomarkers

Acute phase proteins have the potential to be included in an array of biomarkers due to the fact that they, by definition, increase in concentration following disease or injury. The acute phase proteins CRP and SAA are stable in blood samples obtained in field conditions, have a short half life in serum (Tape *et al.*, 1990) and are apparently not affected by differences in season, time of day, gender or age or general anaesthesia (Pepys and Hirschfield, 2003). In human medicine the use of CRP to monitor inflammation (Kordeluk *et al.*, 2016), and to quantify the risk of complications following surgery, is well described (Palani Velu *et al.*, 2016). CRP has been shown to predict postoperative morbidity following oesophageal, gastric and colorectal resections (Warschkow *et al.*, 2012; Platt *et al.*, 2012). In humans, CRP concentration increases in response to infectious, allergic, and inflammatory disease; it is a sensitive marker of tissue necrosis, myocardial infarction, pancreatitis and for the presence of malignancy. Furthermore, CRP concentration has been shown to increase rapidly in response to surgery, burns and bone fractures (Pepys and Hirschfield, 2003; Palani Velu *et al.*, 2016). Consequently the use of APPs in human clinical practice is now the gold standard and routine method for the identification of tissue damage, disease and injury (Pepys and Hirschfield, 2003). Despite this, the use of these biomarkers has not become routine in veterinary medicine (Pepys and Hirschfield, 2003; Jacobsen *et al.*, 2005).

The Aims of this study were as follows:

1. To investigate the effect of surgery on the serum concentrations of SAA, haptoglobin and fibrinogen in horses undergoing surgery for a variety of reasons.
2. To compare the production of SAA and haptoglobin in horses, in response to surgery, to that of another APP, fibrinogen, which is often regarded as a marker for inflammation and is widely used in equine clinical practice.

4.2 Materials and methods

4.2.1 Study animals, case selection and experimental design

This was a prospective clinical study in which 25 horses presented for surgery at the University Veterinary Hospital, University College Dublin, Dublin, Ireland were recruited. The horses were divided into two groups based on whether they were presented for Elective (n=18) or Non-elective (n=7) surgery. The numbers were entirely dictated by the numbers of animals presented to the University Veterinary Hospital during a period of one month in 2003. The surgery was considered elective if the horse presented without clinical evidence of inflammation and had no evidence of abnormalities during full blood counts and serum biochemistry, and non-elective if the horse had clinical evidence of substantial pre-existing disease prior to surgical intervention.

4.2.2 Ethics

Horses were included in the project with the informed consent of their owners and the project was approved by the Faculty of Veterinary Medicine, Ethics and Welfare Committee, University College Dublin, Ireland.

4.2.3 Group 1

Group 1 consisted of 18 horses subjected to elective surgical procedures. These included: closed castration (7), cryptorchidectomy (2), prosthetic laryngoplasty and ventriculocordectomy (3), staphylectomy (1), ovariectomy (2), transphyseal bridging (2), and a modified Forrsell's procedure (1) (Table 4.1.).

4.2.4 Group 2

Group two consisted of seven horses with significant pre-existing disease, subjected to non-elective surgery. These included: tooth repulsion (4), sinusotomy for removal of a sinus cyst (2), and an abscess excision (1) (Table 4.2.).

4.2.5 Surgical procedures and anaesthetic protocol

Details of the general anaesthetic protocol are given in Chapter 2.

4.2.6 Sample collection

Blood was obtained from each horse by jugular venepuncture using a vacutainer system. Each horse was restrained by an experienced handler and subjected to a clinical examination prior to jugular venepuncture. Clinical examination was carried out after blood sampling to reduce any effect of the stress of examination and handling on the concentration of the analytes. Each horse was sampled when it was admitted to the hospital, at the end of anaesthesia, and at 12, 24, 48, and 72 hours following surgery. Further samples were not obtained as the majority of the animals were discharged from the hospital at this point. Samples were obtained to determine full haematology, selected biochemical analysis and concentration of the acute phase proteins; fibrinogen, haptoglobin and SAA. Blood samples preserved in EDTA and lithium heparin were analysed immediately for routine haematological and biochemical analytes; blood in the plain tube was centrifuged at 5000rpm for five minutes, and the serum separated into three, 1.5mL aliquots. These samples were stored at -80°C until they were analysed for concentration of SAA and haptoglobin. Both acute phase proteins are known to be stable when stored at this temperature (Wilkins *et al.*, 1984)

Full details of the sampling methods, haematology, biochemistry and acute phase protein assays are given in chapter 2.

4.2.6.1 Acute phase proteins

The concentration of the acute phase proteins SAA, haptoglobin and fibrinogen were determined using the storage protocol and methodology described in Chapter 2.

Table 4.1. 18 Horses presented for Elective Surgery

Number	Age	Sex	Breed	Length of general anaesthetic	Reason for admission and anaesthesia position (D or L)*
1	1 year	M	TB	34 mins	Castration (D)
2	2 years	M	TB	43 mins	Castration (D)(cryptorchid)
3	5 years	F	TB	27 mins	Castration (D)
4	4 years	M	TB	38 mins	Castration (D) (cryptorchid)
5	4 years	M	TB	63 mins	Arthroscopy(L)
9	2 years		TB	25 mins	Castration(D)
10	6 years	F	Warmblood	83 mins	Crib Bitter(D)
12	3 years	F	TB	75 mins	Intermittent dorsal displacement of the soft palate(D)
14	4 years	F	TB	22 mins	Castration(D)
17	11 years	M	TB	91 mins	Recurrent laryngeal neuropathy(L)
18	3 years	F	TB cross	98 mins	Ovariectomy(D)
19	6 months	M	TB	49 mins	Angular limb deformity(L)
20	6 months	M	TB	59 mins	Angular limb deformity(L)
21	2 years	M	TB	36 mins	Castration(D)
22	5 years	M	Irish Draught	106 mins	Recurrent laryngeal neuropathy(L)
23	7 years	F	TB	22 mins	Castration(D)
24	2 years	M	TB	87 mins	Recurrent laryngeal neuropathy(L)
25	1 year	M	TB	32 mins	Castration(D)

* D=dorsal recumbency, L= lateral recumbency

Table 4.2. Seven Horses Presented for Non-elective Surgery

Number	Age	Sex	Breed	Length of general anaesthetic	Reason for admission and anaesthesia position (D or L)*
6	4 years	M	TB cross	45 mins	Sinus cyst (L)
7	4 years	M	Highland	108 mins	Dental problem (L)
8	6 years	M	TB	124 mins	Dental problem (L)
11	6 years	F	TB cross	62 mins	Dental problem (L)
13	6 years	M	TB	75 mins	Dental Problem (L)
15	4 years	M	TB	43 mins	Sinus cyst (L)
16	3 years	M	TB	117 mins	Neck Abscess (D)

* D=dorsal recumbency, L= lateral recumbency

4.2.6.2 Haematological and clinical biochemical examination

Haematological examination included determination of haemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte count (RBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and a differential white blood cell count. The following biochemical analytes were measured: gamma glutamyl transferase, alkaline phosphatase, creatinine, urea, total protein, albumin, and globulin. Total protein concentration was measured at every time point; however, the following biochemical analytes were only measured at time point one; gamma glutamyl transferase, alkaline phosphatase, creatinine, urea, albumin, and globulin. This was in part due to the resources available for the project and also because the purpose of performing these tests was to determine whether there were any underlying problems not related to the presenting disease (non-elective, group 2) or the surgical intervention. Finally, SAA, haptoglobin and fibrinogen concentration were also determined at every time point.

4.2.7 Clinical examination

Each horse was observed from a distance (usually over the stable door) for a period of not less than one minute to confirm that the horse was behaving in a normal manner, and that there was no external evidence of disease; thereafter the animals were caught and restrained by an experienced handler. A full clinical examination including measurement of heart and respiratory rate, rectal temperature, and palpation of superficial lymph nodes was performed. In addition, the surgical sites were closely examined for the presence of complications, signs of wound infection or any other abnormalities at least twice per day.

4.2.8 Data processing and statistical methods

All data were entered into a Microsoft Excel spread sheet (Microsoft Excel for Mac 2011). Data were checked for errors, consistency and validity using Excel's standard functions. The data were separated by groups (elective and non elective), and once a final database was produced it was imported into Minitab, version 16, for analysis. Microsoft Excel for mac 2011 and Minitab, Version 16

were used to produce graphical summaries of the data. Exploratory data analyses included the production of line graphs and box and whisker plots for each of the analytes in order to determine which variables were of interest and to determine the distribution of these data.

Exploratory analyses were conducted to determine whether the data were normally distributed and a Mann-Whitney U test was used to compare the concentrations of SAA, haptoglobin and fibrinogen between the horses within each group. Wilcoxin signed rank tests (for paired analyses) were used to compare the differences between horses over time (elective and non-elective surgery at selected time points). These tests were chosen because the data were not normally distributed. The following time points were selected for analysis and are included in the results: SAA, admission and 12 hours after surgery; haptoglobin, admission and 24, and 48 hours after surgery; fibrinogen, admission and 12 hours after surgery.

Post hoc power calculations were performed using a 2 sample t-test.

4.3 Results

4.3.1 Acute phase proteins

There were measureable increases in the concentrations of all 3 acute phase proteins in both the non-elective and the elective surgery groups. However, there was considerable variation between each of the three acute phase proteins and between individual horses. Of the three APPs, only SAA concentration was noted to increase and decrease during the sampling period, although this was not the case for two individuals in the elective group and three individuals in the non-elective group. Median acute phase protein results for each time point are given in Table 4.3.

4.3.1.1 Group 1. Elective surgery

a. Serum Amyloid A

There was a significant increase in the median concentration of SAA within 12 hours after surgery ($P < 0.05$) in the horses that underwent elective surgery (Table 4.3). The concentration of SAA reached its peak 24 hours after surgery and began to decline after 48 hours in 23 of the horses. For the remaining two horses

SAA concentration continued to increase. One of these horses experienced some wound drainage and local swelling following castration; the second had more incisional swelling and increased drainage around the site of the laryngotomy than would normally be expected.

b. Haptoglobin

The concentration of haptoglobin increased between 24 and 48 hours after surgery and began decline in 10 of the horses during the sampling period, but did not decline in the remaining 15. A significant difference in median haptoglobin concentration was noted after 48 hours ($P<0.05$).

c. Fibrinogen

The concentration of fibrinogen increased between 24 and 48 hours after surgery in all of the horses in the elective group. The concentration remained elevated or continued to rise in 22 of the 25 horses. There was no significant difference in median Fibrinogen concentration at any time point (Mann Whitney U test).

4.3.1.2 Group 2. Non-elective surgery

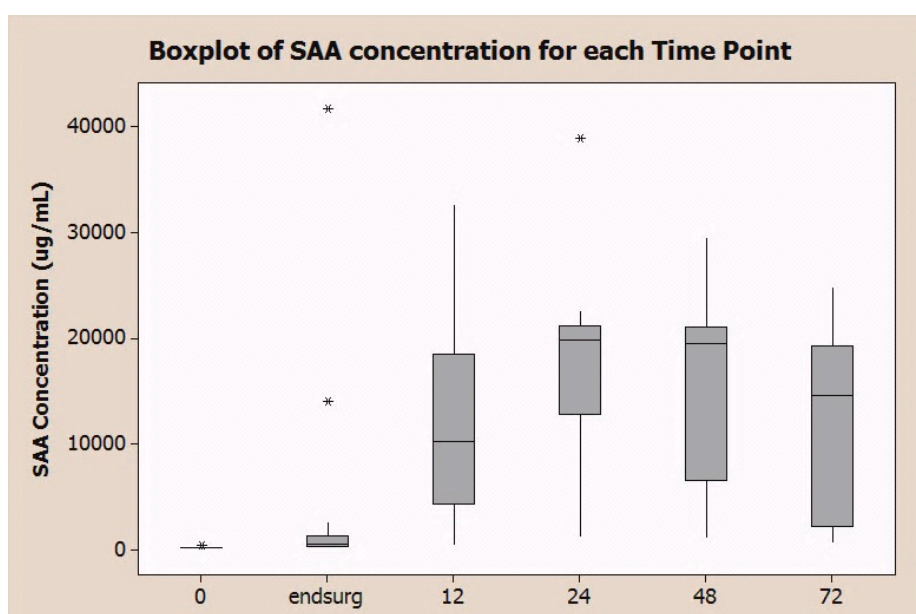
a. Serum Amyloid A

In the non-elective surgery group, basal SAA concentrations were all outwith the reference range; however, there were significant variations in the values with some only mildly increased and others 100 times greater than normal values. There was an increase in SAA concentration in all seven horses after surgery with a statistically significant increase in the median concentration of SAA after 12 hours ($P<0.05$). In four of the horses in the non elective group, SAA concentration began to decline during the sampling period; in the remaining three horses, a dental extraction, an infected sinus cyst and a neck abscess as a result of an infected microchip site, SAA concentration continued to increase. Each of these three horses experienced significant complications, and all three required further surgical therapy.

Table 4.3. Group 1-Elective Surgery. Median results for Acute Phase Protein Concentrations at each time point

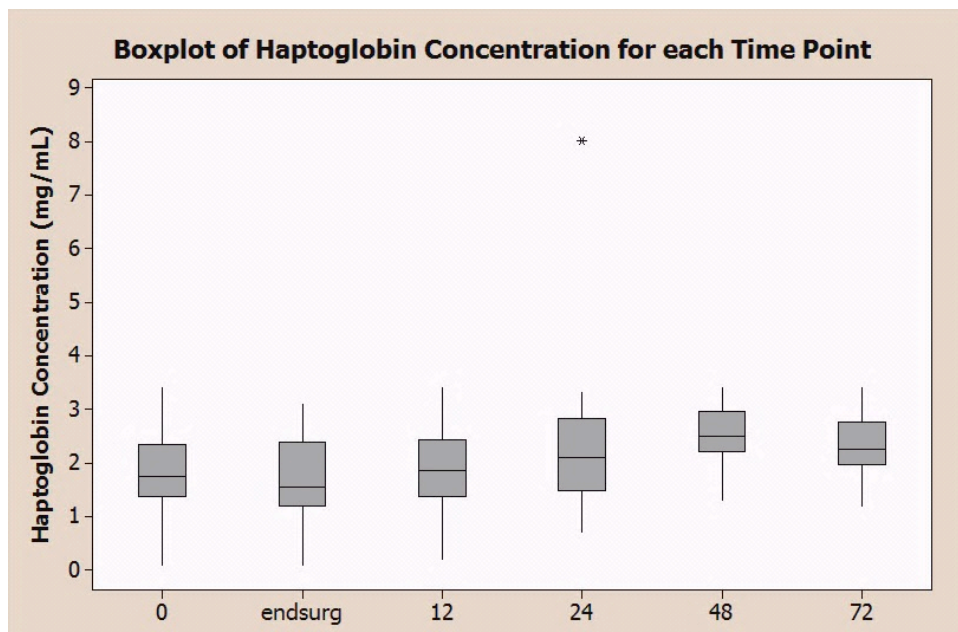
	SAA	Haptoglobin	Fibrinogen
	($\mu\text{g/mL}$)	(mg/mL)	(g/L)
Admission	133(66.5)	2.45(0.75)	4.0(2.0)
End of anaesthesia	453(668)	2.3(0.75)	4.0(2.5)
12 Hours	9800(13500)	1.9(0.95)	4.0(2.5)
24 Hours	19802(7777)	2.1(1.05)	4.0(3.0)
48 Hours	19220(14530)	2.45(0.75)	4.0(2.0)
72 Hours	11585(16884)	2.30(0.75)	4.0(1.5)

Figure 4.1 Box plot showing SAA concentration in the elective surgery group at each time point



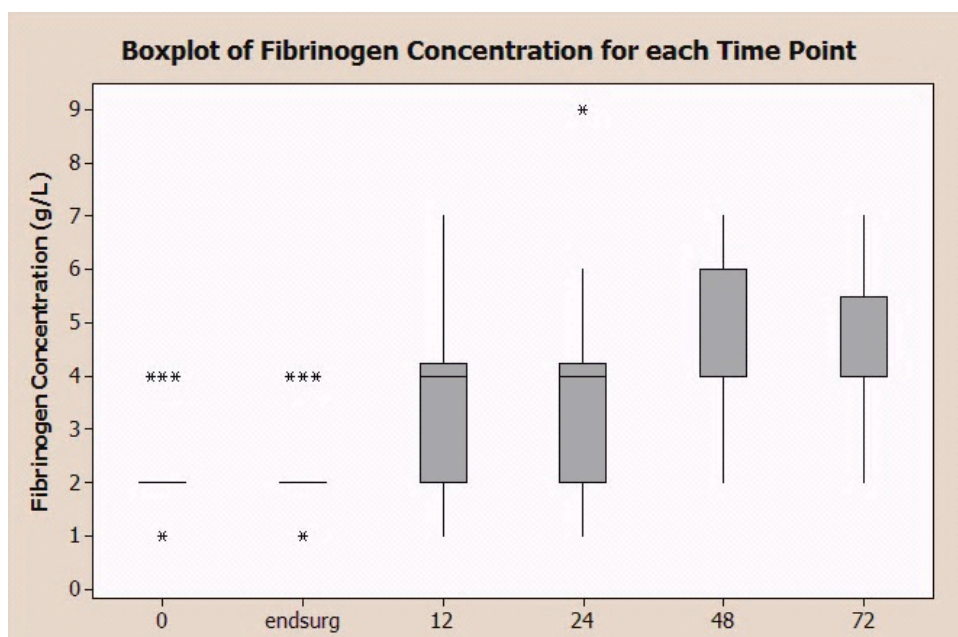
SAA concentration was within the reference range for all horses prior to surgery, although there was considerable variation in response, a significant increase ($P < 0.05$) was noted within 12 hours of surgery. Two horses continued to show increases in SAA concentration during the sampling period, both of which experienced complications of surgery.

Figure 4.2 Box plot showing haptoglobin concentration in the elective surgery group at each time point



A significant difference in mean haptoglobin concentration was noted after 48 hours ($P < 0.05$), however, haptoglobin concentration was quite variable between individual horses and no clear pattern of response was observed.

Figure 4.3 Box plot showing Fibrinogen concentration in the elective surgery group at each time point



The concentration of fibrinogen increased between 24 and 48 hours after surgery in all of the horses in the elective group. The concentration remained elevated or continued to rise in 22 of

the 25 horses. There was no significant difference in median fibrinogen concentration ($P<0.05$) until 48 hours after surgery. The number of individual values was limited.

b. Haptoglobin

The concentration of haptoglobin was highly variable amongst the elective surgery group; however, all of the horses experienced an increased concentration between 24 and 48 hours after surgery. There was a significant increase in the median concentration of haptoglobin after 48 hours ($P<0.05$). In four of the seven horses, haptoglobin concentration began to decline during the sampling period.

c. Fibrinogen

The concentration of fibrinogen increased in all of the horses following surgery, but took between 24 and 48 hours to do so and did not begin to decline during the sampling period for any of the horses. There was a statistically significant difference in fibrinogen concentration after 48 hours ($P<0.05$).

4.3.1.3 Comparison between elective and non-elective surgery

A Wilcoxin signed rank test was used to compare the concentration of the 3 acute phase proteins for the elective and non-elective groups at each time point. There was a significant difference between the concentration of SAA between the two groups but not for haptoglobin or fibrinogen.

4.3.1.4 Acute phase protein kinetics - Summary

There was considerable variation in the response to surgery between the two groups of horses, and between horses in the same group. However surgery led to an increase in SAA, haptoglobin and fibrinogen concentration in all of the horses. A pattern of an increase to a peak, followed by a reduction, or a peak followed by a reduction and then a further rise were noted for SAA concentration. Haptoglobin concentration was too variable to discern a pattern and fibrinogen concentration simply increased and remained high throughout the sampling period.

For SAA concentration, the peak response to surgery was particularly variable in terms of the time of the peak and whether there was a single peak. However, in all of the horses that underwent a decrease and then increase in SAA

concentration, or in which SAA concentration continued to increase beyond the sampling period (elective group n=2, non elective group n=3) evidence of a complication or surgical site infection was noted.

SAA concentration increased for every horse that underwent surgery, all of the horses in the elective group started with normal SAA concentrations and all of the horses in the non-elective group had increased SAA concentrations, although some of these values were only mildly increased. In general, the magnitude of the increase in SAA concentration for the non-elective group was not as great as for the elective group suggesting that there may be a peak total response for SAA. Based on the small numbers of horses in this study, it could be suggested that increased concentrations of SAA always indicate the presence of inflammation, or injury and that continued high concentrations in the post operative period may be suggestive of a complication. However, based on the group with significant pre-existing disease (non-elective group), some of which only had very mild increases in SAA concentration, despite the presence of disease, normal or near normal SAA concentration does not always rule out the possibility of tissue injury.

All of the horses undergoing non-elective procedures (Group 2), that is, the horses with significant pre-existing disease, had a significantly greater median concentration of SAA before, and after the surgery, than the horses in Group 1 (8220µg/mL compared with 133µg/mL) ($P<0.05$).

Although basic descriptive statistical analysis was carried out as described, the variation in SAA concentration between the groups and between individuals within groups made these analyses of limited value. To that end, graphical representation of two horses from each group were produced to illustrate the SAA response to surgery in horses with and without complications (4.7-4.10).

4.3.1.5 Haematology

A range of blood dyscrasias consistent with inflammatory and infectious disease were noted in the horses presented for non-elective surgery, these included increases in total white blood cell count and neutrophilia. Similarly, alterations in mean PCV, total protein and haemoglobin concentration, consistent with minor haemorrhage during surgery and the dilution effect of intraoperative fluid therapy were noted in both elective and non-elective surgery cases.

4.3.1.6 Clinical biochemistry

Clinical biochemistry results for all of the parameters were measured at admission only, as part of a pre-anaesthetic screen. Plasma protein was measured at every time point. There were no significant differences from reference ranges for any of the biochemical parameters assessed.

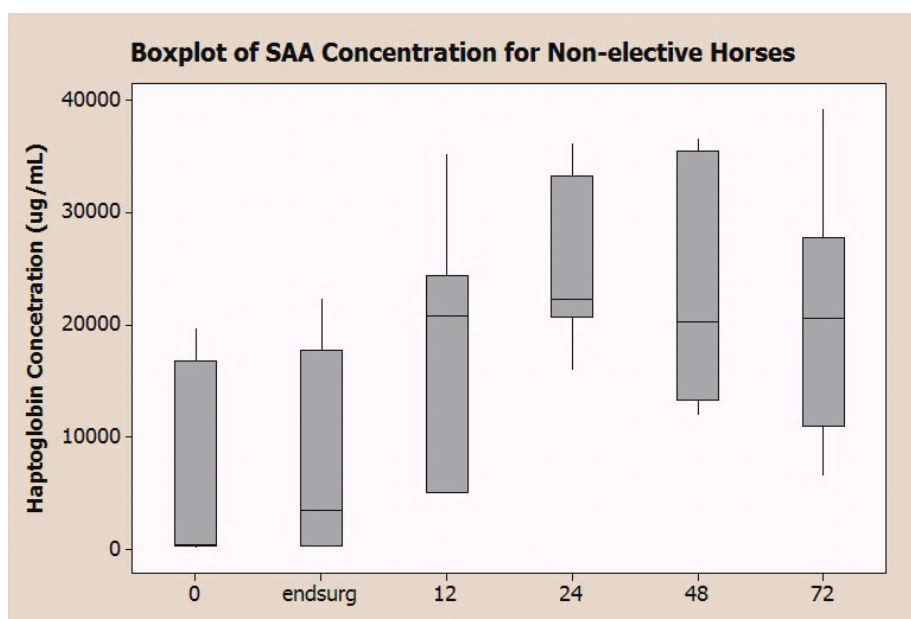
4.3.2 *Post hoc* power calculations

Post hoc power calculations were performed in Minitab, version 16 using a 2-sample t-test. These indicated that with the numbers of animals included in this study a difference in SAA concentration of 179 µg/mL or greater could be detected with an 80% power as significant at $p \leq 0.05$. The biological significance of this, based on previously published results and the results of this study suggest that changes in SAA concentration in response to disease or injury are likely to be of a magnitude much greater than this value.

Table 4.4. Group 2- Non-Elective Surgery. Median results for Acute Phase Protein Concentrations at each time point

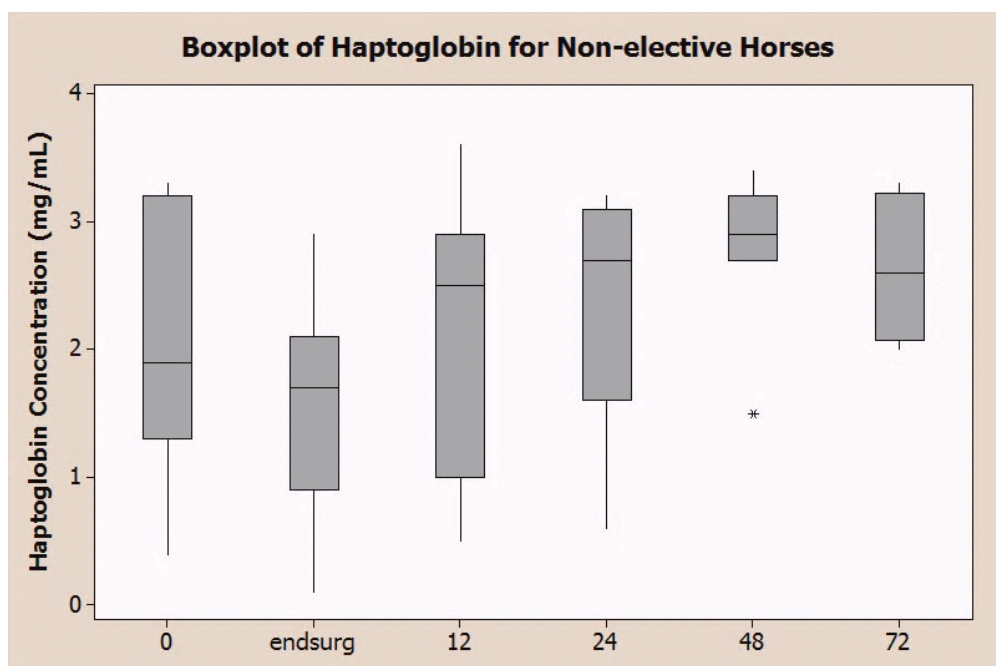
	SAA	Haptoglobin	Fibrinogen
	($\mu\text{g/mL}$)	(mg/mL)	(g/L)
Admission	8220(18923)	1.9(1.40)	2.0(0)
End of anaesthesia	8097(18343)	1.65(0.70)	2.0(0)
12 Hours	21177(22415)	2.45(1.55)	4.0(1)
24 Hours	26097(13787)	2.25(1.75)	4.0(1)
48 Hours	20377(15475)	2.90(0.45)	4.0(2.5)
72 Hours	21770(11465)	2.50(1.20)	4.0(4)

Figure 4.4 Box plot showing SAA concentration in the non-elective surgery group at each time point



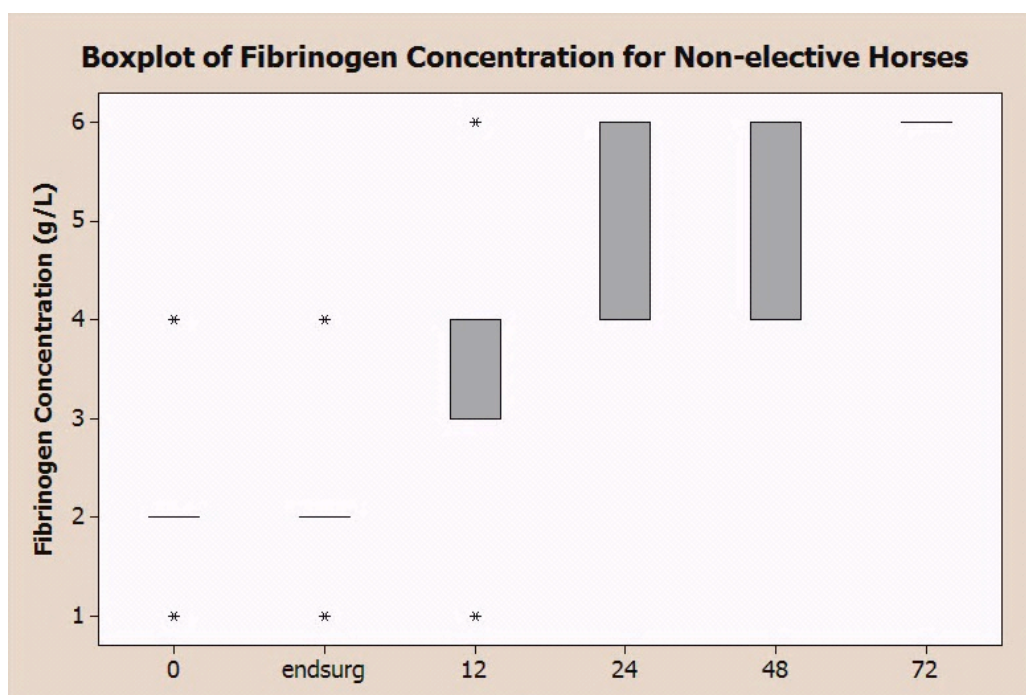
Surgery resulted in an increase in SAA concentration in all seven horses with a statistically significant increase in the median concentration of SAA after 12 hours ($P < 0.05$)

Figure 4.5 Box plot showing haptoglobin concentration in the non-elective surgery group at each time point



There was a significant increase in the median concentration of haptoglobin after 48 hours ($P < 0.05$). However between individual animals there was considerable variation and it was difficult to discern a clear pattern of response

Figure 4.6 Box plot showing Fibrinogen concentration in the non-elective surgery group at each time point



There was a statistically significant difference in fibrinogen concentration after 48 hours ($P < 0.05$). Fibrinogen concentration remained elevated beyond the end of the sampling period for all of the horses.

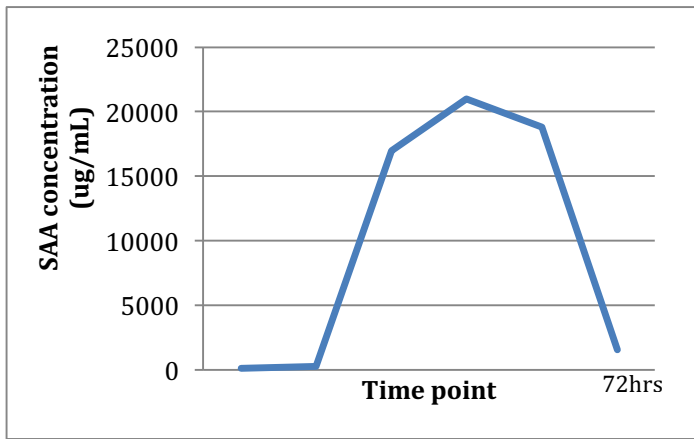


Figure 4.7 Graph showing SAA concentration in a horse subjected to elective surgery without complications. The response occurred within 12 hours of surgery and began to decline during the sampling period.

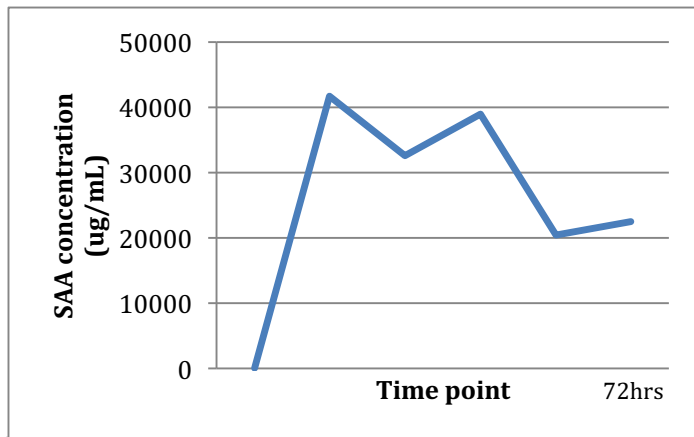


Figure 4.8 Graph showing SAA concentration in a horse subjected to elective surgery with complications. The response occurred within 12 hours of surgery and began to decline before rising to a second peak, then declining and rising again during the sampling period. This horse experienced incisional drainage and swelling post castration.

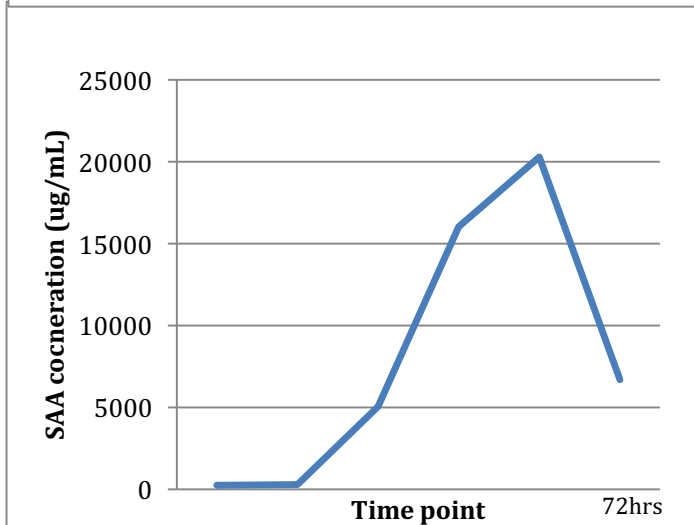


Figure 4.9 Graph showing SAA concentration in a horse subjected to non-elective surgery without complications. The response occurred within 12 hours of surgery and began to decline during the sampling period.

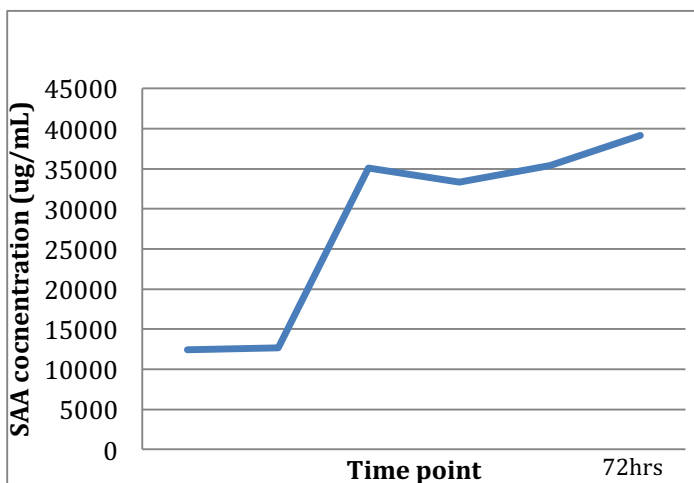


Figure 4.10 Graph showing SAA concentration in a horse subjected to non-elective surgery with complications. The response occurred within 12 hours of surgery and SAA concentration continued to rise during the sampling period. This horse subsequently required further surgery for a neck abscess.

4.4 Discussion

4.4.1 Introduction

The results of this study suggest that surgical trauma produces a measurable response in SAA concentration in both normal individuals and in those with pre-existing disease. Following surgery, SAA concentration increased significantly within 12 hours and reached peak concentration within 24 hours after which point it began to decline in horses without complications, but continued to rise, or dropped and then rose again, in horses with complications. Although baseline SAA concentration was significantly greater in horses with pre-existing disease, the effect of surgical intervention in these animals led to an increase in SAA concentration, although not of the same magnitude as that noted in the elective group. Thus, horses undergoing surgery with significant pre-existing disease (non-elective group) had a much greater median concentration of SAA (8220 μ g/mL) before surgery than the elective surgery horses (133 μ g/mL). Haptoglobin and fibrinogen concentration also responded to surgery and began to increase within 24 hours. Haptoglobin concentration was highly variable between groups and between horses within groups and no clear pattern was apparent. Fibrinogen concentration remained increased without a significant decline in serum concentration for at least 72 hours after surgery. Fibrinogen concentration results did not vary greatly and relatively few values were observed; changes in fibrinogen concentration occurred more slowly when compared to the other APPs suggesting this analyte may be of limited value in the clinical setting despite its common usage.

Based on the small numbers of horses in this study, it could be suggested that increased concentrations of SAA always indicate the presence of inflammation, or injury and that continued high concentrations in the post operative period may be indicative of a complication. However, based on the non-elective group, some of which only had very mild increases in SAA concentration, despite the presence of significant disease, normal or near normal SAA concentration does not always rule out the possibility of tissue injury.

The use of SAA concentration to diagnose and monitor disease in horses is gradually gaining momentum with a number of authors describing the use of these analytes (Pepys *et al.*, 1989; Vandenplas *et al.*, 2005; Pollock *et al.*, 2005; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2009; Andersen *et al.*, 2012; Copas *et al.*, 2013; Belgrave *et al.*, 2013). However, to date it has not gained the widespread acceptance noted in a number of other animal species, including cattle, sheep, pigs and dogs, or people (Pepys *et al.*, 1983, Hind and Pepys, 1987; Hulten *et al.*, 1999; Eckersall *et al.*, 1999b; Stoneham *et al.*, 2001; Hulten and Demmers, 2001; Hulten *et al.*, 2002; Cohen *et al.*, 2005; Vandenplas 2005; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2006; Duggen *et al.*, 2007; Jacobsen *et al.*, 2009; Eckersall and Bell, 2010).

The serum concentrations of fibrinogen and haptoglobin have also been used to diagnose and monitor disease in horses (Allen *et al.*, 1988), and both are sensitive markers that increase in concentration in response to surgical trauma (Allen *et al.*, 1988, Auer *et al.*, 1989, Eurell *et al.*, 1993). However, their concentrations respond relatively slowly following disease, trauma or infection and vary widely in normal horses (Hillstrom *et al.*, 2010).

The concentration of SAA has been shown to increase in horses with septic arthritis, infectious respiratory disease, after castration, and after surgery of variable intensity (Hulten *et al.*, 2002, Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2009), but the distinction between the effects of elective and non-elective surgery had not previously been investigated. The results of this study agree with previous work indicating that surgical trauma produces an acute phase response but that the scale of response varies on the markers used and between animals (Allen *et al.*, 1988, Pepys *et al.*, 1989; Ellis and Humphreys, 1992; Hulten *et al.*, 1999; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2009) and, at least speculatively, on the presence of, and maybe, duration of, pre existing disease or inflammation.

4.4.2 Developing the test, using SAA concentration in surgical practice

The value of the measurement of SAA concentration to a surgeon in clinical practice would be in determining which horses were clearly in the recovery phase and not affected by complications such as surgical site infection. In this

initial study, SAA concentration rose sharply and quickly following surgery and began to reduce by 24 hours after the conclusion of the surgical procedure in horses without complications. The clinical signs of early surgical site infection can be subtle and are typically only apparent after the infection is well established. The ability to identify these animals prior to the development of fulminant infection, allowing rapid instigation of treatment, could significantly reduce morbidity and in some instances mortality (Rodriguez *et al.*, 2009). Unfortunately none of the horses in this study were sampled after 72 following surgery, and it would have been very interesting to follow the changes in APP concentration further into the recovery period, particularly in the horses that suffered complications.

Other studies, performed subsequent to the work presented here, have suggested that SAA is the most sensitive method for identifying inflammation in horses (Hillstrom *et al.*, 2010). Based on the findings presented in Chapter 3 and in this chapter, one could conclude that clinically normal horses have very low SAA concentrations and that SAA concentration increases rapidly, sometimes by a factor of at least 10 times normal, in response to an inflammatory stimulus

A number of specific questions arise, which are not resolved by the present study, with respect to the clinical use of SAA concentration;

1. Are there differences in SAA concentration, including speed of response and magnitude, based on different surgical procedures?
2. What effect does the presence of surgical complications, including surgical site infection, have on SAA concentration?
3. Given that it is known that individual differences exist in SAA responses, are there any particular SAA profiles that can be associated with the development of a particular complication?

4.4.3 Limitations and further investigation

This part of the study was very much limited by the type and number of horses available for sampling, and by the types of pathologies represented in the non-elective surgery horses and the limited number of elective procedures. It could be of value to extend these studies with larger numbers of animals presented for different procedures or perhaps grouped according to the affected body system.

In humans CRP concentration is frequently measured in the post-operative period in order to identify individuals with complications and to inform therapeutic interventions (Palani Velu *et al.*, 2016). From a clinical perspective it may be that the magnitude and intensity of the acute phase response is affected by the nature of the surgical procedure (Jacobsen *et al.*, 2009). Thus, in view of the drive to develop the use of minimally invasive surgery in veterinary medicine, it may be that SAA concentration could be used to determine the degree of tissue trauma caused by a particular intervention or as an aid to identification of post-operative complications such as wound infection (Busk *et al.*, 2010). The decline in SAA concentration, noted in some of the animals in this study, following surgical trauma suggest that this analyte could form part of a minimum database during postoperative monitoring. In human medicine, panels of biomarkers are frequently used to produce a single diagnostic or predictive paradigm for a particular disease or surgical outcome (Hsu *et al.*, 2014; Palani Velu *et al.*, 2016). However, in human medicine, relatively specific surgical specialist groups means that large numbers of similar cases can be accumulated quickly, which is in stark contrast to the situation in equine veterinary hospitals, in which the case load tends to be more seasonal and diverse.

During examination and statistical analyses of the results, it became apparent that the variation in APP response between individuals and between groups led to major limitations when using standard statistical comparisons and to that end examples of individual horse responses are included alongside a description of the response.

It will be important to investigate a number of specific uses for the measurement of SAA concentration. These include, but are not limited to, monitoring response to treatment of septic arthritis. In such cases, repeated synoviocentesis, to monitor intra-articular inflammation, can be detrimental. However, plasma SAA concentration could be monitored and correlated to the degree of lameness with relative ease. The use of antimicrobials is a topic that has received a great deal of interest, and public scrutiny, since the emergence of a number of multi-resistant, and often nosocomial organisms (Boerlin *et al.*, 2001; O'Mahony *et al.*, 2005). A horse with an increasing plasma SAA concentration in the postoperative period should be considered for closer

scrutiny or further perioperative antimicrobial therapy whereas perhaps a declining profile may inform a prompter cessation of antimicrobial treatment.

It could be postulated that induction and recovery from anaesthesia and surgical positioning could lead to an acute phase response (Ellis *et al.*, 1992). In humans and animals the effect of anaesthesia on the acute phase response is believed to be negligible (Pepys *et al.*, 1989; Pepys and Hirschfield 2003). Allen *et al.*, (1988) demonstrated that anaesthesia had no significant effect on fibrinogen production; its' potential effects on SAA were not investigated, however in the study by Pepys *et al.*, (1989) general anaesthesia without surgery had no effect on SAA concentration. In the study described here, both groups of horses were subjected to the same anaesthetic protocol to minimise any potential effects on the results and to at least make any effect uniform across the horses in the study. In both groups, it was not possible to distinguish any discernable evidence of an effect of general anaesthesia on the acute phase protein concentrations.

An obvious next step might include the measurement of acute phase protein concentration in horses subjected to anaesthesia without surgery, i.e., during advanced imaging techniques such as MRI in which horses are routinely anaesthetised for long periods without surgical intervention. Unfortunately, these types of imaging modalities were not yet widely available at the time when the study presented above was performed. Nevertheless, even positioning in dorsal or lateral recumbency for short periods of time during anaesthesia can lead to increased concentrations of enzymes indicative of muscle damage (creatinine kinase and alkaline phosphatase), (Smith *et al.*, 1996). It is reasonable to assume that an inflammatory response, and therefore an acute phase response, could accompany these types of changes. Similarly, the possible dilution effect of the intravenous fluids given during the surgery on the concentration of the acute phase proteins was not investigated. However, there was a decrease in the mean haemoglobin concentration of the horses undergoing elective surgery, probably as a result of haemodilution.

There are clearly many other factors, common to equine medicine and surgery, that could potentially influence the acute phase response, for example, the use of antimicrobials, non-steroidal anti-inflammatory drugs, invasive versus non-invasive surgical procedures and experience of the surgeon. In conclusion, separating the effects of anaesthesia, positioning and recovery, and the use of

different pharmacological agents from the effect of surgical intervention is likely to be extremely challenging.

No attempt was made to evaluate the prognostic value of the various acute phase reactants; this was due to the diverse range of surgical conditions affecting the horses presented and the relatively small numbers of animals in each group during the study period.

There is evidence that a specific combination of cytokines is required to induce increases or decreases in acute phase reactants (Ramadori *et al.*, 1985; Perlmutter *et al.*, 1986; Gauldie *et al.*, 1987; Bauman *et al.*, 1987). The significance of this is that particular pathological conditions which lead to the production of a unique combination of cytokines may in turn have a unique acute phase profile (Ganapathi *et al.*, 1991). As the activity of SAA in diseased horses is further characterised and perhaps other equine acute phase markers identified, it may become possible to identify an acute phase signature for particular pathological conditions and to further use these markers to make decisions regarding treatment and prognosis.

Having established that SAA is an effective indicator of inflammation in horses with surgical and therefore acute trauma, the next phase of this work has focused on other causes of inflammation, low grade inflammatory conditions and infectious disease, and investigates the use of SAA and other acute phase reactants as prognostic indicators in horses (Chapters 5, 6 and 7).

CHAPTER 5

Surgical colic and the acute phase response in horses

Can we improve clinical decision making at admission?

5.1 Introduction

Few clinical syndromes affecting equidae engender as much concern, anxiety and fear amongst horse owners, trainers and keepers as colic. The term colic refers to any syndrome which includes clinical signs attributable to dysfunction or derangement of the gastrointestinal tract and therefore refers to a group of diseases with a variety of aetiologies, and with a wide spectrum of severity and outcome (Mair, 2002; Marshall and Blikslager, 2012). Fortunately, the majority of horses affected by colic have low-grade transient abdominal pain that is self-limiting or is rapidly ameliorated following the administration of medical therapy by a veterinary surgeon (Proudman, 1992). However, a small, but significant, minority of horses affected with colic require rapid surgical intervention (Hillyer *et al.*, 1997).

The purpose of surgery in the colicking horse is three fold: 1. Initially to obtain or confirm the diagnosis of a lesion requiring surgical intervention; 2. To determine the prognosis following treatment; and, finally, 3. To correct, if it seems feasible, the abnormality or determine that euthanasia is the most appropriate course of action.

Clinical signs of colic are attributable to varying degrees of intestinal injury, which, although principally associated with intestinal ischemia and obstruction, can also occur when the intestinal lumen remains patent (Cohen, 2002). A spectrum of lesions with variable amounts of vascular compromise and intestinal patency are regularly encountered. It is widely recognised that determination of the degree of intestinal injury (Snyder, 1989), which occurs prior to and following surgical correction of a lesion is critical when attempting to decide which horses should be treated, and in turn deciding their prognosis. Surgical intervention in horses with gastrointestinal disease is a major undertaking financially (Hillyer *et al.*, 1997), in terms of the personnel required and surgical skill and experience. Additionally, performing surgery on horses, which subsequently perish as a result of intestinal injury and its consequences, has significant implications for equine welfare.

As previously discussed in Chapter 1, a number of groups have focused on the identification of a range of laboratory analytes that might provide useful information on survival rates at the time of presentation. However, to date, no

single variable, or group of variables have been shown to be any more effective than measurement of heart rate at presentation alone (Orsini *et al.*, 1989; Freden *et al.*, 1989; Pascoe *et al.*, 1990; Reeves *et al.*, 1990; Furr, *et al.*, 1995; Blikslager and Roberts, 1995; Thoefner *et al.*, 2000; Senior *et al.*, 2011). This is in stark contrast to the situation in a number of clinical syndromes in humans, e.g., myocardial disease, gastrointestinal and pancreatic surgery in which the measurement of a number of acute phase reactants is routinely used to help determine which treatment modality is most appropriate (Waleczek *et al.*, 1991; Schillinger *et al.*, 2002; Dhankhar *et al.*, 2011; Palani Velu *et al.*, 2016). Following on from Chapters 3 and 4, in which it was established that the resting concentration of the acute phase proteins Serum Amyloid A (SAA), haptoglobin and fibrinogen are measureable and not highly variable in clinically normal horses, and that these analytes respond rapidly following disease and surgical intervention (Pollock *et al.*, 2005), it seems reasonable to speculate that measurement of these particular APPs could be used to determine the degree of tissue damage, and therefore likelihood of survival, of horses presented with surgical colic. Identification of individuals with widespread intestinal ischaemia, necrosis, and therefore severe systemic compromise, with its associated grave or hopeless prognosis for survival, prior to the onset of treatment would have considerable implications during decision making by veterinary surgeons, and horse owners/keepers and trainers alike. Perhaps most importantly, the ability to identify these horses without the need for invasive and often pointless surgical intervention would be of unquestionable value.

5.1.1 Developing a clinical test

The focus of the work presented in this thesis was to explore whether acute phase protein measurements, specifically SAA, should be adopted from a position of sporadic use as part of a general panel of biochemical analytes, without specific purpose, to a position whereby it could be used as part of a targeted panel of biomarkers and clinical parameters to inform decision making in a clinical setting for a specific disease or syndrome.

In order for a test to function clinically, it is critical to define the parameters for which the test is intended. In the case of surgical colic these could include:

1. Ability rapidly to differentiate between horses with a poor or hopeless prognosis and those that are likely to respond to treatment
2. Ability to differentiate between horses with medical and surgical colic
3. A test in which results could be available quickly and “horse-side”

5.1.2 Summary and aims

The Aims of this chapter were as follows:

1. To determine at intervals, and analyse serum concentration of SAA, haptoglobin and fibrinogen in horses with surgical colic at the point of presentation to specialist referral centres to resolution of clinical signs, euthanasia or death following evaluation and treatment in the centres concerned.
2. To explore whether APPs, specifically SAA and haptoglobin, can be used accurately to predict the outcome and likelihood of survival following surgical intervention in horses with colic and could be used during decision making at the time of initial presentation.

5.2 Materials and methods

5.2.1 Experimental design

This was a prospective clinical study in which horses were recruited at three university based equine clinics, The Faculty of Veterinary Medicine, University College Dublin, Ireland, The Large Animal Hospital, Royal Veterinary and Agricultural University, Copenhagen, Denmark and the School of Veterinary Medicine, University of Glasgow.

5.2.2 Ethics

The project was approved by the ethics and welfare committees at each institution. Owners and keepers, or their agents, were asked to give written consent, in the form of a consent form which was clearly explained and signed by both the lead clinician and owner, keeper or their agent, prior to collection of these clinical data.

5.2.3 Study animals

The animals studied in this Chapter represented a wide range of equine breeds, types, and uses. Detailed information on these animals relevant to their outcome is given in Tables 5.1 and 5.2.

5.2.3.1 Case selection

The selection criteria were as follows: Any horse presented to the equine emergency service at any one of the above three clinics between 2003 and 2006 which was determined to have a lesion which required surgical intervention and was thereafter subjected to surgery no matter the surgical outcome and in which the author, was either principal or assistant surgeon. During the study period, animals presented for the investigation of colic that did not require a surgical consultation or subsequent surgical intervention were not included. In addition, animals presented and subsequently subjected to euthanasia on financial grounds were not included.

5.2.4 Sampling procedures

Signalment, historical data, initial examination, surgical findings including the position and type of lesion and procedure performed, results of routine ancillary diagnostics and outcome were recorded. Blood samples for the acute phase reactants SAA, haptoglobin and fibrinogen, total protein (TP) concentration, packed red cell volume (PCV) were collected using the methods described in Chapter 2, at the following time intervals: presentation, at the end of the anaesthetic period or the point of euthanasia, and then at 12, 24, 48 and 72 hours post surgery assuming the horse survived to each time point. Heart rate was also recorded at each time point.

5.2.5 Anaesthesia

All of the horses described in this chapter were subjected to general anaesthesia. Broadly similar protocols for general anaesthesia were used in all three institutions, these represented the standard operating procedure at the time of sampling. Details of the protocols are given in Chapter 2. The broad lack of effect of anaesthesia, viewed against the perspective of an increase in values

due to the surgical procedure itself, on the acute phase response has been documented and was discussed briefly in Chapter 4.

5.2.6 Data processing

All data were entered into a Microsoft Excel spread sheet (Microsoft Excel for mac). Data were checked for errors, consistency and validity using Excel's standard functions. The data were separated into groups based on the outcome and once a final database was produced it was imported into STATA, version 12.1, for analysis. Minitab, version 16, and Microsoft Excel for mac, were used to produce graphical summaries of the data. Simple line graphs were initially produced to explore variables of interest. Thereafter, box plots were produced to further scrutinise these data, these are included.

5.2.7 Statistical methods

Full details of the statistical tests performed are given in Chapter 2.

The relationship between each explanatory variable and each of the two possible outcomes 1). surgical survival and discharge, 2). Death or euthanasia during anaesthesia or during the post operative period without discharge from the hospital, was examined using univariable logistic regression in order to identify variables for inclusion in multivariable model(s).

Comparisons were made between each variable in each of the two outcome groups for the values of each analyte or clinical variable obtained at the point of admission. The reason for choosing the point of admission was that this is the key decision making time when dealing with an animal affected by a surgical colic. Restricting analysis to one time point also minimised the risk of Type I error by reducing the number of comparisons made.

5.2.7.1 Univariable analysis

Examination of Explanatory Variables

The relationship between the following explanatory variables and the outcome variable, euthanasia/death or survival to discharge, were examined: SAA concentration, haptoglobin concentration, fibrinogen concentration, total protein concentration (TP), Packed red cell volume (PVC), heart rate (HR) in

beats per minute, and age. All continuous variables were categorised into quartiles in order to examine whether this form of the variable produced a better fit in the univariable logistic regression model. The categorical or continuous form of each variable that fitted the model best was taken forward to the multivariable modelling stage. Where appropriate, 4-level categorical forms of variables were collapsed to either 3- or 2-level (binary) variables. Wald P values were used to summarise the fit of categorical variables.

5.2.7.2 Multivariable analysis and model building

Potential explanatory variables were ordered based on a combination of factors: those with the lowest p value and highest odds ratio, biological importance and log likelihood value. A manual forward stepwise process was used to build the optimal multivariable model(s). Variables were retained within the models if their associated p-value was below 0.05.

Any confounding effect of non-significant variables was examined by forcing each into the final multivariable model(s) one at a time. Significant confounding was regarded as an alteration in the odds ratio of variables retained within the multivariable model(s) of more than 20% (Dohoo *et al.*, 2010). Assessment of correlation was an automated feature of model building. Manual assessment of correlation was not done as there was no indication from any of the models that there was any significant correlation between the variables included in the final models.

5.2.7.3 Receiver operating characteristic curves (ROC)

ROC analysis is one of the most popular graphical tools for evaluating the diagnostic power of a biomarker. It can be used to determine the ability of a test to discriminate between animals that are likely to survive a disease or syndrome and those that will not. The strength of the ROC analysis is that it provides the clinician with ability to view the trend of sensitivity over all values of the variable, and thus provides information about the relationship between the sensitivity and the specificity of a biomarker at all possible values. ROC analysis assesses the “performance” of the diagnostic test in terms of the sensitivity and 1-specificity (1-Sp) and therefore allows the user to determine the proportion of animals that are correctly classified by the test; this is summarised using the area under the curve (Greiner *et al.*, 2000). Receiver

operating characteristic (ROC) curves were created to estimate the predictive ability of multivariable models and the RocTab function in STATA was used to identify the optimal cut-off in different APPs which best differentiated between horses that were discharged or not.

5.2.7.4 Goodness of fit testing

Goodness of fit testing was performed on the final models using an Homer-Lemeshow goodness of fit test in STATA version 12.1

5.2.7.5 *Post hoc* sample size calculations

Post hoc power calculations were performed using a 2 sample t-test in Minitab V 16.

Table 5.1. Signalment, diagnosis, surgical procedure performed and clinic for the 21 horses in group 1

Horse ID D=dublin C=Copenhagen G=Glasgow	Surgical diagnosis	Procedure	Age	Sex
23 (D)	Epiploic foramen entrapment	Resection and anastomosis	15	M
24 (D)	Strangulating Lipoma	Resection and anastomosis	17	M
25 (D)	Strangulating Lipoma	Resection and anastomosis	25	F
26 (D)	Right Dorsal Displacement	Correct displacement, colon dump	5	M
27 (D)	Right Dorsal Displacement	Correct displacement, colon dump	4	M
28 (D)	Right Dorsal Displacement	Correct displacement, colon dump	2	M
29 (D)	Strangulating Lipoma	Resection and anastomosis	16	F
30 (D)	Large Colon Torsion	Correct displacement	8	F
31 (D)	Small intestinal volvulus	Decompress lesion	4	F
32 (C)	Left dorsal displacement	Correct displacement, colon dump	11	M
33 (C)	Right Dorsal Displacement	Correct displacement, colon dump	3	F
34 (C)	Ileal impaction	Decompress lesion	0.5	M
35 (C)	Ceacal impaction	Typhlotomy	5	M
36 (C)	Small intestinal eventration	Resection and anastomosis	2	M

Horse ID	Surgical diagnosis	Procedure	Age	Sex
D=dublin				
C=Copenhagen				
G=Glasgow				
38 (C)	Entrapping mesodiverticular band	Decompress lesion	3	F
39 (G)	Peritonitis	Open lavage	2	M
40 (G)	Right Dorsal Displacement	Correct displacement, colon dump	11	M
41 (G)	Strangulating lipoma	Resection and anastomosis	7	F
42 (G)	Ascarid impaction	Soften and decompress via enterotomy	0.5	M
43 (G)	Strangulating inguinal hernia	Resection and anastomosis	18	M

Table 5.2. Signalment, diagnosis, clinic and reason for euthanasia for the 38 horses in group 2

Horse ID D=Dublin	Diagnosis	Reason for Euthanasia	Age	Sex
1 (D)	Small intestinal volvulus	Extensive lesion	12	F
2 (D)	Large colon torsion	Severe intestinal compromise, beyond boundaries in which resection possible	16	F
3 (D)	Strangulated inguinal hernia	Severe compromise of terminal ileum, not amenable to resection	7 months	F
4 (D)	Small intestinal volvulus	Extensive lesion	9	M
5 (D)	Peritonitis ruptured stomach	Severe abdominal contamination -prognosis hopeless	22	M
6 (D)	Strangulating lipoma	Extensive lesion	26	F
7 (C)	Enterocolitis	Extensive lesion, evidence of peritonitis	4	M
8 (C)	Pelvic flexure rupture	Severe abdominal contamination -prognosis hopeless	8	F
9 (C)	Epiploic foramen entrapment jejunum	Extensive lesion	7	M
10 (C)	Strangulating lipoma	Extensive lesion	10	F
11 (C)	Strangulating lipoma	Extensive lesion	13	F
12 (C)	Small intestinal volvulus	Extensive lesion	aged	M
13 (C)	Jejunojenuo intussusception	Extensive lesion	2 months	M
14 (C)	Ruptured stomach	Severe abdominal contamination -prognosis hopeless	3	M
15 (C)	Epiploic foramen entrapment jejunum	Extensive lesion	7	M
16 (G)	Entrapping mesenteric rent - Jejunum	Extensive lesion	11	M

Horse ID D=Dublin	Diagnosis	Reason for Euthanasia	Age	Sex
17 (G)	Strangulating lipoma	Extensive lesion	17	F
18 (G)	Small colon enterolith and rupture	Severe abdominal contamination - prognosis hopeless	5	M
19 (G)	Epiploic foramen entrapment jejunum	Extensive lesion	10	F
20 (G)	Large colon torsion	Severe intestinal compromise, beyond boundaries in which resection possible	13	F
21 (G)	Strangulating lipoma	Extensive lesion	20	M
22 (G)	Strangulating lipoma	Extensive lesion	19	F
44 (D)	Colon torsion - Resection	Ileus, severe cardiovascular compromise (24 hours after surgery)	12	F
45 (D)	Epiploic formamen entrapment - Resection and anastomosis	Ileus, severe cardiovascular compromise (48 hours after surgery)	4	M
46 (D)	Focal eosinophilic bands Decompression	Ileus, severe cardiovascular compromise (12 hours after surgery)	1.5	M
47 (D)	Small intestinal volvulus - Resection and anastomosis	Died in recovery	2	M
48 (C)	Peritonitis ruptured stomach	Severe ileus (48 hours)	5	F
49 (C)	Colon torsion - Resection	Ileus, severe cardiovascular compromise (48 hours after surgery)	10	F
50 (C)	Epiploic formamen entrapment - Resection and anastomosis	Ileus, severe cardiovascular compromise (12 hours after surgery)	6	F
51 (C)	Right dorsal displacement of the large colon and caecal torsion - correct displacement, typhlotomy	Caecal rupture (48 hours after surgery)	17	F

Horse ID D=Dublin	Diagnosis	Reason for Euthanasia	Age	Sex
52 (G)	Small intestinal volvulus - Ressection and anastomosis	Rectal tear (24 hours after surgery)	6	M
53 (G)	Colon torsion - Resection	Ileus, severe cardiovascular compromise (12 hours after surgery)	14	F
54 (G)	Small intestinal intussusception - Resection and anastomosis	Ileus, severe cardiovascular compromise (24 hours after surgery)	6 months	F
55 (G)	Small intestinal volvulus - jejunocecal anastomosis	Severe ileus (48 hours after surgery)	6	M
56 (G)	Epiploic formamen entrapment - Ressection and anastomosis	Ileus, severe cardiovascular compromise (24 hours after surgery)	3	M
57 (G)	Right dorsal displacement - Correct displacement, colon dump	Died in recovery	19	F
58 (G)	Entrapping mesodiverticular band - jejunum- resection and anastomosis	Ileus, severe cardiovascular compromise (12 hours after surgery)	7	M
59 (G)	Large colon torsion	Peritonitis (48 hours after surgery)	13	F

5.3 Results

Fifty nine cases, ranging in age from two months to 26 years were identified. There were 25 females and 34 males. Based upon outcome, two groups emerged;

- (1) Horses that underwent surgery and survived to be discharged from the clinic at various time intervals after treatment (n=21)
- (2) Horses that died or were euthanised either during general anaesthesia or during the postoperative period prior to discharge (n=38)

5.3.1 Group 1. Horses subjected to surgery which were subsequently discharged from the hospital

Twenty-one horses, presented for investigation of clinical signs of colic and subsequently subjected to exploratory abdominal celiotomy, survived to be discharged from one of the three hospitals. The signalment of these animals, surgical diagnosis, procedure performed and in which clinic, is given in Table 5.1. Results for the three APPs and for HR, PCV and TP at admission are given in Table 5.3

There was considerable variation in the concentration of SAA at admission, which ranged from 113 µg/mL to 10876 µg/mL, however, 10 of the 21 horses had an SAA concentration within the normal reference range (0-22 µg/mL). There were three outliers with an SAA concentration more than 100 times normal, each of these horses was subsequently noted to have intraoperative evidence of significant tissue damage (typhlitis, peritonitis and necrosis of the jejunum). The remaining horses had mild increases in SAA concentration (203µg/mL-547µg/mL). In all 21 horses, changes in SAA concentration followed a broadly similar pattern whereby it increased during the initial 12-24 hours before beginning to decline. Consequently, mean SAA concentration increased from the point of admission, reached peak concentration 24 hours after admission after which point it began to decline (Figure 5.1). Haptoglobin concentration was within the reference range for 18 of the 21 horses at admission; thereafter the concentration increased from the end of anaesthesia and began to decline after 48 hours (Figure 5.2). The same three outlying horses identified as a result of a considerably increased SAA concentration, also had an increased haptoglobin

concentration. Fibrinogen concentration was within the reference range for 18 of the 21 horses at admission and began to increase from the end of anaesthesia but did not decline during the sampling period (Figure 5.3). The three outlying horses, noted as a result of increased concentration of SAA and haptoglobin, also had a fibrinogen concentration outwith the reference range. Heart rate and total protein concentration followed a general pattern of reducing from a high at admission for all of the horses (Figures 5.4 and 5.5). Total protein concentration was variable without evidence of a pattern (Figure 5.6).

5.3.2 Group 2 horses that died or were euthanised

Thirty eight horses, presented for investigation of clinical signs of colic and subsequently subjected to exploratory abdominal celiotomy, were determined, by a European or Royal College of Veterinary Surgeons Recognised Specialist surgeon (the author), to have lesions which were not amenable to surgical intervention and were therefore subjected to euthanasia during general anaesthesia (n=22) or, were considered to have a lesion amenable to treatment and were therefore recovered from anaesthesia but subsequently died or were euthanised in one of the three hospitals (n=16) (these two groups were combined because they had the same outcome and it was therefore considered appropriate to analyse them as one group). The signalment of these animals, surgical diagnosis and details of which clinic they presented to, is given in Table 5.2. Results for the three APPs and for HR, PCV and TP are given in table 5.4.

The concentration of SAA at admission was outwith the reference range for all of the horses in group 2. In the subgroup of horses not recovered from anaesthesia, the concentration of SAA was increased by a factor of 100 times for a number of the horses.

5.3.3 Graphical comparisons between groups

Box plots were produced using Minitab, version 16 for each of the explanatory variables, at admission, for the two groups of horses. These variables were the acute phase proteins, SAA, haptoglobin and fibrinogen, and the clinical and haematological analytes, HR, PCV and TP. The purpose of these plots was to assess which variable or variables were likely to be of most interest when producing models to predict survival.

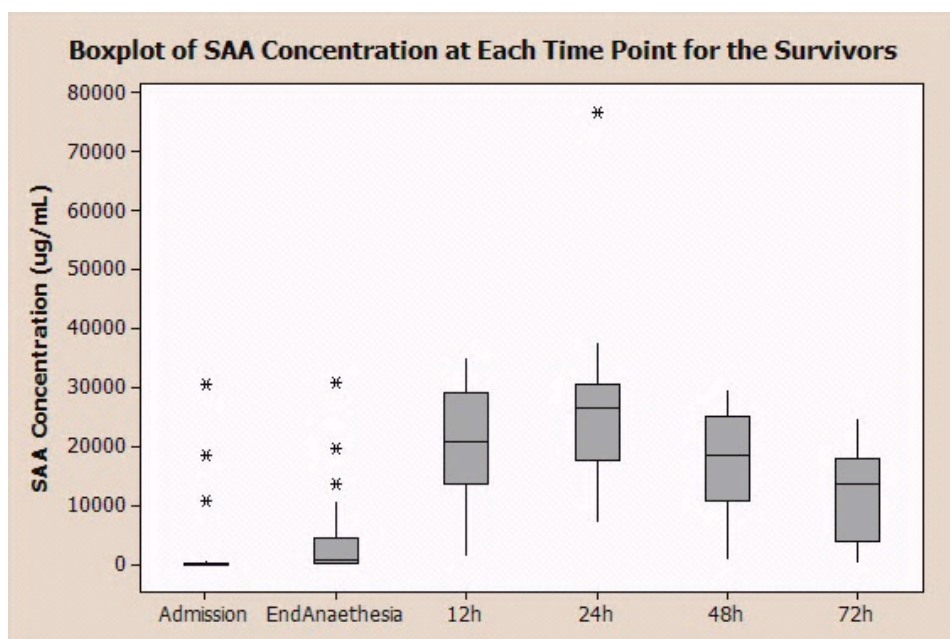
Table 5.3 Results of analytes collected at admission for horses in Group 1 (survivors)

Horse ID	HR	SAA (µg/mL)	Haptoglobin (mg/mL)	Fibrinogen (g/L)	PCV	TP
23	64	346	2	2	46	78
24	76	178	1.3	2	58	70
25	68	123	1.4	2	31	69
26	58	184	1	2	31	58
27	62	547	1	2	45	62
28	72	139	2.2	2	35 *	
29	68	370	1.1	1	54	55
30	86	127	2	2	65	46
31	76	356	1	2	32	83
32	52	129	1.2	2	35	78
33	52	113.5	1.5	2	37	52
34	84	124	1.5	2	37	41
35	48	10876	3.2	6	49	52
36	62	176	1.1	2	45	69
37	68	203	1.3	2	48 *	
38	80	183	1.6	2	45	62
39	92	3765	3.9	7	46	54
40	58	1275	1	2	35	79
41	76	234	1.6	2	25	62
42	48	282	0.9	2	26	64
43	68	645	2.9	4	25	79

Table 5.4 Results of analytes collected at admission for horses in Group 2 (non-survivors)

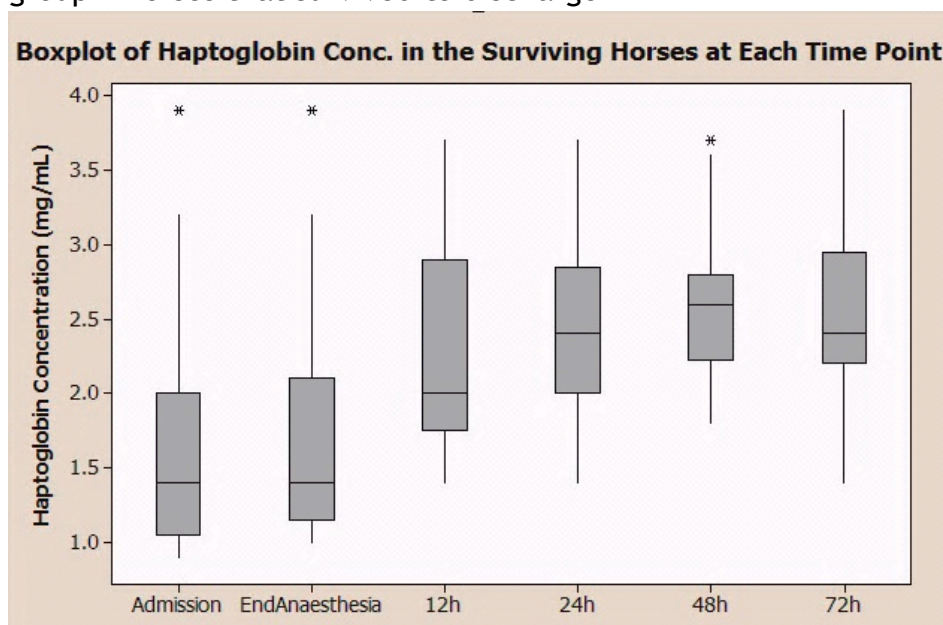
Horse ID	HR	SAA (µg/mL)	Haptoglobin (mg/mL)	Fibrinogen (g/L)	PCV	TP
1	98	2441	2.5	2	48	73
2	112	3247	2.7	2	52	83
3	68	2999	3	2	39	66
4	88	2172	2.4	3	54	54
5	120	41364	4.5	6	58	72
6	60	3245	2.9	4	51	70
7	120	45375	3.4	4	34	69
8	64	1958	2.4	4	39	67
9	44	1260	3.2	4	43	58
10	80	3739	3	4	61	63
11	68	3136	2.9	3	55	64
12	82	1953	2.4	3	57	77
13	80	4273	2.8	2	53	80
14	32	52568	2.8	6	47	93
15	52	7150	2.7	2	49	97
16	102	3484	3.2	3	54	54
17	28	63790	4.2	6	48	52
18	64	3055	3	4	43	81
19	48	1582	2.9	4	55	58
20	100	3940	2.1	5	67	72
21	80	2638	2	3	42	57
22	92	2463	1.8	2	37	85
44	88	658	2	2	55	89
45	68	453	1.8	2	62	77
46	76	3290	2.1	2	48	74
47	80	789	2.4	2	48	72
48	80	4573	2.6	2	58	77
49	108	936	3.3	2	62	85
50	40	1228	2	2	34	50
51	52	783	1.4	2	30	61
52	78	933	3.6	2	48	70
53	100	952	2.7	2	57	85
54	88	765	3	2	37	52
55	58	367	2	2	41	54
56	78	402	2.4	2	30	49
57	60	347	2	2	30	58
58	88	983	1.8	2	48	57
59	88	8761	2	2	55	74

Figure 5.1 Box plot showing SAA Concentration at each time point for the group 1 horses that survived to discharge



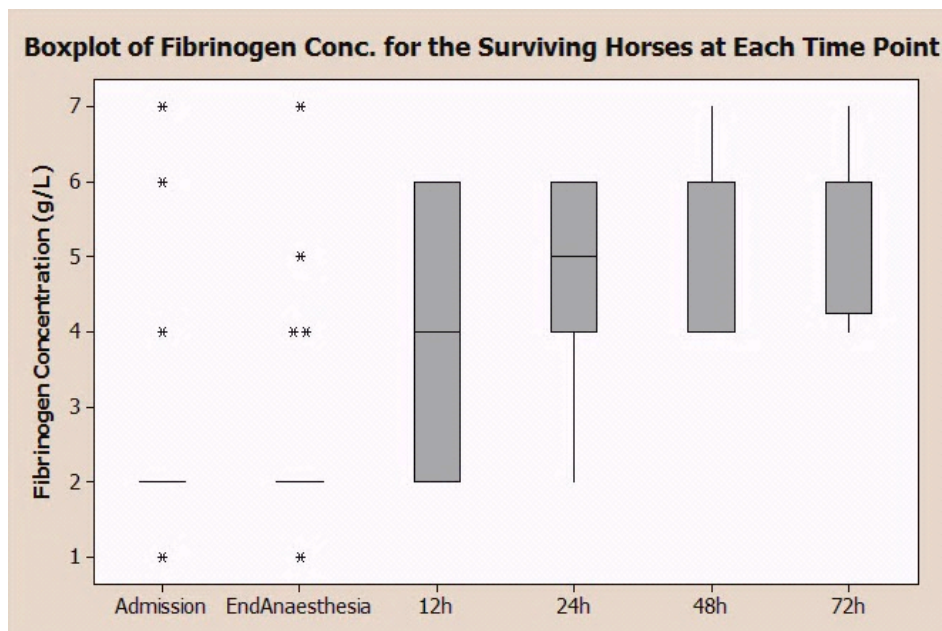
There were three outliers, nevertheless SAA concentration followed a broadly similar pattern in all of the horses, increasing for 24 hours before beginning to decline.

Figure 5.2 Box plot showing haptoglobin concentration at each time point for the group 1 horses that survived to discharge



There was considerable variation in haptoglobin concentration and the same outliers were noted as with SAA concentration.

Figure 5.3 Box plot showing Fibrinogen Concentration at each time point for the Group 1 horses that survived to discharge



Fibrinogen concentration was within the reference range for 18 of the 21 horses at admission and began to increase from the end of anaesthesia but did not decline during the sampling period. Three outliers were noted at admission.

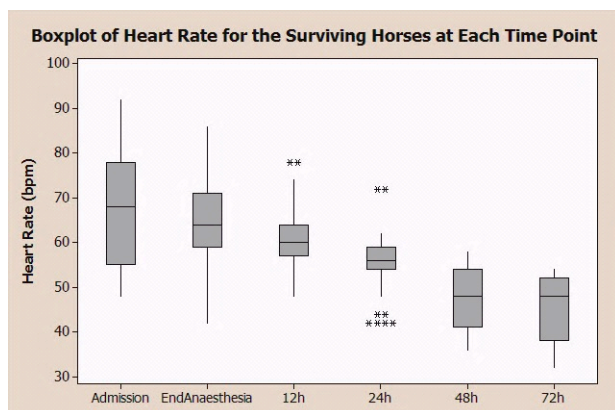


Figure 5.4 Box plot showing HR at each time point for the Group 1 horses that survived to discharge.

As previously noted in the literature, heart rate is commonly noted to decline from the point of presentation in surviving horses.

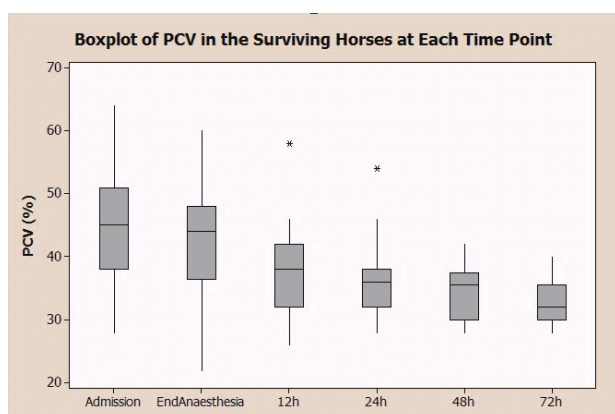


Figure 5.5 Box plot showing PCV at each time point for the Group 1 horses that survived to discharge.

As also previously noted in the literature, PCV also declined from the point of presentation in the surviving horses.

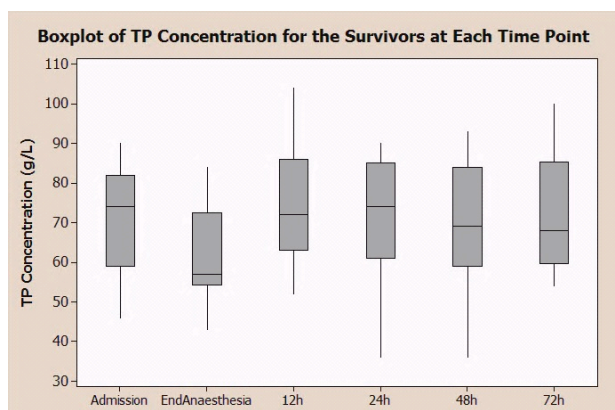
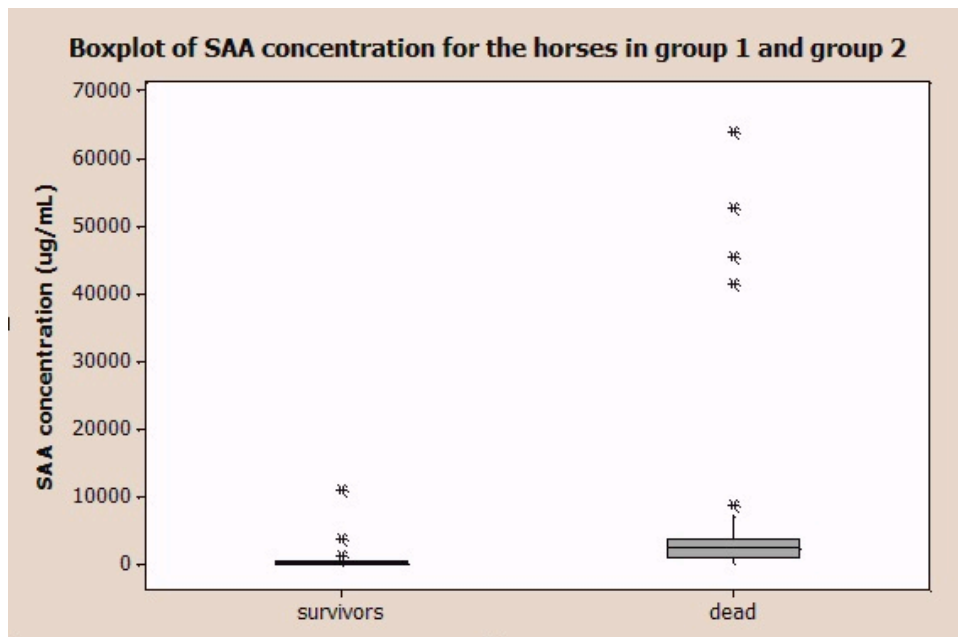


Figure 5.6 Box plot showing TP Concentration at each time point for the Group 1 horses that survived to discharge.

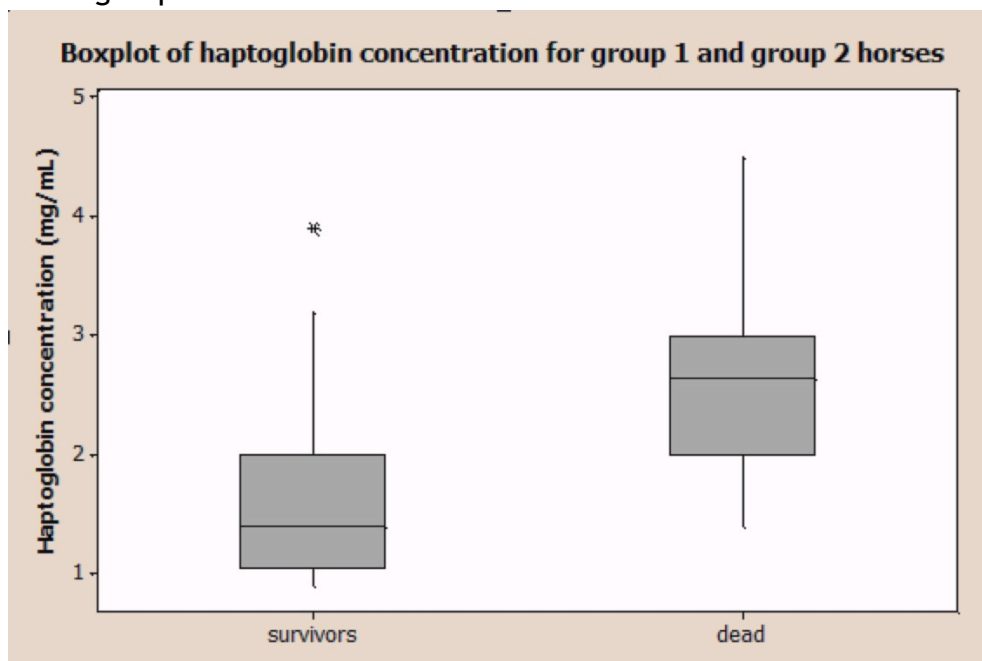
The TP results were difficult to relate directly to the clinical findings.

Figure 5.7 Box plot showing SAA concentration for the horses in group 1 and group 2



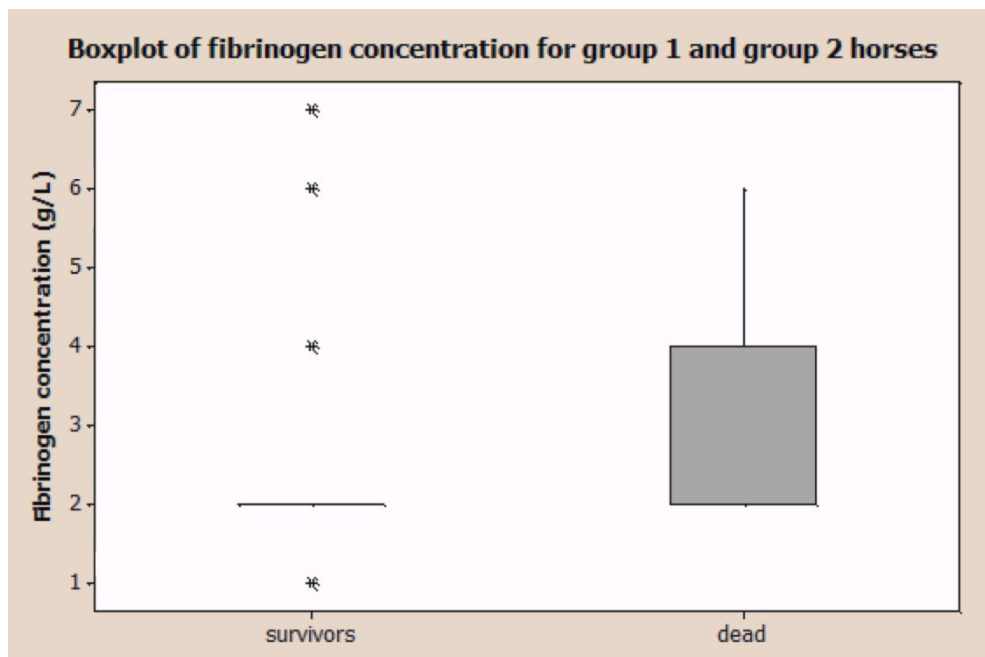
Samples for SAA concentration at admission only were compared. In group 1, a number of outliers were identified in horses with particular evidence of tissue necrosis. In group 2, a wide range of SAA concentrations were identified at admission reflecting the variety of surgical lesions identified and considerable tissue necrosis and inflammation.

Figure 5.8 Box plot showing haptoglobin concentration for the horses in group 1 and group 2



Samples for haptoglobin concentration at admission only were compared. In group 1, the same outliers were identified as with SAA concentration. There was considerable variation amongst the group 2 samples; however, these were generally of a greater concentration than those in group 1.

Figure 5.9 Box plot showing fibrinogen concentration for the horses in group 1 and group 2



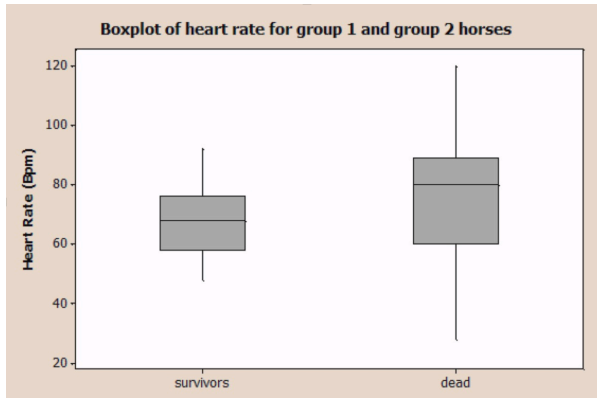


Figure 5.10 Box plot showing heart rate for the horses in group 1 and group 2.

Heart rate at admission was generally higher for the non-survivors.

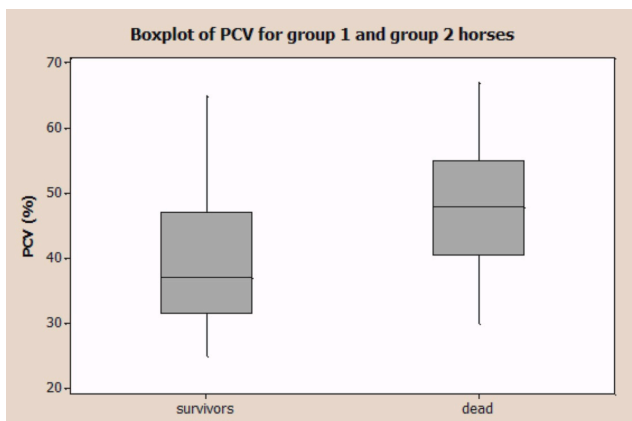


Figure 5.11 Box plot showing PCV for the horses in group 1 and group 2.

PCV at admission was generally higher for the non-survivors.

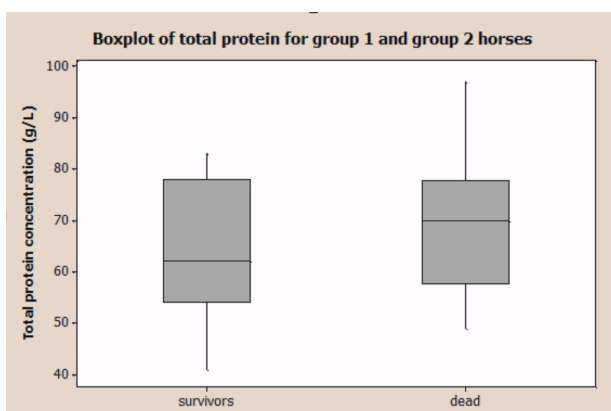


Figure 5.12 Box plot showing total protein concentration for the horses in group 1 and group 2.

TP concentration was broadly similar for both groups.

5.3.4 Statistical modelling

In this study, horses presented for evaluation of clinical signs of colic that were subsequently subjected to an emergency abdominal celiotomy were divided into two groups based on outcome. A number of alternative statistical analyses were performed using multivariable logistic regression models and receiver operating characteristic curves (ROC) in order to assess which variables were most strongly associated with the outcome, in this case death or survival to discharge. For all models, clinical data at admission were used, given that the purpose of this study was to determine whether this information could be used to decide whether an individual horse should be subjected to surgical exploration.

The following statistical models were produced in order to determine which explanatory variable or variables were associated with the outcome and could therefore have potential to predict which horses would not survive.

5.3.4.1 Horses which survived to be discharged versus horses which died or where euthanised (59 observations).

a. Univariable analysis

Seven explanatory variables were screened at the univariable level, both as continuous variables and as categorical variables. Details of confidence intervals, odds ratios and P values are given in Table 5.5. SAA and haptoglobin concentration and PCV were found to be significantly associated with death (SAA concentration, $p=0.03$, haptoglobin concentration $p<0.001$, PCV $p=0.01$). Fibrinogen concentration, age, sex, heart rate, and total protein were not significantly associated with outcome (Table 5.5).

b. Multivariable analyses

SAA, haptoglobin and fibrinogen concentration were included in a final multivariable model (Table 5.6). In this model, Category 2 SAA concentration ($>350\mu\text{g/mL}$) at admission remained a statistically significant predictor for survival of horses presented for the investigation of colic with a surgical lesion ($P = 0.001$) haptoglobin ($P=0.003$) and fibrinogen ($P=0.008$) concentration were also significantly associated with the outcome variable (Table 5.5).

Table 5.5 Results for the univariable analyses for each of the seven explanatory variables.

Variable	OR	CI	P value	Dead	Alive
SAA (µg/mL) (CONT)	1.0005	1.00005-1.0009	0.03	38	21
SAA(CAT)			<0.001		
113.5-347	1	*	*	1	14
356-983	38.5	3.75-395.41	0.002	11	4
1228-8247	196	11.12-3453.722	<0.001	14	1
>63790	84	6.76-1045.31	0.001	12	2
Haptoglobin (mg/mL) (CONT)	7.59	2.59-22.27	<0.001	38	21
Haptoglobin (CAT)			<0.001		
0.9-1.6	1	*	*	1	15
1.8-2.4	80	7.48-856.01	<0.001	16	3
2.5-2.9	150	8.38-2685.50	0.001	10	1
>3	82.5	6.62-1028.84	0.001	11	2
Fibrinogen (g/L)(CONT)	1.31	0.82-2.09	0.255	38	21
Fibrinogen (CAT)			0.1		
1.0-2.0	1	*	*	22	18
3.0-3.0	3	0.72-12.42	0.13	5	0
>4	1.22	0.65-2.28	0.53	11	3
HR (bpm)(Cont)	1.02	0.99-1.05	0.10	38	21
HR (CAT)			0.009		
28-60	1	*	*	10	6
64-68	0.33	0.8-1.54	0.12	6	11
79-92	2.6	0.52-13.0	0.25	13	3
>92	5.4	0.54-53.0	0.15	9	1
PCV (%) (CONT)	1.08	1.02-1.14	0.01	38	21
PCV (CAT)			0.02		
25-37	1	*	*	7	11
39-47	2.2	0.50-9.75	0.3	7	5
48-54	6.29	1.30-30.54	0.02	12	3
>55	9.43	1.60-55.45	0.01	12	2

Variable	OR	CI	P value	Dead	Alive
TP (g/L)(CONT)	1.04	0.99-1.08	0.14	38	21
TP (CAT)			0.15		
41-57	1	*	*	9	6
58-59	0.86	0.21-3.58	0.83	9	7
70-77	7.33	0.74-72.63	0.09	11	1
>78	1.2	0.27-5.40	0.81	9	5
Age in years (CONT)	1.05	0.41-8.01	0.25	38	21
Age in years (CAT)			0.48		
0.2-4	1	*	*	10	9
5.0-7.0	1.8	0.40-8.07	0.44	8	4
8.0-14.0	3.3	0.69-15.74	0.69	11	3
>15	1.62	0.39-6.68	0.39	9	5

Wald p-values for categorical variables are in **bold**

Table 5.6 Results for the multivariable analysis including SAA, haptoglobin and fibrinogen

Variable	OR	CI	P value	Dead	Alive
SAACAT2 (ref <350ng/ml)					
>350ng/ml	1	*	*	1	14
	60.73	4.97-742.63	0.001	37	7
Haptoglobin	20.28	0.09-0.70	0.003	38	21
Fibrinogen	0.25	0.08-0.17	0.008	38	21

c. Receiver operating characteristic curves

Receiver operating characteristic curves (ROC) were produced for the final multivariable model, (with and without fibrinogen) (Figures 5.13 and 5.14) and for SAA and haptoglobin concentration alone respectively, in order to estimate an appropriate cut point for optimal sensitivity and specificity when using SAA (Figure 5.15) and / or haptoglobin (Figure 5.16) to predict survival in horses with clinical signs of surgical colic.

The area under the curve for SAA concentration was 0.89 and for Haptoglobin concentration was 0.84. This suggests that when using these tests one could expect 89% and 84% of horses to be classified correctly.

ROctab results for SAA concentration suggested that a range of SAA concentrations between 347 µg/mL and 453 µg/mL would result in the correct classification of approximately 88% of animals, with a sensitivity of approximately 97% and a specificity of between 66 and 77%. A range is given because in this range sensitivity and specificity vary by very small amounts. For haptoglobin a concentration greater than 1.8 mg/mL would result in the correct classification of 88% of cases with a sensitivity of 97.37% and a specificity of 71.43%. A fibrinogen concentration greater than 3 g/L was associated with the correct classification of 57.63% of cases with a sensitivity of 42.11% and a specificity of 85.71%.

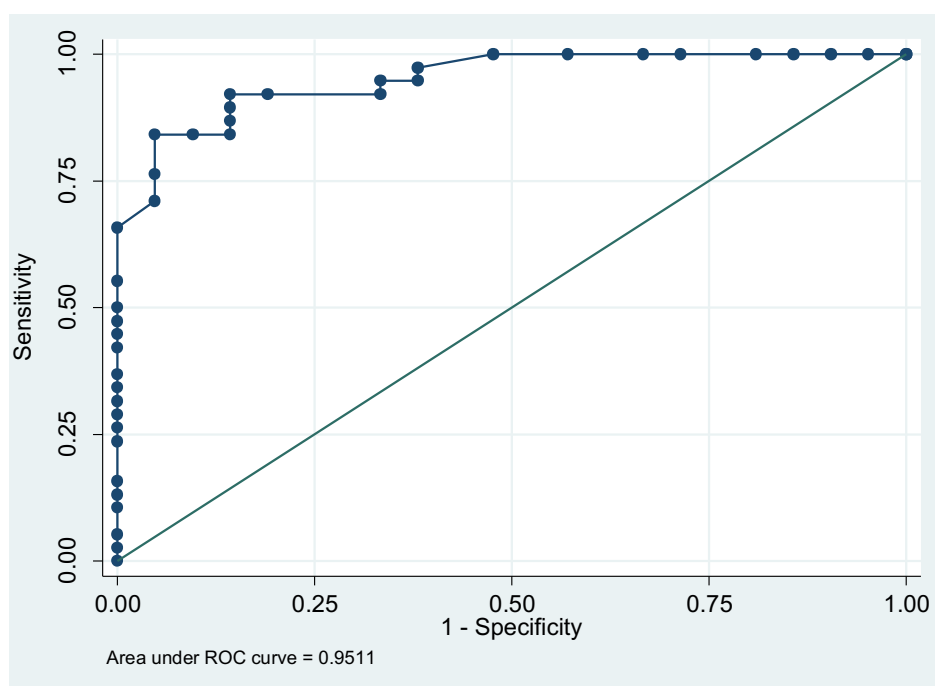
5.3.5 Goodness of fit testing

Hosmer-Lemeshow goodness of fit testing was performed on the final model. Hosmer-Lemeshow goodness of fit tests failed to identify any evidence of a lack of fit of the final model (P-value =0.98).

5.3.6 *Post hoc* sample size calculations

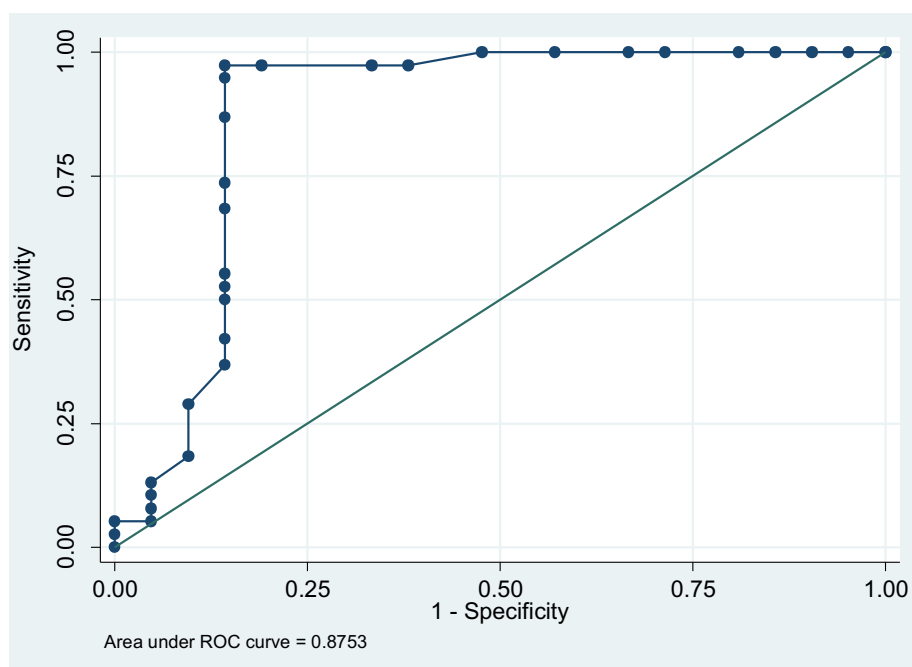
Post hoc power calculations were performed in Minitab, version 16 using a 2-sample t-test. These indicated that with the numbers of animals included in this study a difference of 107 µg/mL or greater in SAA concentration could be detected with an 80% power as significant at $p < 0.05$.

Figure 5.13 ROC curve for the final model including fibrinogen concentration



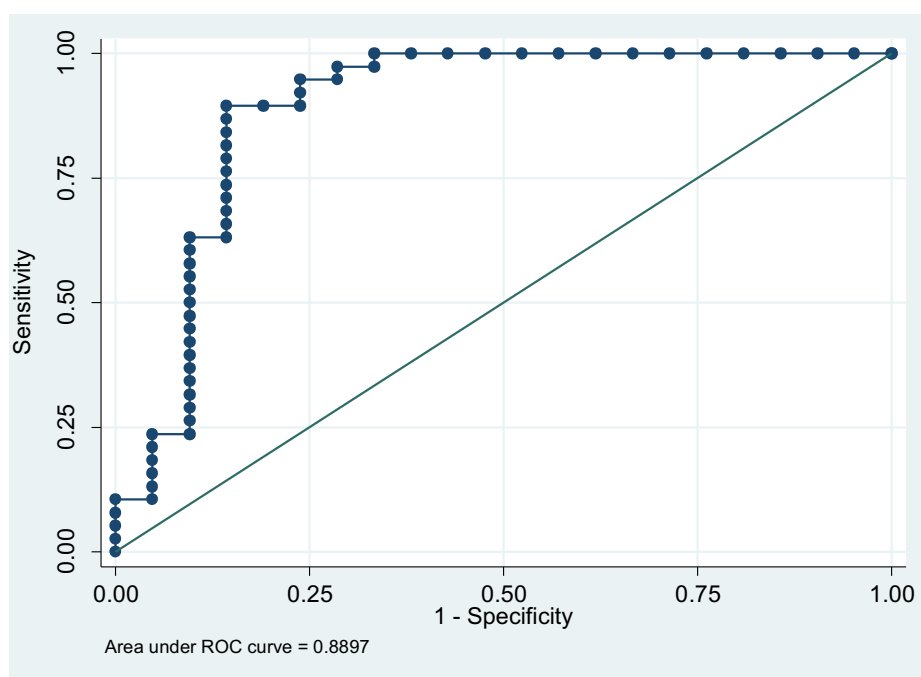
The ROC curve is a measure of performance of the test (in the final model including fibrinogen), with the area under the curve indicating the percentage of horses (95%) classified correctly (in this case animals which do not survive).

Figure 5.14 ROC curve for the final model excluding fibrinogen concentration



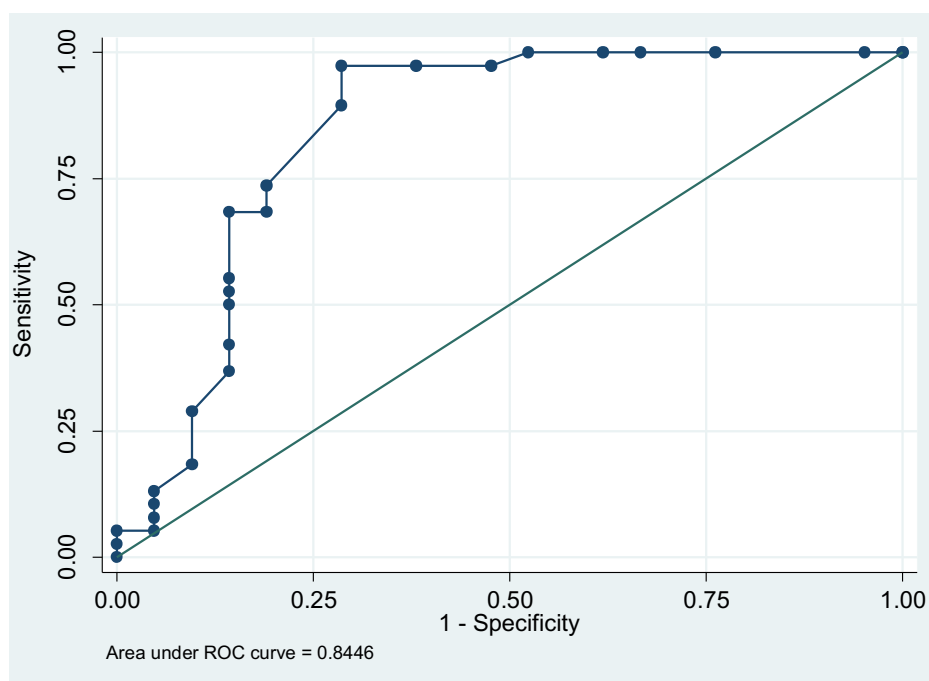
The ROC curve is a measure of performance of the test (in the final model excluding fibrinogen), with the area under the curve indicating the percentage of horses (88%) classified correctly (in this case animals which do not survive).

Figure 5.15 ROC curve for SAA concentration



The ROC curve is a measure of performance of the test (in this case SAA concentration), with the area under the curve indicating the percentage of horses (98%) classified correctly (in this case animals which do not survive).

Figure 5.16 ROC curve for haptoglobin concentration



The ROC curve is a measure of performance of the test (in this case haptoglobin concentration), with the area under the curve indicating the percentage of horses (84%) classified correctly (in this case animals which do not survive).

5.4 Discussion

5.4.1 Review of findings

In the results of these studies, the ability of acute phase proteins, heart rate and two haematological parameters, packed red cell volume (PCV) and total protein (TP) measurement, to predict the outcome of horses presented for the investigation of surgical colic were assessed. These data were grouped according to the outcome; either the horses survived to be discharged, or were euthanised or died prior to discharge. All of the horses in the group that did not survive had an SAA concentration outwith the reference range, by a factor of between 2 and 100 times, at admission. There was considerable variation in the concentration of SAA at admission in the group that did survive to be discharged; however, in this group, SAA concentration was considerably less than for those in the non-surviving group (except for 3 outliers with evidence of marked tissue necrosis). In the horses in group 1, in which serial samples were obtained, the behaviour of SAA concentration followed the same general pattern of an increase over a period of 24 hours followed by a decrease. As described in chapter 4, these findings suggest that SAA is a good marker of tissue necrosis and of surgical trauma. Haptoglobin concentration was within the normal range for the majority of the horses in group 1 (those that survived), although there were several outliers. Amongst group 2, the non-surviving group, the majority of horses had a haptoglobin concentration outwith the reference range. Fibrinogen concentration was more similar across both groups than the other two APPs, although there were considerably more outliers.

A multivariable model suggested that there were significant differences in the concentration of SAA, haptoglobin and fibrinogen between the two groups and that these markers therefore had the potential to be useful during the decision making process.

ROC curves were produced for the final model, including and not including fibrinogen, and for SAA and haptoglobin. The areas under the ROC curves (AUC) represent a global summary of diagnostic accuracy. Guidelines suggest that a non-informative test has an AUC of 0.5, an accurate test has an AUC of 0.5 to 0.7, a very accurate test has an AUC of 0.7 -0.9 and a highly accurate test has an AUC of 0.9 to 1. The area under the curve for SAA concentration was 0.89 and

for Haptoglobin concentration was 0.84. This suggests that when using these tests one could expect 89% and 84% of horses to be classified correctly.

The original intention had been to compare serial APP samples from the surviving group with a sub group of horses which survived surgery but were not discharged from the hospital. These analyses were undertaken; however, due to small numbers in each set and the risks of multiple comparisons, these were left out of the thesis.

5.4.2 Comparison of these findings with previous work

Previous work to identify and assess variables that could be used to predict outcome, and therefore inform decision making in horses with surgical colic, have focused on a number of areas. Many of these are related to clinical parameters measured during initial clinical examination, including heart rate, presence or absence of gastrointestinal borbyrigmi (sounds), results of rectal examination, passage of a nasogastric tube, peritoneal fluid examination and the measurement of PCV and TP (Freden *et al.*, 1989; Furr *et al.*, 1995; Blikslager *et al.*, 1995; Thoenes *et al.*, 2000; Senior *et al.*, 2011).

There is very little specific work on the use of acute phase proteins to predict survival in horses affected by colic (Sandholm *et al.*, 1995), although one study suggests that non-survivors had statistically greater concentrations of SAA than survivors in a large group of horses (Vandeplas *et al.*, 2005). Recent work by Pihl *et al.*, (2015) suggests that SAA and other clinical parameters could be used to differentiate between horses that could be treated medically from those which should be subjected to surgical intervention.

5.4.3 Limitations and factors that may have influenced the results

There are some significant limitations associated with the work presented in this Chapter. Principally these relate to the relatively small number of horses. In addition, the fact that these data were collected in three different university hospitals could have influenced the outcome for the horses based on local decision-making and treatment. A similar anaesthetic protocol and the fact that all of the procedures were undertaken by the same surgeon, either as principal or assistant, may have reduced the effect. Consideration was given to including centre as a random effect in the multivariable models, but given the size of the

databases and resultant statistical power it was not considered appropriate. In the recent similar multicenter study by Pihl et al, (2015), there was no effect of centre.

The effect of anaesthesia on the acute phase response has often been cited as a potential problem when interpreting results from horses with inflammatory disease that have also been subjected to general anaesthesia. While the effect of anaesthesia on SAA concentration was not specifically investigated in our study, previous studies by Allen and Kold (1988) indicate that anaesthesia has no significant effect on fibrinogen production and on SAA production (Pepys and Hirschfield, 2003). The three groups of horses across the three hospitals were subjected to the same or a very similar anaesthesia protocol in an attempt to minimise any effects on the results.

One of the aims of this study was to produce a model or models that could be used to inform decision making in horses presented for investigation and treatment of colic. As stated in the introduction, colic is not a disease in itself, but rather a term that describes a set of clinical signs associated with gastrointestinal dysfunction. It could be argued that there is considerable variation between the diagnosis, and therefore the pathological lesions, of some of the horses described in this study. There is no doubt that the power of this work could be increased through the inclusion of larger numbers of horses affected by the same or very similar lesions. Nevertheless, all of the horses described are unified by a common pathology: loss of gastrointestinal integrity leading to the urgent requirement for surgical exploration as the apparent option for treatment.

In some of the analyses reported in this study, the explanatory variables which remained statistically significant in the final multivariable model had a cut point or range of cut points which would make them of limited biological value in the clinical situation. In most cases this was because the cut point value was very close to normal, or very abnormal, as to be a value which would not be commonly encountered in practice. The best example of this was haptoglobin concentration, with the model suggesting that a value greater than 1.8 mg/mL was associated with death.

There were a considerable number of outlying results amongst the APPs, and this could have had an effect on the interpretation of mean results and in the comparisons. These outliers simply reflect the individual nature of the acute phase response in all species and of the underlying pathologies. For this reason, full results were included alongside a description of the behaviour or individual APPs in individual horses. As in human medicine, there is clearly an argument for assessing repeated APP results at the individual level (Pepys and Hirschfield, 2003).

The use of Kaplan-Meier survival analysis was considered, however, given the small numbers in each group and the fact that comparisons were made using the admission samples only, it was considered that as this test is a measure of the fraction of animals surviving for a certain amount of time after treatment it was less appropriate for this study than the models that were utilised.

5.4.4 What the clinician requires - Statistical significance versus biological significance

For any a test to be adopted and effective in the clinical setting, a number of criteria must be satisfied. These include but are not limited to:

1. The test must be easy to perform and results must be available quickly; in the case of colic, decision making is typically measured in minutes (such an SAA test has recently become commercially available - StableLab ©).
2. The test must be sensitive and specific, it is critical to identify as many animals that require intervention as possible. The costs in welfare and financial terms of operating on horses that do not require surgery are high.
3. The test should be interpreted against a background of other clinical, haematological and biochemical examinations and tests.
4. The test should be as binary in its output as possible; unfortunately horses with clinical signs of colic are difficult to categorise.
- 5 The test should be necessary in strengthening clinical decisions in circumstances where there is some doubt on the basis of pre-existing tests.

When attempting to define and validate a marker or markers for clinical decision-making, there is always a danger of assessing only one variable in

isolation. There is no doubt that, just as in human medicine, acute phase protein concentration should not be used in isolation. In many years of research into clinical decision making in equine colic, few clinical or haematological and biochemical analytes have been able to compete with the predictive value of heart rate at the time of admission. Perhaps this is related to the association between heart rate and endotoxaemia (Senior *et al.*, 2011). Surprisingly, HR did not feature strongly in many of the models produced during this study; perhaps this is because it is more labile than other markers of survival.

In chapter 4 it was shown that SAA concentration in horses, just like CRP concentration in humans, is a non-specific, rapidly responding marker for tissue injury, necrosis and surgical trauma. The findings of this study, in horses with colic, confirm that this is the case. However, based on the findings presented here, it would be unwise to base a decision on whether to operate on a horse or subject it to euthanasia using APP concentration alone. Nevertheless, the measurement of APP concentration, and in particular SAA, is likely to be a very useful adjunct to other parameters during the investigation and treatment of colic.

5.4.5 Future work

Future work should concentrate on larger number of horses, grouped by diagnosis/surgical lesion or specific syndrome. The inclusion of horses from a variety of hospitals would also be of value and appropriate statistical modelling that accounts for clustering of horses would be appropriate. Given the myriad of objective and subjective measurements commonly recorded in a modern specialist veterinary clinic, it is certain that there are other appropriate explanatory variables that should be included in future models. In addition, proteomic and metabolomics methodology is likely to identify other acute phase proteins in the horse in the years ahead, some of which could be included in a testing regimen to develop an acute phase signature for predicting the outcome for horses with surgical colic.

Across the field of medicine and veterinary medicine, models to predict survival are often generated from data such as those presented in this Chapter. These models may effectively describe the results for the data from which they have been generated, as is the case here. However, the true test comes when the

model continues to predict outcome effectively when used with a different data set from that from which it was produced (Berrisford *et al.*, 2005; Zhang *et al.*, 2013). This is an obvious next step for the models described here.

5.4.6 Conclusion

In conclusion, the findings presented here suggest that the acute phase proteins SAA and haptoglobin could contribute to decision making in horses presented for the evaluation of surgical colic. These markers are likely to be most effective when used in combination with more traditional markers of severity such as PCV, TP and heart rate as well as a thorough historical and clinical assessment of the patient. The findings demonstrate that clinicians can and should combine statistically significant and biologically logical findings during acute clinical decision-making. It may be that future work will identify particular colic syndromes with a particular acute phase profile or signature. The use of these acute phase markers to identify horses with a very poor or hopeless prognosis at the point of admission would be of considerable benefit to equine welfare.

Chapter 6

The effect of gastric ulceration on the acute phase response-

Chronicity and the acute phase response

6.1 Introduction

Thoroughbred horses in race training are subject to a variety of stressors. These range from the training regimen itself, to frequent transport, mixing with unfamiliar individuals, different surroundings, and exposure to infectious agents (Cappelli *et al.*, 2013). The presence of these stressors has a variety of effects, including sub-optimal performance (Morris and Seeherman, 1991), the development of clinical and subclinical disease, modelling of soft tissue and bone, that can lead to increased risk of injury, and ultimately, for some horses, retirement from racing (Verheyen and Wood, 2004; Smith and Goodship, 2008). Wastage from the thoroughbred industry, as a result of sub-optimal performance, or the presence of disease or injury, overt or subclinical, is of significant concern to the industry itself and the public at large (Jeffcott *et al.*, 1982; Wilsher *et al.*, 2006; Parkin and Rossdale, 2006).

Evidence for a link between race training and gastric ulceration has emerged in recent years with estimates of prevalence of gastric ulceration ranging from 66% (Hammond *et al.*, 1986) to over 90% (Murray *et al.*, 1989) in racing Thoroughbreds. Conversely, prevalence of ulceration is reported to be negligible in horses turned out to pasture and considerably lower in horses used for pleasure riding (37%), (Murray *et al.*, 1989). The effects of moderate to severe grades of gastric ulceration appear to be variable. However, there is compelling evidence that treatment frequently leads to improved performance and amelioration of clinical signs such as poor appetite, loss of condition, and colic (Murray, 1988; Murray *et al.*, 1989; Murray, 1992). At present, definitive diagnosis of gastric ulceration can only be achieved through examination of the stomach with an endoscope, with a minimum insertion tube length of 3 metres. In order to effectively evaluate the majority of the glandular and squamous portions of the equine stomach, horses need to be starved for 24 hours. Prior to the procedure, the requirement to withhold feed in horses under training, coupled with the necessity to use expensive equipment, has led to the practice of presumptive treatment as a means of diagnosis. Effective treatment involves the use of the proton pump inhibitor omeprazole (Murray *et al.*, 1997), and although effective, there are significant financial implications.

The ability to identify horses that have a high probability of being affected by gastric ulceration without the need for endoscopic examination would be of considerable benefit.

6.1.1 Pathophysiology of gastric ulceration

Equine Gastric Ulcer Syndrome (EDUS) is thought to be due to an imbalance between inciting and protective factors in the equine stomach (Murray and Grodinsky, 1989). A myriad of inciting factors have been identified in horses and other species. These include; physiological stress, excessive acid production and exposure, diet and dietary management, volatile fatty acid, lactic acid and bile acid production, exercise, use of steroidal and non steroidal anti-inflammatory drugs, and perhaps infection. To date a firm link between gastric ulceration and *Helicobacter spp.* or other bacterial pathogens is yet to be firmly established. Nevertheless, these organisms may play a role in delayed gastric healing (Nord, 1988; Green *et al.*, 1991; Sanchez, 2004; Nadeau and Andrews, 2009). The pattern is complicated as inciting, as well as protective, factors may be different in the glandular versus squamous epithelial regions of the equine stomach (Argenzio, 1999).

Protective factors include mucosal prostaglandin E2 and epidermal growth factor production, maintenance of gastric blood flow, the gastric mucous/bicarbonate layer production and gastrointestinal motility (Sanchez 2004). It is known that horses secrete hydrochloric acid continuously and therefore, any mechanism that increases exposure of non-protected gastric wall to this acid is likely to lead to an increased risk for ulcerative damage.

Ulceration of the squamous portion of the equine stomach has been compared to gastro-oesophageal reflux disease (GERDS) in humans (Argenzio, 1999). While a sphincter protects the distal portion of the human oesophagus, there is no such barrier between the squamous portion of the equine stomach and gastric acid. In part, this may account for the anatomical location of equine ulcers along the margo plicatus or boundary between glandular and squamous epithelial regions (Sanchez, 2004). Ulceration of the squamous portion of the equine stomach is more common than the glandular portion. Hydrochloric acid damages the outer cell layer and consequently diffuses into the stratum spinosum leading to the inhibition of sodium transport resulting in cellular swelling and ulceration (Nadeau *et al.*, 2003).

6.1.2 Mechanisms of gastric healing

The physiology of healing following gastric ulceration in horses, particularly within the gastric squamous epithelial area, is complex and poorly understood. However, healing is a dynamic process and appears to begin almost immediately after the onset of ulceration (Murray *et al.*, 2001). Epithelial proliferation at the margin of ulcers is accompanied by vascular proliferation in the lamina propria and takes place in response to an increasing concentration of epidermal growth factor (EGF) (Jeffrey *et al.*, 2001). A considerable inflammatory response accompanies healing in equine gastric squamous epithelium. This includes influx of leucocytes, angiogenesis, hyperplasia, wound contracture and re-epithelialisation (Tarnawski *et al.*, 1990; Szabo *et al.*, 1994) similar to healing in equine skin wounds. Upregulation of COX-2 has been noted in horses with experimentally induced ulcers in the gastric squamous epithelium (Morrissey *et al.*, 2010); however, limited information is available on the types of inflammatory mediators present during ulcer healing.

6.1.3 Summary and aims

In summary, it is clear that a profound inflammatory response accompanies the development and subsequent healing of equine gastric ulcers. As a consequence, it seems reasonable to postulate that components of that response could be used as biological markers in horses affected by EGUS.

In light of this the aims of this chapter were as follows:

1. To test the hypothesis that the concentration of serum amyloid A (SAA), haptoglobin and fibrinogen increase in response to the presence of ulceration of the equine stomach such that these markers could be used to identify individuals that require further screening.
2. To determine, with measurement of the APPs selected for examination in the present study, whether there were any associated changes in routine biochemistry or haematology associated with the subclinical disease presented by gastric ulceration.

6.2 Materials and methods

6.2.1 Study animals

This was a prospective clinical study in which 100 Thoroughbred racehorses in training were recruited. 100 horses were chosen for initial examination as this was the number that could logistically be subjected to serial gastroscopic examination in the period of time available for the project, and based on the number of trainers that responded to adverts in the lay press and following a direct approach. The aim was to make measurements on a representative population of Thoroughbred horses. The horses were selected from nine separate training establishments in County Kildare, Ireland. Six of these trained exclusively national hunt horses, two trained exclusively flat horses and one yard contained a mixture of flat and national hunt horses. Animals were randomly selected, within yards, using a random number generator system based on stable door number. The number of horses from each yard was limited to the number of animals that could be comfortably subjected to gastroscopy in one day. Details of signalment are given in Appendix 2. Of the 100 Thoroughbred horses identified, there were 77 males (70 Geldings, 7 colts) and 23 females. The ages of these horses ranged from 2 to 10 years. Each horse was subjected to clinical examination, jugular venopuncture, and gastroscopy on three separate occasions. Gastroscopy was performed three times in an attempt to determine whether there was variation, over time, in the appearance of gastric pathology, if present, and to assess any effect this might have on the concentration of the three acute phase proteins. The second examination took place four weeks after the first and the third and final examination took place six weeks after the second. These data were collected between May 2003 and September 2003.

6.2.2 Ethics

Ethical approval for this study was granted by the Ethics and Welfare Committee, School of Veterinary Medicine, University College Dublin. A member of the Health Research Board (undertakes a similar role as Home Office Inspector in UK) sat on the committee and oversaw ethical approval for the study. Racehorse trainers were contacted through the National Trainers Federation of Ireland, and asked to give written consent in the form

of a consent form that was clearly explained and signed prior to collection of these clinical data. Information regarding clinical findings was shared with the trainers following data collection.

6.2.3 Sampling procedures

Signalment, historical data, clinical examination, results of serial gastroscopy and performance data were recorded in an Excel spread sheet (Microsoft Excel for Mac). The level of work for each horse was recorded at each sampling point as; rest, light work, moderate work, or full work and coded as 1, 2, 3, or 4, respectively. In addition, the official handicap for the horse was recorded as increasing, decreasing or unchanged and coded 1, 2 or 3, respectively.

Full details of the sampling methods, haematology, biochemistry and acute phase protein assays are given in Chapter 2.

6.2.4 Gastroscopy

The trainers were instructed to starve each horse for at least 24 hours prior to examination, muzzles were provided for this purpose. In all but four horses, which required sedation, gastroscopy was performed with the aid of a nose twitch. The insertion tube of a three metre video endoscope was inserted via the ventral meatus of the right nostril and advanced into the pharynx. At this point, the head was flexed and the horse encouraged to swallow in order to facilitate passage of the insertion tube of the endoscope via the oesophagus to the stomach. The stomach was insufflated with room air to facilitate examination of as much of the interior surface as possible. Saline was flushed into the stomach, via a trans-endoscopic catheter, in a proportion of horses to remove any ingesta that were adherent to the gastric wall. The entire stomach was examined and the scope was then retroflexed to allow examination of the cardia and fundus. Where possible the pylorus was also imaged. During removal of the endoscope, air was insufflated to allow examination of the oesophagus. Each examination was recorded on a VHS video recorder and tapes were stored for future analysis. The stomachs, or a video recording of the examination, were examined by two individuals experienced in the examination of the equine stomach, and the level of ulceration, if any, was scored using the system described by Murray *et al.*,

(1996)(Table 6.1). Full details of the scoring technique including pictures of each grade are given in Chapter 2. For the purpose of this project horses with a score of 4 or above were considered to be positive for gastric ulceration. This decision was based on the fact that grade 4 on the scale described by Murray *et al.*, (1996) is the first grade in which the integrity of the squamous epithelium or glandular mucosa is compromised and there is true ulceration. At the time the project was carried out, gastric ulceration had only recently been identified in horses ante mortem. Indeed the gastroscope used in this study was the only one in Ireland at the time. Furthermore, at the time it was accepted practice to conflate the scores for the squamous and glandular mucosa.

6.2.5 Data processing

All data were entered into a Microsoft Excel spread sheet (Microsoft Excel for mac). Data were checked for errors, consistency and validity using Excel's standard functions. The data were separated into groups based on the outcome variable of interest, gastric ulceration score of 4 or above, and once a final data base was produced it was imported into Stata, version 12.1, for analysis. Minitab, version 16, was used to produce graphical summaries of the data.

6.2.5.1 Statistical methods

Full details of the statistical tests performed are given in Chapter 2.

6.2.5.2 Mann Whitney U test

A Mann Whitney U test was used to compare the concentration of SAA between the horses with and without clinically significant gastric ulceration (Score of 4 or more) for each time point because these data were not normally distributed.

6.2.5.3 Wilcoxin signed rank test

A Wilcoxin signed rank test was used to determine whether any of the differences between the median results for ulcer score and SAA concentration were statistically significant between time points (Figures 6.1 and 6.2)

6.2.5.4 Univariable analysis logistic regression

Examination of Explanatory Variables

The relationships between the following explanatory variables and the outcome variable, gastric ulcer score of 4 or above, were examined: SAA concentration, haptoglobin concentration, fibrinogen concentration, age, sex, type of race (Flat and National Hunt), yard, and work level (rest, light work, moderate work, or full work). Analysis was undertaken based on horse-sampling episodes, such that each horse was entered more than once. Horse was then analysed as a random effect.

6.2.5.5 Multivariable analysis and model building

Potential explanatory variables were ordered based on a combination of factors: those with the lowest p-value and highest odds ratio, biological importance and log likelihood value. A manual forward stepwise process was used to build the optimal multivariable model(s). Variables were retained within the models if their associated p-value was below 0.05.

Any confounding effect of non-significant variables was examined by forcing each into the final multivariable model(s) one at a time. Significant confounding was regarded as an alteration in the odds ratio of variables retained within the multivariable model(s) of more than 20% (Dohoo *et al.*, 2010).

For the outcome variable, gastric ulceration score of 4 or more, single-level, two-level (examination within horse or yard) and three-level models (examination within horse and horse within yard) were created to identify the best fit for the models and to assess whether unexplained variance was still present at the level of horse and or yard. Multi-level analyses also allowed for the effect of any elements within yard or horse to be accounted for when coefficients for fixed effects were estimated.

It was recognised that yard and race type overlap to a large extent, however these were included as yard was considered a proxy for management and race type as a proxy for exercise experience. It was acknowledged that these cannot be truly untangled, however, using yard as a random effect sought to compensate for variation at an inter-yard level. Race type was included as a

fixed effect to explore systematic differences in stress that was hypothesised to exist between the disciplines.

6.2.5.6 Goodness of fit testing

Goodness of fit testing was performed on the final single-level model using a Homer-Lemeshow goodness of fit test in STATA version 12.1.

6.2.5.7 Receiver operating characteristic curves (ROC)

ROC curves were produced for each of the three time points for the outcome variable gastric ulcer score 4 plus and the explanatory variable SAA concentration. Further information is contained in Chapter 2.

6.2.5.8 Sample size calculation

A *post hoc* power calculation with a sample size of 100 divided into 51 cases and 49 controls identified that the study would have sufficient power (>80%) to identify odds ratios of 3.5 or more with 95% confidence, assuming prevalence of exposure in the control population between 18 and 55%.

Table 6.1. The Scoring System used for Gastric Ulceration in Horses
Scoring system described by Murray *et al.*, (1996).

Score	Squamous mucosa	Glandular mucosa
0	Normal	Normal
1	Mild hyperkeratosis, hyperaemia	Focal areas of hyperaemia
2	Moderate hyperkeratosis, hyperaemia, 1-2 small erosions	Multifocal hyperaemia, up to 3 small lesions
3	Multifocal small erosions, with hyperaemia +/- hyperkeratosis	Multifocal hyperaemia, up to three small lesions
4	1-4 small ulcers, minimal thickening at margin, +/- small erosions	> 3 small lesions, multifocal hyperaemia
5	Deeper appearing ulceration, mild to moderate thickening of margins, +/- bleeding	1-2 moderate sized lesions, hyperaemia
6	Multifocal ulceration, mild to moderate thickening of margins, +/- bleeding	1-2 large lesions, hyperaemia
7	More extensive, deep-appearing ulceration, +/- bleeding	1-2 large lesions, + smaller lesions, hyperaemia
8	Focal large, deep-appearing ulceration, +/- multifocal erosions/ulcers, +/- bleeding. More extensive and with more changes in surrounding tissue than for 7	1-2 large, deep-appearing lesions, +/- smaller lesions, hyperaemia
9	Extensive, deep ulceration with bleeding, covering larger area than for 8	3-4 large, deep-appearing lesions, +/- smaller lesions, hyperaemia
10	Most severe, most extensive, deepest-appearing ulceration. Active bleeding or evidence of recent bleeding. Majority of mucosa ulcerated	5 or more large, deep-appearing lesions, +/- small lesions, hyperaemia

6.3 Results

6.3.1 Overview of gastroscopic findings

All 100 horses were available and amenable for gastroscopy at time point 1. At time point 2, three of the horses were either not available or refractory to endoscopy, and at time point 3, 21 of the horses were either not available or refractory to endoscopy. In the horses that were available, and in which gastroscopy could be performed, the technique was carried out with a minimum of three people, including a horse handler, one individual operating the endoscopic controls and a final individual passing the scope via the ventral nasal meatus of the right nares.

The horses were separated into groups based on the presence or absence of gastric ulceration. A gastric ulcer score of 4 or above was used as the cut off point for a diagnosis of gastric ulceration. The results are shown in Table 6.2.

Of the 100 horses, gastroscopy was accomplished in 76 horses on all 3 occasions, and in 24 horses on only two occasions. This was either because the horse was refractory to endoscopy or had been declared to race and was subsequently not available. 15 horses had a gastric ulcer score of less than 4 on all three occasions, 29 horses had a gastric ulcer score of 4 or more on only one occasion, 35 horses had a gastric ulcer score of 4 or more on two occasions and 21 horses had a gastric ulcer score of 4 or more on all three occasions. No oesophageal abnormalities were identified in any of the horses at any of the time points.

No management changes were instituted in response to the diagnosis of gastric ulceration and no treatment was carried out. The reason for this was that, at the time the project was undertaken, the significance of gastric ulceration in performance horses was poorly understood and no effective treatment was available and licensed for use in the horse.

6.3.2 Gastric pathology

There was a high prevalence of gastric ulceration with 51-67% of horses affected at any one time. A full spectrum of gastric pathology affecting both the glandular and squamous epithelium of the stomachs was identified.

The lesions identified were predominantly within the squamous epithelium adjacent to the *margo plicatus*. Lesions ranged from mild hyperkeratosis, and hyperaemia of the mucosa through moderate ulceration to extensive, deep ulceration with active bleeding. In severe cases, lesions were noted extending dorsally towards the gastric fundus. In a number of individuals, there was evidence of a proliferative margin to the ulcer, indicating healing.

6.3.3 Supplementary clinical examination results

Each of the horses that were available for gastroscopy (numbers given above), were subjected to a full clinical examination prior to passage of the insertion tube of the endoscope. Clinical information, including heart and respiratory rate, rectal temperature, mucous membrane colour, capillary refill time (CRT), and results of palpation of superficial lymph nodes was recorded. As part of the standard history taking procedure, questions were asked regarding changes in amount and type of food consumption, appearance and regularity of faecal material, evidence of colic or a change of behaviour, resistance to tacking up and girthing or unwillingness/resistance to work. In addition, the trainers were asked to provide a recent history of any veterinary treatment or the presence of any clinical signs of disease. Eleven horses had transient rises in heart rate during the examination, three horses had been noted to be lame in the previous month but had not been treated. In the remaining horses, all clinical parameters were within normal limits.

6.3.4 Acute phase protein results

There was a wide range of results for the acute phase proteins, particularly SAA which varied from low normal to abnormal results well outside the reference range. A considerable number of outliers were identified for each time point; based on the data available, no other reason or reasons, other than EGUS, for this range of results was identified.

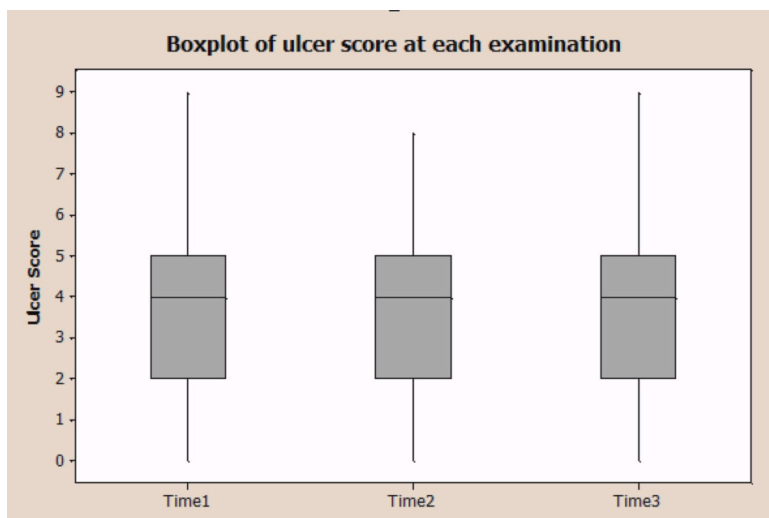
Median results and ranges for the acute phase proteins and ulcer scores for each time point are shown in Table 6.3.

Table 6.2. Gastric Ulceration Findings Over Time

	Time point 1	Time Point 2	Time Point 3
Total Horses	100	79	75
< 4	49	26	31
≥ 4	51	53	44

Table demonstrating that 51% (51 of 100 horses) of the horses at time point 1 had evidence of clinical gastric ulceration, 67% (53 of 79 horses) of the horses at time point 2 had evidence of clinical gastric ulceration and 58% (44 of 75 horses) of the horses at time point 3 had evidence of clinical gastric ulceration.

Figure 6.1 Box plot of ulcer score at each time point



A Wilcoxin signed rank test determined that there was no significant difference between the median results for ulcer score at each time point ($P=0.257$). The range of ulcer scores was broadly similar for each group.

6.3.5 Initial review of data

As a first step in examining the data, simple graphical representations of selected explanatory variables of potential interest versus the outcome variable, gastric ulcer score of 4 or more, were produced. This was done for a number of reasons: firstly, examining the data in this way facilitated identification of errors or missing values in the data; secondly it helped to guide further examination of the data by identifying patterns and generating potential hypotheses for links between possible explanatory variables of interest and the outcome variable, gastric ulcer score of 4 or more.

Of the 100 horses included in the study, a full set of data, that is ulcer score, and APP concentration results were available for all of the horses at the initial examination. At the second examination, 79 horses had a full data set, and by the final examination 75 horses had a full data set.

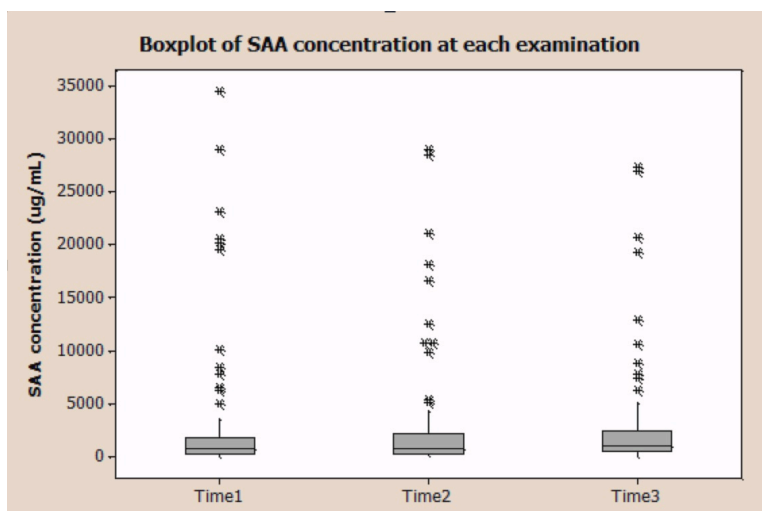
6.3.5.1 Method

Box plots were produced using Minitab V. 16 for SAA, haptoglobin and fibrinogen against gastric ulcer score (Figures 6.3-6.11). Based on these graphical representations, it was apparent that the SAA concentration was the explanatory variable likely to be of interest in relation to the outcome variable, gastric ulceration. Box plots were also produced for ulcer score and SAA concentration across the three time points (Figures 6.1 and 6.2). Following this, the results for SAA concentration at each time point were separated based on ulcer of 0-3 or 4 -9 (Table 6.4).

Table 6.3 Acute Phase Protein Results and Ulcer Scores in 100 Thoroughbred Horses on 3 Occasions over 6 weeks

	SAA (ug/mL)	Haptoglobin (mg/mL)	Fibrinogen (g/L)	Ulcer score
	Median	Median	Median	median
	Range	Range	Range	range
Time Point 1	767 284-34420	1.8 0.5-3.2	2.0 1.0-4.0	4 1-9
Time Point 2	732 140-29010	1.8 0.1-3.2	2.0 1.0-4.0	4 0-8
Time Point 3	446 18.61-27335	1.7 0.1-4.0	2.0 1.0-4.0	4 0-9

Figure 6.2 Box plot showing SAA concentration for each time point



A Wilcoxin signed rank test determined that there was no significant difference between the median results for SAA concentration at each time point ($P=0.117$). The range of SAA concentrations was broadly similar for each group, however there were a considerable number of outlying results at every time point.

Table 6.4 Median SAA results and ulcer scores for each time point for horses affected by clinical levels of gastric ulceration (gastric ulcer score >4) and for horses not affected by gastric ulceration

	SAA (ug/mL)	Ulcer score
	Median Range	Median Range
Time Point 1	1269	5
Ulcerated (n=51)	126-34420	4-9
Time Point 1	384	2
Non- ulcerated (n=49)	1.0-6475	0-3
Time Point 2	756	5
Ulcerated (n=53)	102-28450	4-9
Time Point 2	734	2
Non- ulcerated (n=26)	170-29010	0-3
Time Point 3	1927	5
Ulcerated (n=44)	204-27337	4-9
Time Point 3	614	2
Non- Ulcerated (n=31)	172-12915	0-3

A Mann Whitney u test was used to compare the concentration of SAA in the ulcerated versus non-ulcerated groups at each time point. There was a significant difference between these values for time point 1 and 3 ($P<0.000$ and $P=0.001$), but no significant difference for time point 2 ($P=0.50$).

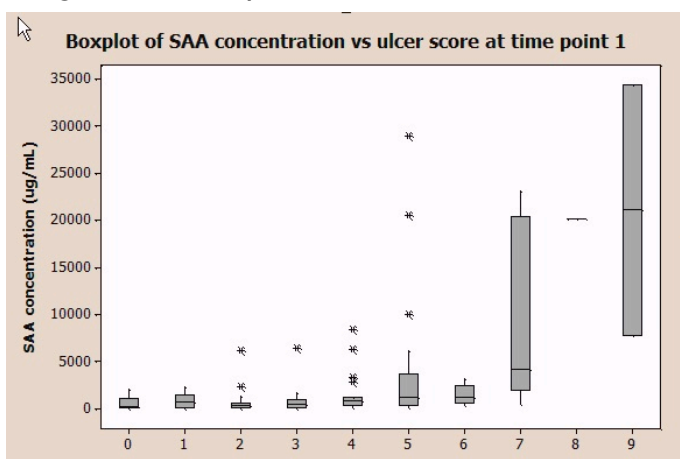
6.3.6 Statistical association between explanatory and outcome variables

There was a significant association between SAA concentration (p-value = 0.008) and an ulcer score of 4 or above across all the observation time points. Fibrinogen and haptoglobin concentration were not associated with ulcer score at any of the time points. There was a significant difference between SAA concentration for horses with and without clinical gastric ulceration for the horses at time point 1 and 3, but not time point 2 (Table 6.4).

6.3.7 Further hypotheses

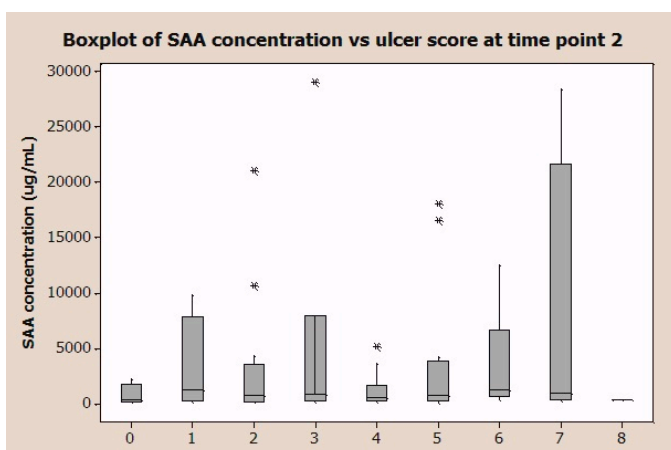
Due to the fact that, of the three acute phase proteins measured, only SAA concentration was significantly associated with the outcome variable of gastric ulcer score of 4 or more, the data were re-organised and examined based on whether or not there was an ulcer score and an SAA concentration for each of the time points. There was a complete set of data for 57 of the 100 horses. Based on the results presented thus far further questions arose. Graphical representations of ulcer score versus SAA concentration were reviewed to explore this data (Figures 6.1 and 6.2).

Figure 6.3 Box plot of SAA concentration vs Ulcer Score at time point 1



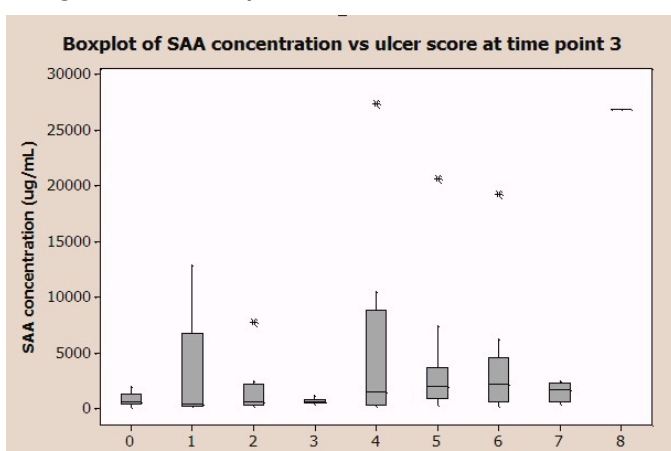
SAA concentration appeared to increase with ulcer score, however there were a considerable number of outliers. SAA concentration was significantly associated with an ulcer score of 4 or above for all time points (p-value = 0.008).

Figure 6.4 Box plot of SAA concentration vs Ulcer Score at time point 2



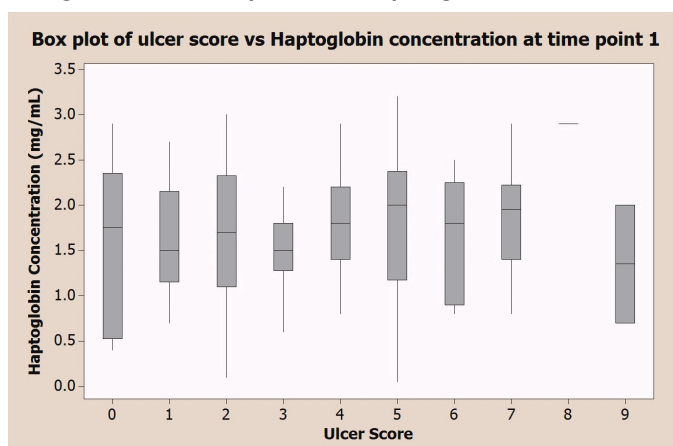
SAA concentration was significantly associated with an ulcer score of 4 or above for all time points (p-value = 0.008). Again there were a considerable number of outlying results.

Figure 6.5 Box plot of SAA concentration vs Ulcer Score at time point 3



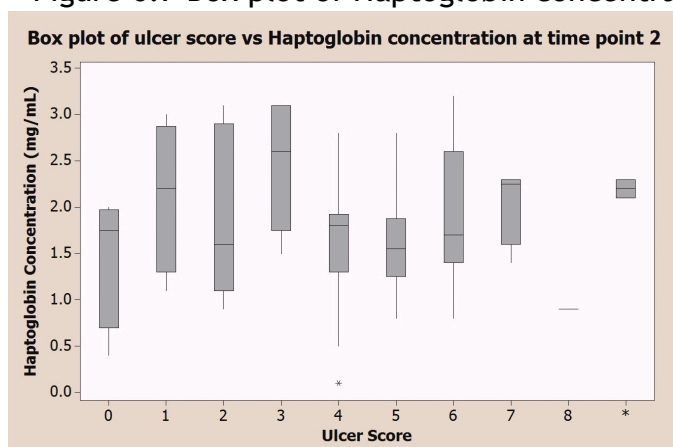
SAA concentration was significantly associated with an ulcer score of 4 or above for all time points (P value = 0.008). A number of outliers are visible; the reason for these is unknown.

Figure 6.6 Box plot of Haptoglobin concentration vs Ulcer Score at time point 1



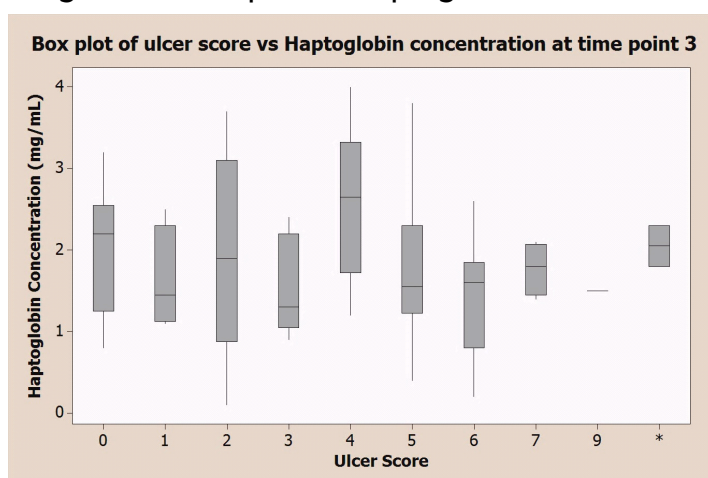
Haptoglobin concentration was not significantly associated with any ulcer score. The results appeared similar across the range of scores.

Figure 6.7 Box plot of Haptoglobin concentration vs Ulcer Score at time point 2



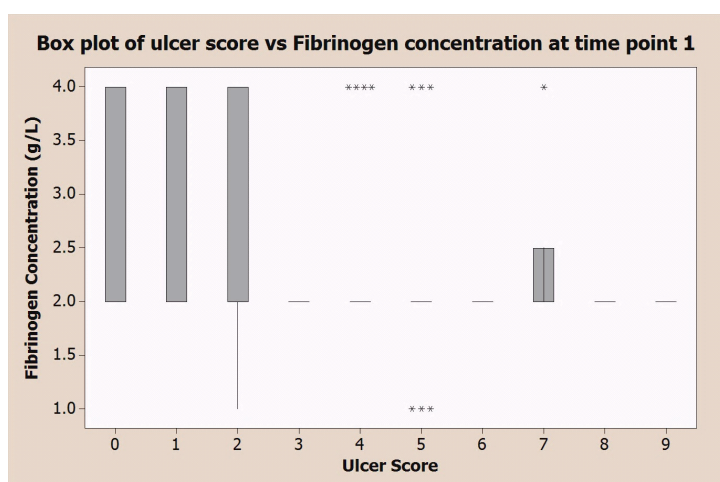
Haptoglobin concentration was not significantly associated with any ulcer score. The results appeared similar across the range of scores.

Figure 6.8 Box plot of Haptoglobin concentration vs Ulcer Score at time point 3



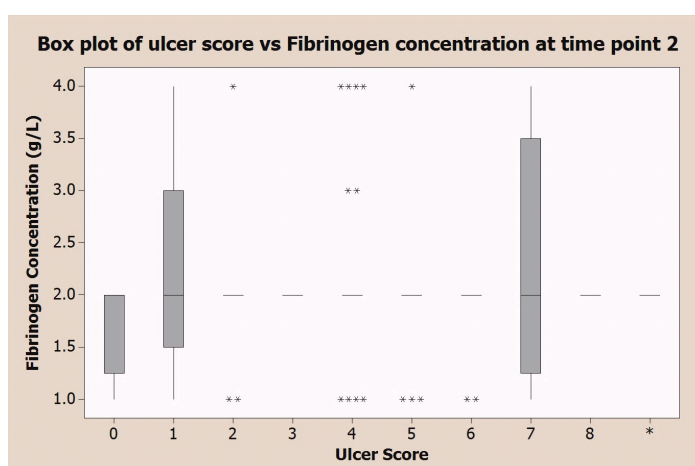
Haptoglobin concentration was not significantly associated with any ulcer score. The results appeared similar across the range of scores.

Figure 6.9 Box plot of Fibrinogen concentration vs Ulcer Score at time point 1



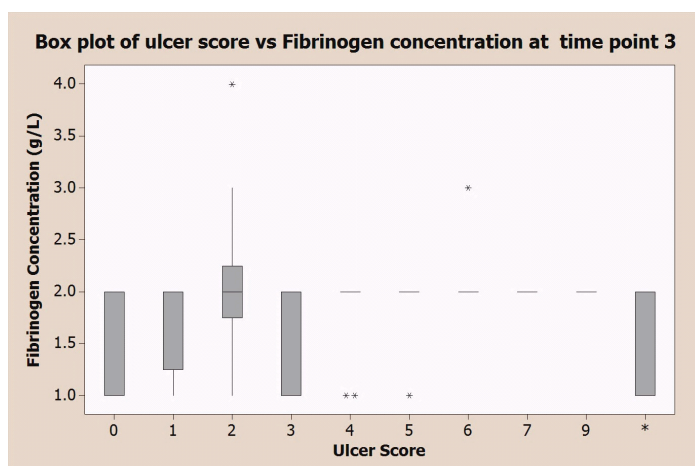
Fibrinogen concentration was not significantly associated with any ulcer score. The results were very variable across each ulcer score.

Figure 6.10 Box plot of Fibrinogen concentration vs Ulcer Score at time point 2



Fibrinogen concentration was not significantly associated with any ulcer score. The results were very variable across each ulcer score.

Figure 6.11 Box plot of Fibrinogen concentration vs Ulcer Score at time point 3



6.3.8 Model building

Univariable analysis

Of the nine explanatory variables screened at the univariable level, three were taken forward for consideration in each of the manual forward stepwise analyses. These variables were SAA concentration, race type and work level.

Multivariable analysis

In the final multivariable models, SAA concentration and race type were found to be significantly associated with the outcome variable, gastric ulcer score of 4 or more.

Because the relationship between the explanatory variable SAA concentration and the outcome variable, gastric ulcer score of 4 or more, was non-linear, SAA concentration was separated into quartiles (Table 6.5). For each multivariable model the 1st and 2nd quartiles, represented an SAA concentration of 0.5 µg/mL to 771µg/mL. The third quartile represented an SAA concentration of 772µg/mL to 2185µg/mL and the 4th quartile represented an SAA concentration of 2186µg/mL to 34420µg/mL.

A single level, multivariable logistic regression model was created, (Table 6.5) describing the association between the explanatory variables SAA and race type and the outcome variable, ulcer score 4 plus. SAA concentration in the 3rd (P=0.03, O.R. 2.04, C.I.1.08-3.85) and 4th (P=0.001, O.R. 4.32, C.I. 2.14-8.72) quartiles was significantly associated with a gastric ulcer score of 4 or above as was race type with flat race training significantly associated with an ulcer score of 4 or above (P=0.002, O.R. 4.87, C.I. 1.78-13.36).

Two-level, multivariable logistic regression models were created to describe the association between the explanatory variables SAA and race type and the outcome variable, ulcer score 4 plus. In these models, horse or yard were included as a random effect to account for the fact that multiple examinations were performed on each horse at the three different time points (horse level *Rho* = 0.37, p-value = 0.001), and to account for the fact that the 100 study horses were distributed between nine different yards (Yard level *Rho* = 0.08, p-value = 0.027), (Tables 6.6 and 6.7).

In the first two level model, in which any clustering within horse was accounted for, SAA concentration in the 3rd (P=0.049, O.R. 2.34, C.I.1.00-5.89) and 4th (P=0.001, O.R. 7.67, C.I. 2.67-22.04) quartiles were significantly associated with a gastric ulcer score of 4 or above as was race type with flat race training significantly associated with an ulcer score of 4 or above (P=0.007, O.R. 7.54, C.I. 1.72-32.93), (Table 6.7).

In the second two level model, in which any clustering within yard was accounted for, SAA concentration in the 3rd (P=0.046, O.R. 1.99, C.I.1.01-3.92) and 4th (P=0.001, O.R. 4.70, C.I. 2.23-9.92) quartiles were significantly associated with a gastric ulcer score of 4 or above as was race type with flat race training significantly associated with an ulcer score of 4 or above (P=0.022, O.R.4.76, C.I. 1.25-18.04), (Table 6.7).

A three level multivariable logistic regression was also performed taking into account clustering within horse within yard (Table 6.8a). This model describes the association between the explanatory variables SAA and race type and the outcome variable, ulcer score 4 plus. In this model, quartile 4 was compared with an aggregate of quartiles 1-3 indicating that SAA concentration significantly predicts an ulcer score of 4 or more (P =0.001, O.R. 5.69, C.I. 2.12-15.27). When the effect of race type (Flat vs National Hunt) was included the effect remained significant (P=0.024, O.R. 7.47, C.I. 1.30-43.01).

The final model was an alternative analysis using three-level, multivariable logistic regression (Table 6.8b) to describe the association between the explanatory variable SAA and the outcome variable, ulcer score of 4 or more. In this model SAA concentration per increase of 1000 units associated with an ulcer score of 4 or above (P <0.005, O.R. 1.12, C.I. 1.04-1.22). For every extra 1000 unit increase in SAA concentration, the likelihood of an ulcer score of 4 or more increased by 1.12 times (12%). When the effect of race type (Flat vs National Hunt) was included the effect remained significant (P=0.02, O.R. 7.01, C.I. 1.35-36.29).

Hosmer-Lemeshow goodness of fit testing was performed on the final single-level model. Hosmer-Lemeshow goodness of fit tests failed to identify any evidence of a lack of fit of the final model (p-value =0.99).

Table 6.5 Single-level, multivariable logistic regression model describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus (based on 106 non ulcerated and 148 ulcerated observations).

Variable	Odds ratio	P-value	95% confidence interval
SAA 1 st and 2 nd quartiles (0.5 to 771)	1(reference)		
SAA 3 rd quartile (772 to 2185)	2.04	0.03	1.08 - 3.85
SAA 4 th quartile (2186 to 34420)	4.32	<0.001	2.14 - 8.72
Flat race training			
No	1 (reference)		
Yes	4.87	0.002	1.78 - 13.36
SAA concentration in the 3rd and 4th quartiles and Race type, in this case flat race training, were significantly associated with an ulcer score of 4 or more.			

Table 6.6 Two-level, multivariable logistic regression model describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus (based on 106 non ulcerated and 148 ulcerated observations).

Variable	Odds ratio	P-value	95% confidence interval
SAA 1 st and 2 nd quartiles (0.5 to 771)	1(reference)		
SAA 3 rd quartile (772 to 2185)	2.34	0.049	1.00 - 5.48
SAA 4 th quartile (2186 to 34420)	7.67	<0.001	2.67 - 22.04
Flat race training			
No	1(reference)		
Yes	7.54	0.007	1.72 - 32.93

Horse is included as a random effect to account for the fact that multiple examinations were performed on each horse at three different points in time. (Horse level $Rho = 0.37$, p-value = 0.001).

Table 6.7 Two-level, multivariable logistic regression model with yard fitted as a random effect, describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus (based on 106 non ulcerated and 148 ulcerated observations).

Variable	Odds ratio	P-value	95% confidence interval
SAA 1 st and 2 nd quartiles (0.5 to 771)	1(reference)		
SAA 3 rd quartile (772 to 2185)	1.99	0.046	1.01 - 3.92
SAA 4 th quartile (2186 to 34420)	4.70	<0.001	2.23 - 9.92
Flat race horse			
No	1(reference)		
Yes	4.76	0.022	1.25 - 18.04
Yard is included as a random effect to account for the fact that the 100 study horses were distributed between nine different yards. (Yard level $Rho = 0.08$, p-value = 0.027).			

Table 6.8a Three-level, multivariable logistic regression model describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus (based on 106 non ulcerated and 148 ulcerated observations).

Variable	Odds ratio	P-value	95% confidence interval
SAA 1 st , 2 nd and 3 rd quartiles (0.5 to 2185)	1(reference)		
SAA 4 th quartile (2186 to 34420)	5.69	0.001	2.12 - 15.27
Flat race horse			
No	1(reference)		
Yes	7.47	0.024	1.30 - 43.01

Yard is included as a random effect to account for the fact that the 100 study horses were distributed between nine different yards and horse is included as a random effect to account for the fact that multiple examinations were performed on each horse at three different points in time. (Examination nested within horse which is nested within yard). (Horse level $Rho = 0.3843$, yard level $Rho = 0.0729$, overall p-value for random effects = 0.0016).

Table 6.8b Alternative analysis, three-level, multivariable logistic regression model describing the association between explanatory variables SAA and race type and the outcome variable, ulcer score four plus (based on 106 non ulcerated and 148 ulcerated observations).

Variable	Odds ratio	P-value	95% confidence interval
SAA (per 1000 units)	1.12	0.005	1.04 - 1.22
Flat race horse			
No	1(reference)		
Yes	7.01	0.02	1.35 - 36.29

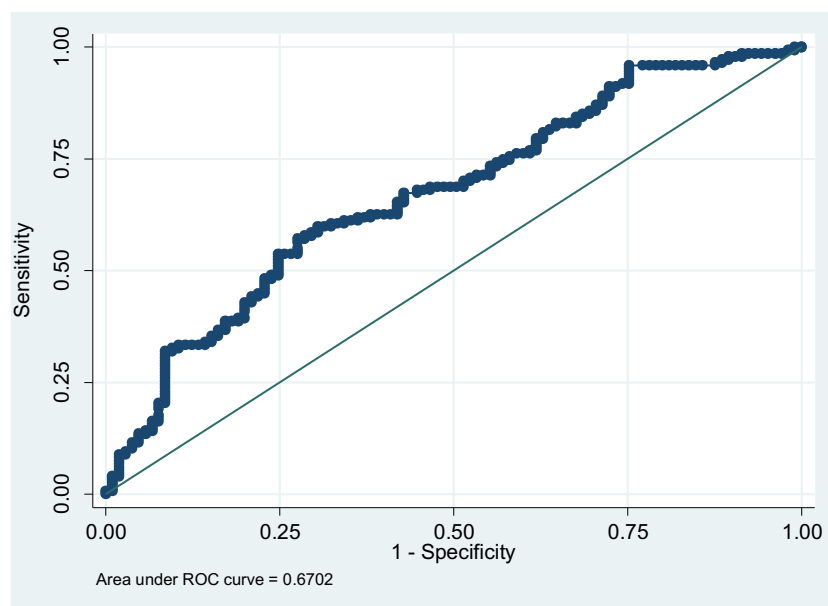
Yard is included as a random effect to account for the fact that the 100 study horses were distributed between nine different yards and horse is included as a random effect to account for the fact that multiple examinations were performed on each horse at three different points in time. (Examination nested within horse which is nested within yard). (Horse level $Rho = 0.3385$, yard level $Rho = 0.0729$, overall p-value for random effects = 0.0046).

6.3.9 Receiver operating characteristic curves (ROC)

The aim of the study was to determine whether SAA concentration could be used in a clinical setting to identify horses that should be subjected to gastroscopy. In order for this test to be clinically robust, it would require to have a high sensitivity for detecting horses with ulceration, in other words there would need to be few false negatives when using SAA as a test for ulceration. Receiver operating characteristic curves (ROC) were produced to determine what concentration of SAA could be considered as a likely cut point for a gastric ulcer score of 4 or above for all horses (Figure 6.12) and for flat and national hunt trained horses respectively (Figures 6.13 and 6.14). Roctab results suggested that a concentration range for SAA of 642µg/mL to 950µg/mL has the best combination of sensitivity and specificity and is likely to correctly classify over 60% of horses. A range is given because at these concentrations sensitivity and specificity vary by very small amounts.

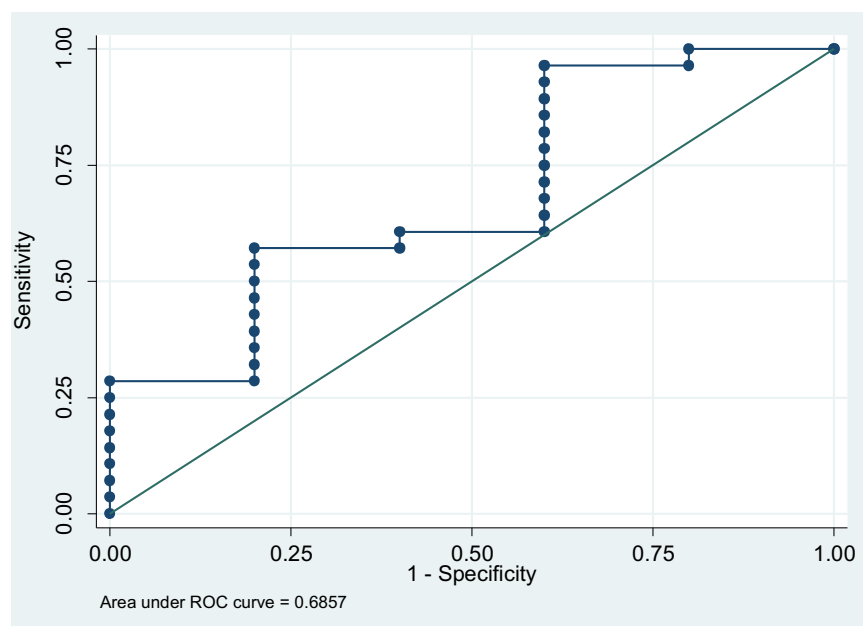
The areas under the ROC curves (AUC) represent a global summary of diagnostic accuracy. Guidelines suggest that a non-informative test has an AUC of 0.5, an accurate test has an AUC of 0.5 to 0.7, a very accurate test has an AUC of 0.7 - 0.9 and a highly accurate test has an AUC of 0.9 to 1. In the study described, the areas under the ROC curves were 0.67, 0.69 and 0.67 respectively. This indicates that 67%, 69% and 67% of animals would be classified correctly using this model.

Figure 6.12 ROC Curve demonstrating the relationship between Ulcer Score 4 and above and SAA concentration at Time Point 1



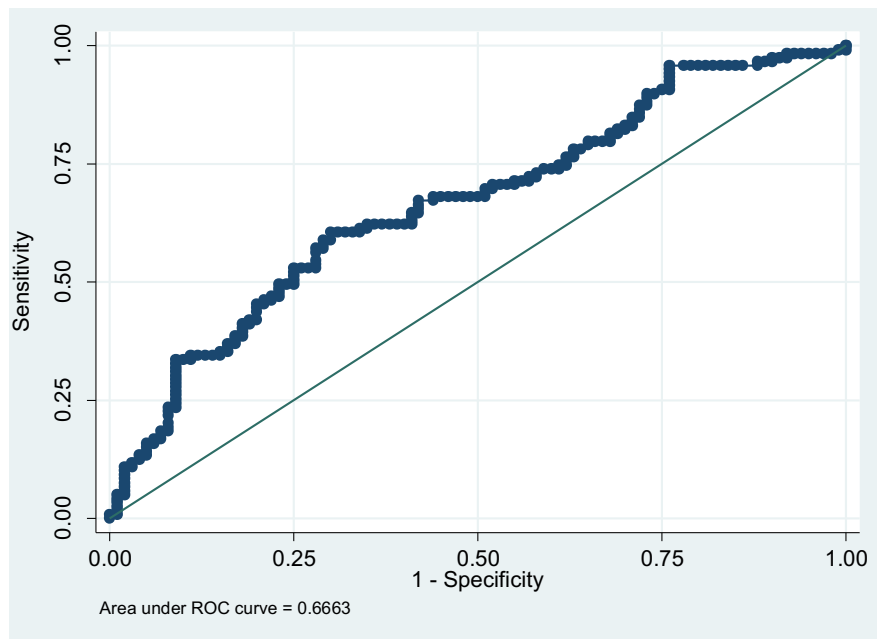
The ROC curve is a measure of performance of the test, with the area under the curve indicating the percentage of horses (67%) classified correctly (in this case animals with a gastric ulcer score of 4 or more).

Figure 6.13 ROC Curve demonstrating correlation between Ulcer Score 4 and above and SAA concentration for horses trained for Flat racing



The ROC curve is a measure of performance of the test, with the area under the curve indicating the percentage of horses (69%) classified correctly (in this case animals with gastric ulceration).

Figure 6.14 ROC Curve demonstrating correlation between ulcer score 4 and above and SAA concentration for horses trained for National Hunt racing



The ROC curve is a measure of performance of the test, with the area under the curve indicating the percentage of horses (67%) classified correctly (in this case animals with gastric ulceration).

6.3.10 Power calculations

Power calculations performed following data collection based on a prevalence of gastric ulceration in the range present over the 3 time periods (51-69%) suggested that between 66 and 83 horses were required to identify significant odds ratios of the magnitude observed in the final models.

6.4 Discussion

6.4.1 Summary of findings

In this Chapter a population of 100 Thoroughbred horses in training were subjected to serial gastroscopy, and acute phase protein analysis. There was a high prevalence of gastric ulceration with 51-67% of horses affected at any one time. A number of alternative analyses were performed using multivariate logistic regression methods and at all three examination time points there was a statistically significant, although variable, association between SAA concentration and a gastric ulcer score of 4 or above. In addition to this Flat Racehorses were shown to be at increased risk of having a gastric ulcer score of 4 or above. Work level was also shown to be a risk factor for the presence of gastric ulceration of grade 4 or above but was only present at the univariable level. A cut point was established using ROC curves whereby an SAA concentration range of 642µg/mL to 950µg/mL could indicate the presence of a gastric ulcer score of 4 or above with a sensitivity of 63% and a specificity of 62%. The results describe the extent to which SAA concentration is associated with gastric ulceration. However, the association was not strong and varied between time points. A considerable number of outlying results were identified at each time point; the reason or reasons for these values were not apparent and there was scant evidence of other disease processes amongst the horses aside from low-grade lameness. It had been further hypothesised that changes in the concentration of the three acute phase proteins might occur as the grade of ulceration changed, hence the reasoning for serial examination of the stomachs. However, based on the work presented here, it was not possible to determine an association between these factors.

6.4.2 Relevance to previous and on-going work

Previous work concurs with the findings presented here that there is a high prevalence of gastric ulceration in horses in race training (Vatistas *et al.*, 1999; Begg and O'Sullivan, 2003; Luthersson *et al.*, 2009). The effects of moderate and severe levels of gastric ulceration on performance are poorly understood; nevertheless, there is growing evidence to suggest that gastric ulceration can be performance limiting in horses (Nieto *et al.*, 2009; Tamzali *et al.*, 2011). This work represents details of some of the first horses to be examined gastroscopically in Ireland, and it is interesting that the prevalence results are similar to those recorded by other workers.

In humans, comparable syndromes to equine gastric ulceration, such as gastro-oesophageal reflux disease (GERDs), are commonly encountered in athletes and symptoms are frequently proportional to exercise intensity (Worobetz and Gerrard, 1985; Collins *et al.*, 2003). The development of GERDs may be related to posture during a number of sporting disciplines, and this effect may be replicated in horses during faster gaits such as canter and gallop during which increased intra-abdominal and intragastric pressure push gastric acid into the squamous portion of the stomach (Lorenzo-Figueras and Merritt, 2002). For this reason, a number of workers have also postulated that gastric ulceration could be used as a measure of overtraining in horses (De Graaf-Roelfsema *et al.*, 2010).

Gastroscopy remains the gold standard technique for detection of equine gastric ulceration. Several other tests for detection of gastric ulceration in the horse have been described, including measurement of urine and plasma sucrose concentration (O'Conner *et al.*, 2004; Hewetson *et al.*, 2006), the measurement of faecal occult blood (Pelligrini and Carter, 2007), and serum alpha-1-antitrypsin (Taharaguchi *et al.*, 2007). All have variable levels of complexity and ability to detect horses with gastric ulceration, and to date none of these tests have gained real acceptance and are not part of the routine screening process for performance horses. In humans, the use serum alpha-1-antitrypsin has been used as a biomarker of ulceration and SAA has been used to detect gastric neoplasia (Chan *et al.*, 2007; Hsu *et al.*, 2007).

6.4.3 Factors that may have influenced results

There are a number of factors that may have affected the results of the study described in this Chapter. Most notably, determination of training level and intensity is subjective, and quantification of the level of work in Thoroughbred racehorses remains a real challenge. To this end, work level was left out of the final models. In the case of these 100 horses across nine distinct training establishments, it is likely that the degree of training intensity across the 4 categories of work that were recorded varied from yard to yard. Furthermore while there was an association between a gastric ulcer score of 4 or above and SAA concentration, the measurement of SAA concentration is not a specific test and for that reason, it might be more appropriate as a screening tool to identify horses which then should be subsequently subjected to a period of starvation and gastroscopy. Such a test might be most appropriately and effectively used in animals that had been pre-screened for other causes of inflammation common in performance horses, such as respiratory disease. The considerable number of outlying results suggests that there may have been other factors contributing to the high concentration of SAA recorded in some of the horses. While a number of the horses had a history of low-grade lameness and several had evidence of transient upper respiratory tract infection during the course of the study, it was not possible to associate any of these findings with particularly high SAA concentrations. The gastric ulcer scoring system used during this work to describe ulceration of the squamous and glandular portions of the equine stomach was that defined by Murray *et al.*, (1996) and has been largely superseded by the scoring system from 0 to 4 as prepared by the Equine Gastric Ulcer Council (Andrews *et al.*, 1999). However, it was felt that it was more appropriate to use the system that was in widespread use at the time the work was carried out. Furthermore the conflation of squamous and glandular results into one score, as was practised at the time, and would not be standard practice today, may also have affected the results. However, the vast majority of horses had only squamous ulceration and consequently this conflation is likely to have had limited effect. The videos were watched by the author and another clinician and a combined score agreed, no effort was made to determine how repeatable the scores were, however generally there was a high level of agreement between the scorers. Because a score of 4 or more was required to classify the horse as ulcerated, it was considered reasonable to assume that considerable

tissue damage had to be present and the risk of including animals that were only mildly affected by gastric pathology was therefore low.

It is worth considering the relationship between SAA concentration and the inflammatory events surrounding the development and healing of equine gastric ulcers. Firstly, equine gastric ulcers are examples of chronic disease and as a result do not really fit the profile for the use of an acute phase protein. Consequently, if the use of acute phase proteins to detect gastric ulceration became widespread in clinical practice, such a test could only be used if the presence of other pro inflammatory inciting factors or events had been ruled out. The association between work level and race type at a univariable level, suggest other variables may have a role in to play in induction of SAA production in Thoroughbreds. Work investigating the inflammatory process during wound healing in horses suggests, somewhat counter intuitively, that an increasing intensity of inflammation is associated with effective healing (Wilmink *et al.*, 2003). It could therefore be postulated that SAA production might increase as the ulcer begins to heal, perhaps accounting for the variation in association between ulcer grade and SAA concentration at different time points. Future work could concentrate on different groups of animals, for example larger numbers of horses with increasing or decreasing ulcer scores. It would also be important to investigate the effect of different training regimens, the age at which animals enter training and the effect of other equine performance disciplines, some of which are believed to have different prevalence of gastric ulceration (Andrews *et al.*, 1999).

6.4.4 Performance of models

As with all epidemiological models, it would be of value to test the models against observations from a new group of horses to assess the ability of the model to predict animals with a gastric ulcer score of 4 or more. Furthermore, given the myriad of objective and subjective measurements commonly recorded in a modern racing establishment, it may be that there are other appropriate explanatory variables that should be included in future models. In addition, proteomic and metabolomic methodology is likely to identify other acute phase proteins in the horse in the years ahead, some of which could be included in a testing regimen to develop an acute phase signature for gastric ulceration.

6.4.5 Conclusion and future hypotheses

The results of this study suggest that SAA concentration is associated with equine gastric ulceration, although the association is variable and many other factors, which were not identified, may have a role to play. Based on evidence generated by these studies, it is currently impossible to recommend a testing regimen based around the concentration of currently recognised APPs that would substantially alter current diagnostic approaches to EGUS.

The question of why equine gastric ulceration, an apparently chronic process, is associated with increased SAA concentration remains.

Although complex, the ability to measure quantitatively the intensity of work and performance, and to determine its effects on both ulceration and the acute phase response is of considerable importance. As with the situation in human medicine, whereby a particular acute phase protein or proteins are used to diagnose, prognosticate and monitor response to treatment for a particular disease or syndrome, the recognition of gastric ulceration by a repeatable acute phase profile or "signature" in horses would be a major advance.

A number of other "stress" related conditions are commonly encountered in horses, including flexor tendon injuries and fatigue fractures, and express their effects on bone and soft tissue (Smith and Goodship, 2008; Whitton *et al.*, 2010). It may be that these produce a reliable, distinct and characteristic acute phase profile. Thus, the future of disease and welfare surveillance could be irrevocably changed, reducing the need for invasive and expensive diagnostic testing. As demonstrated in Chapter 3, acute phase proteins including SAA are excellent markers of normality in the Thoroughbred.

The health of equine athletes in training is of considerable public interest and the presence of subclinical disease, such as gastric ulceration, has implications for welfare and performance. Wastage from the thoroughbred racing industry as a consequence of poor performance is common and may be related to financial pressures precluding or limiting veterinary intervention. In this respect, the results described above are a significant first step in the use of applied acute phase protein analysis in identifying important stressors of horses and assessing the general health of the horse.

Chapter 7

Acute Phase Proteins: Detection of Sub-Clinical Infectious Disease

7.1 Introduction

A major challenge in the clinical setting is the identification of subclinical disease. Animals that are either incubating or in the early stages of infection may appear clinically normal but nevertheless act as reservoirs and serve to disseminate infectious agents throughout the environment. Delays in identifying such an individual may lead to the development of significant pathology, resulting in irreversible damage for the individual, and an increased risk of infection for other, in contact animals (Yager, 1987; Giguere *et al.*, 1997; Giguere, 2001). A number of infectious agents in horses are insidious in onset and become increasingly difficult to treat as fulminant disease develops. The consequences of such infection may be death or permanent damage with resulting effects on long-term health and performance (Wilsher *et al.*, 2006; Richard *et al.*, 2010; Fraipoint *et al.*, 2011). The ability to detect infected individuals prior to the onset of clinical signs has advantages both in terms of animal welfare and from a commercial perspective given the potential value of lifetime earnings of a high quality Thoroughbred.

A number of markers of disease have been described in horses, including the acute phase proteins SAA, haptoglobin and fibrinogen (Copas *et al.*, 2013). Several of these have been used as an aid to diagnosis in neonatal foals with clinical signs of disease (Chavatte *et al.*, 1991; Hulten and Demmers, 2002; Turk and Habus, 2010; Copas *et al.*, 2013) and in other species including cattle, pigs, small animals and wild animals (Zhu *et al.*, 2004; Akerstedt *et al.*, 2007; Gerardi *et al.*, 2009; Szczubial *et al.*, 2012; Korman *et al.*, 2012; Stanton *et al.*, 2013). However, most of these are used mainly after the onset of clinical signs. In human medicine, the use of markers of subclinical disease is well established for a variety of conditions including atherosclerosis, neoplasia and some infectious diseases. In the human clinical setting the use of CRP as a nonspecific marker for inflammation, infection and other forms of tissue damage is considered to be clinically useful (Pepys and Hirschfield, 2003). Critically, CRP and other human acute phase proteins are rarely considered to be diagnostic alone, instead changes in their concentrations are used to identify individuals who require further screening. Diagnosis can then be made following clinical examination and after further diagnostic testing (Pepys and Hirschfield, 2003). In addition, these markers are used to follow the progress of recovery following treatment, to

determine individual patient prognosis and to identify patients with neoplastic metastasis (Paracha *et al.*, 2000; De Backer *et al.*, 2002; Anuradha *et al.*, 2012).

7.1.1 Background to the study

A unique opportunity arose during these studies when a yard in which the author was researching (Chapter 3) experienced an outbreak of *Rhodococcus equi* (*R. equi*). The surveillance programme on the yard involved monthly blood sampling of all foals, hence samples were available for testing from animals with subclinical infections prior to the onset of disease. In foals, disease caused by *R. equi* is characteristically insidious in onset (Yager, 1987; Giguere *et al.*, 1997; Giguere, 2001) and the resulting respiratory infection leads to an extensive bronchopneumonia (Hondalus, 1997). Dissemination of infection from the lung to other sites has been described, leading to a variety of clinical syndromes including: septic arthritis, septic physitis, discospondylitis, serositis, cutaneous ulcerative lymphangitis, ulcerative colitis and/or mesenteric lymphadenitis (Zink *et al.*, 1986; Hondulas, 1997; Muscatello, 2012). Diagnosis of *R. equi* is not straightforward (Anzai *et al.*, 1997; Bertone, 1998; Ramirez *et al.*, 2004). Haematological examination may indicate the presence of a neutrophilia, increased fibrinogen concentration and thrombocytophilia, and it is these analytes that have been used to detect infected foals until now (Giguère, *et al.*, 2003). A number of serological tests have been described, although none have gained widespread acceptance for early detection and diagnosis of the disease (Vivrette *et al.*, 2000; Sellon *et al.*, 2001; Arriaga *et al.*, 2002; Martens *et al.*, 2002; Giguere, 2003; Cohen *et al.*, 2005). Prognosis is dependent on early recognition of individuals with clinical signs and treatment of the disease. A number of long-term effects on future health and performance have been described including reduced performance and failure to compete (Ainsworth *et al.*, 1998; Bertone, 1998).

The welfare and financial consequences for stud farms producing foals affected by *R. equi* are significant and, as such, a great deal of research has been devoted to investigating the epidemiology, diagnosis and treatment of this disease (Giguere *et al.*, 1997; Giguere 2001; Cohen *et al.*, 2005). The need for early detection, prior to the onset of fulminant disease, is vital.

The use of acute phase proteins as markers of subclinical infectious disease in the horse has produced some conflicting results, with Hulten *et al.*, (2002) suggesting that SAA was a sensitive marker for infectious respiratory disease in foals and Cohen *et al.*, (2005) concluding that results were highly variable when SAA was used in isolation to diagnose *R. equi* infections.

The aims of this study were as follows:

1. To determine whether SAA, haptoglobin and fibrinogen could be used in a group of foals, clinically normal at the time of sampling, which subsequently developed fulminant signs of *R. equi* infection.
2. To compare SAA, haptoglobin and fibrinogen as potential markers of subclinical *R. equi* infection, to the traditional haematological markers (haemoglobin concentration, WBC count and platelet count) of infectious respiratory disease.
3. To explore whether SAA could be used to identify foals that should subsequently undergo diagnostic testing for the presence of subclinical disease, in this case *R. equi*.

7.2 Materials and methods

7.2.1 Experimental design and study animals

This study included two groups of Thoroughbred foals, and had a prospective and retrospective element. Group 1, the normal group, consisted of 17 clinically normal Thoroughbred foals between the ages of 3 and 7 months of mixed sex, selected randomly from one well-managed stud farm in Co. Kildare, Ireland. Full details of this group can be found in Chapter 3. These foals were sampled prospectively.

Group 2 consisted of 12 Thoroughbred foals from the same commercial stud farm as, but kept geographically remote from, Group 1. They were aged between three and six months and were part of a larger group of approximately 200 foals. These foals had been subjected to a regular screening regimen, including daily examination for unthriftiness, malaise, respiratory signs, and inappetance. Blood sampling was conducted at monthly intervals for haematological and clinical

biochemical parameters to monitor the traditional markers of infection with *R. equi*. These samples were obtained retrospectively.

All of the foals were kept as “foals at foot”, that is to say with their natural mother or with a foster mother. Each foal was stabled throughout the night and kept at pasture during the day.

7.2.2 Ethics

Ethical approval for this study was granted by the Ethics and Welfare committee, School of Veterinary Medicine, University College Dublin. This particular equine premises was chosen due to the numbers of horses available and the fact that a routine sampling regimen was already in place, allowing surplus samples to be collected for the purpose of this study with minimal disruption to the animals and to the routine.

7.2.3 Sampling procedures

Samples from the infected foals were made available retrospectively for acute phase protein analysis following confirmation of *R. equi*. The foals in Group 2 were clinically normal at the time of blood sampling, but within 4 weeks of the date of blood sample acquisition developed clinical signs of infection with *R. equi*, confirmed by bronchoalveolar lavage (BAL) and culture of the organism from the BAL fluid.

7.2.3.1 Sample collection

Blood was obtained from each horse by jugular venepuncture using a vacutainer system. Each horse was restrained by the regular handler and subjected to a clinical examination prior to jugular venepuncture. Full details of the sampling methods, haematology, biochemistry and acute phase protein assays are given in chapter 2. The author personally collected all of the samples from the Group 1 foals in August 2003, Group 2 foals were sampled by the yard veterinary surgeon 3 months after the Group 1 samples were obtained.

7.2.3.2 Acute phase proteins

The following acute phase proteins were measured: SAA, haptoglobin and fibrinogen.

7.2.3.3 Haematological examination

Haematological examination included determination of haemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte count (RBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and a differential white blood cell count.

7.2.3.4 Clinical examination

Each foal was observed from a distance (usually over the stable door) for a period of not less than one minute; thereafter the foals were caught and restrained by the regular handler. A full clinical examination including measurement of heart and respiratory rate, rectal temperature, and palpation of superficial lymph nodes was performed.

7.2.4 Diagnostic techniques for the detection of *Rhodococcus equi*

Of approximately 200 foals subjected to monthly blood sampling, as part of a routine screening regimen, the clinical signs of *R. equi* were detected and the disease confirmed by positive culture of bronchoalveolar (BAL) lavage fluid and BAL cytology in 12 foals. Any foal with any of the clinical signs associated with *R. equi* infection was subjected to BAL. Full details of the techniques and methodology used to confirm *R. equi* are given in Chapter 2.

7.2.4.1 Bronchoalveolar lavage

In the 12 foals in Group 2, there were broadly similar findings in the BAL cytology. Multiple gram positive rod- to coccoid- shaped, pleomorphic (water melon seed like) organisms were noted within neutrophils and macrophages; there were multiple degenerative neutrophils and alveolar macrophages.

7.2.4.2 Microbial culture

The inclusion criteria dictated that all affected animals must have a positive culture of BAL fluid for *R. equi*. In all 12 foals, *R. equi* grew on sheep blood agar at 30°C. Gram stains of the cultured organisms yielded multiple gram-positive rods and cocci and cytology of the BAL fluid demonstrated macrophages containing multiple cocci-shaped bacteria (See Chapter 2).

7.2.5 Data processing and statistical methods

7.2.5.1 Data processing

All data were entered into a Microsoft Excel spread sheet (Microsoft Excel for mac). Data were checked for errors, consistency and validity using standard functions in MS Excel. The data, separated into the two groups were imported into Minitab version 16, and Microsoft Excel for mac.

7.2.5.2 Statistical methods

Statistical analysis was performed using Minitab, version 16.0. The results from the variables of interest from foals affected by *R. equi* were compared with the results from the normal foals, Group 1, presented in Chapter 3.

Analysis of each variable was performed using a Mann-Whitney U test and comparisons were made for SAA, haptoglobin, fibrinogen, haemoglobin, white blood cell count and platelet numbers.

7.2.5.2.1 Normality testing

Given the small number of animals it was considered inappropriate to perform testing for normality and the data were analysed using a non-parametric test.

7.3 Results

7.3.1 Clinical findings

The foals in Group 1 were clinically normal and displayed behaviour common to animals of their type and age. The foals in Group 2 (the *R. equi* group), which were all clinically normal, as assessed by full clinical examination by the yard veterinary surgeon, at the point of blood sampling, developed clinical signs between 3 and 4 weeks after blood sampling. Consequently all of these foals developed reduced activity, lethargy, diminished willingness to nurse the mare, and in 10 of the 12 an increased respiratory rate (42-48bpm) was noted (tachypnoea). Two of the 12 foals had enlargement of one or more synovial structure (1 tarsocrural joint and femeropatellar joint, 1 femeropatellar joint only), and two had diarrhoea. One foal had a concurrent cough with intermittent bilateral mucopurulent nasal discharge. This foal, the most severely affected,

was additionally subjected to thoracic radiography, which demonstrated the presence of an alveolar pattern and multiple coalescing areas of consolidation (Figure 7.1).

7.3.2 Acute phase protein profile

In the Group 1 (normal foals) (Table 7.1), median SAA concentration was 143.80µg/mL, median haptoglobin concentration was 3.25mg/mL and median fibrinogen concentration was 2.0g/L \pm (Figures 7.2, 7.3. 7.4). All of the results fell with the previously described reference ranges. An in depth discussion of these results can be found in Chapter 3.

In the Group 2 foals (infected with *R. equi*) (Table 7.1), median SAA concentration was 406.0 µg/mL, median haptoglobin concentration was 3.70mg/mL and median fibrinogen concentration was 4.0g/L (Figures 7.2, 7.3. and 7.4). The results for SAA concentration were all outwith the reference range for the horse. Two of the foals had particularly high values.

7.3.3 Haematology results

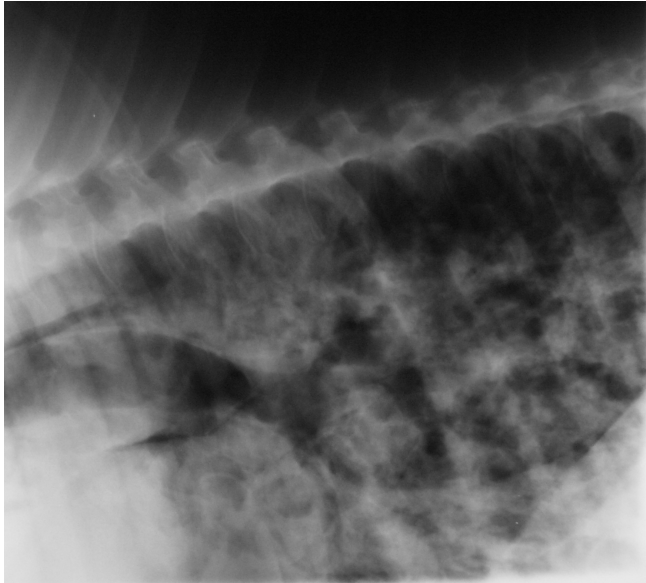
In the Group 1 foals (normal) (Table 7.1), median Hb concentration was 131g/L, mean white blood cell count was 10.10×10^9 /L, and median platelet count was $131.50 \pm$ (Figures 7.5, 7.6, 7.7).

In the Group 2 foals (infected with *R. equi*.) (Table 7.1); median Hb concentration was 121.0g/L \pm 54.59, mean white blood cell count was 11.75×10^9 /L, and mean platelet count was 563.0 (Figures 7.5, 7.6, 7.7).

7.3.4 Graphical review of data

As a first step in examining the data, box plots were produced showing each variable the concentration of the acute phase proteins, SAA, haptoglobin and fibrinogen, and the haematological parameters WBC count, Hb concentration and platelet count, by group (Figures 7.2-7.7).

Figure 7.1 Lateral radiograph of the thorax of one of the foals in Group 2



There is a diffuse alveolar pattern with evidence of a number of discrete and coalescing areas of consolidation typically associated with abscessation.

Table 7.1 Results for APPs and selected haematological parameters for the foals in groups 1 and

Foal ID	Age		Sex		SAA (ng/mL)		Hapto (mg/mL)		Fb (g/L)		Hb (g/L)		WBC ((x10 ⁹ /L))		Platelets	
	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1
	3 months	6 months	F	F	1183	176.7	4.6	2.9	2	2	141	128	20	11	702	128
	4 months	5 months	M	F	451	130	3	3.2	4	4	143	153	13.3	11.2	1271	153
	6 months	7 months	M	M	10570	139.3	3.7	3.9	3	2	141	122	14.3	8	608	122
	5 months	8 months	M	M	38260	94.5	2.2	2.4	2	2	15.1	128	16.6	9.7	836	128
	3 months	8 months	F	M	395	155.5	3.8	4.1	4	4	138	135	11.9	11.4	518	135
	3 months	5 months	F	F	417	189	2.8	3.4	4	2	123	136	11.5	8.5	259	136
	4 months	6 months	F	M	1030	103.5	3.9	2.4	2	2	111	112	10	7.7	442	112
	5 months	6 months	M	F	388	113.5	3.9	3.8	4	3	115	126	10.8	9	1802	126
	3 months	7 months	F	F	368	242	3	2.4	2	2	119	128	11.6	10.1	514	128
	3 months	7 months	M	F	375	196.1	3	2.7	4	2	139	143	17.5	15	926	143
	4 months	6 months	F	M	358	53.5	4	3.3	2	2	101	135	7.4	8.8	270	135
	5 months	5 months	F	M	375	146.5	3.7	4.6	2	2	101	121	7.3	11.8	204	121
		5 months		F		174.1		3		2		141		8.6		141
		5 months		M		115		3.6		3		137		10.1		137
		5 months		M		141		2.1		4		114		11.6		114
		5 months		F		148.5		4.2		2		152		14.3		152
Median	N/A	NA	N/A	NA	406	143.8	3.7	3.25	4	2	121	121	11.75	10.1	563	131.5

7.3.5 Statistical analysis and conclusions

In the Group 1 foals, SAA, haptoglobin and fibrinogen concentration were all within normal ranges (Chapter 3 and published ranges), and within the limits of the sample size there was no effect of sex, or age on SAA or haptoglobin concentration.

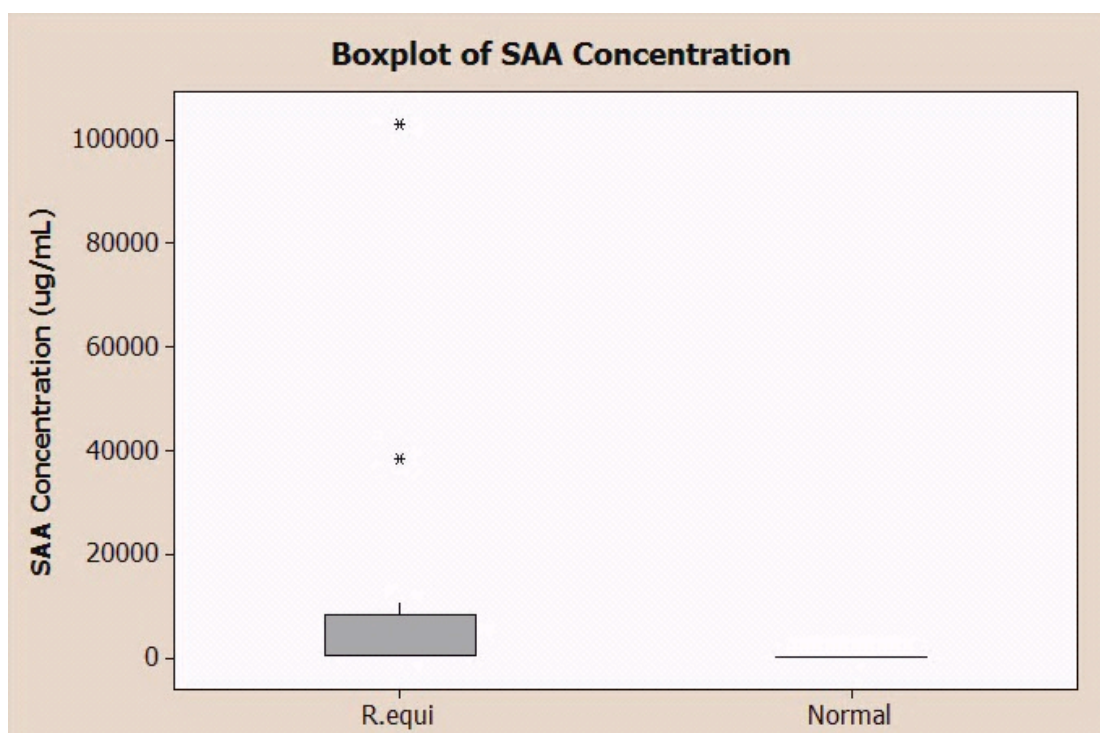
All Group 2 foals had a significantly higher concentration of SAA, and fibrinogen than the normal foals ($p=0.000$ and $p=0.0087$).

There were no significant differences between haptoglobin concentration between the group 1 and group 2 foals.

The selected haematological values for the normal group (Group 1) foals were all within the reference ranges specified by the laboratory. There was no association with age group or sex for platelet numbers, Hb concentration or WBC count.

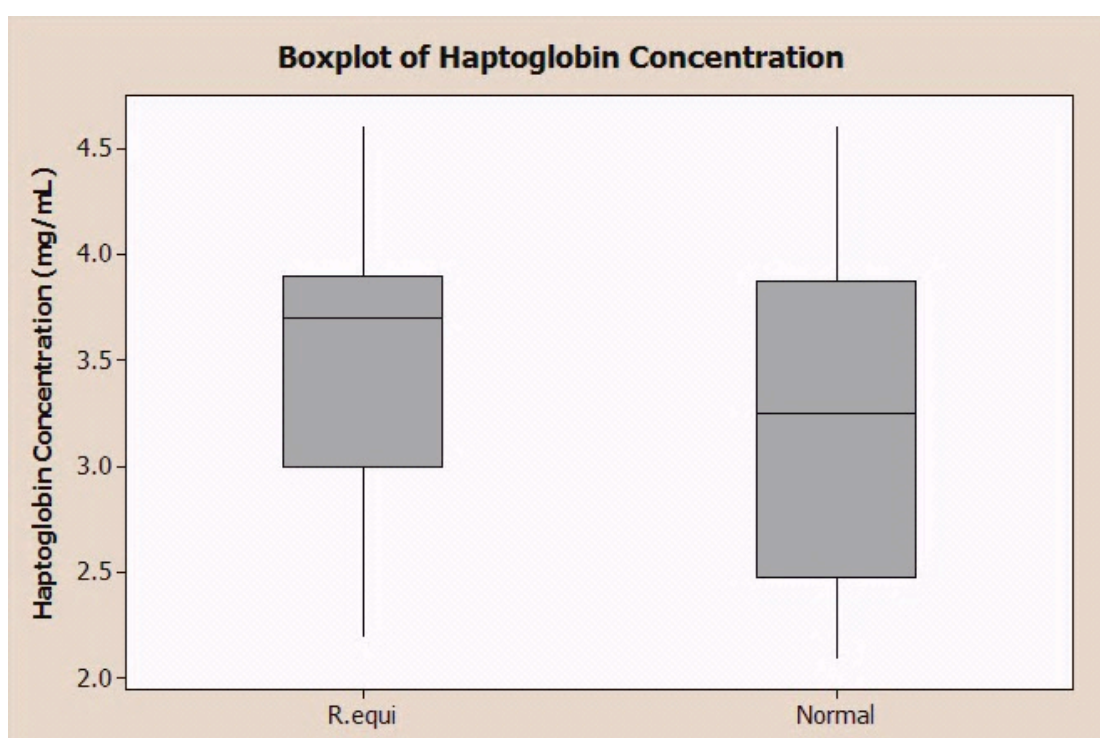
The foals in Group 2 had a significantly higher number of platelets than normal foals (Group 1) ($p=0.001$), There were no statistical differences between WBC or Hb concentration between the foals in groups 1 and 2.

Figure 7.2 Box plot of SAA concentration for the 2 groups of foals



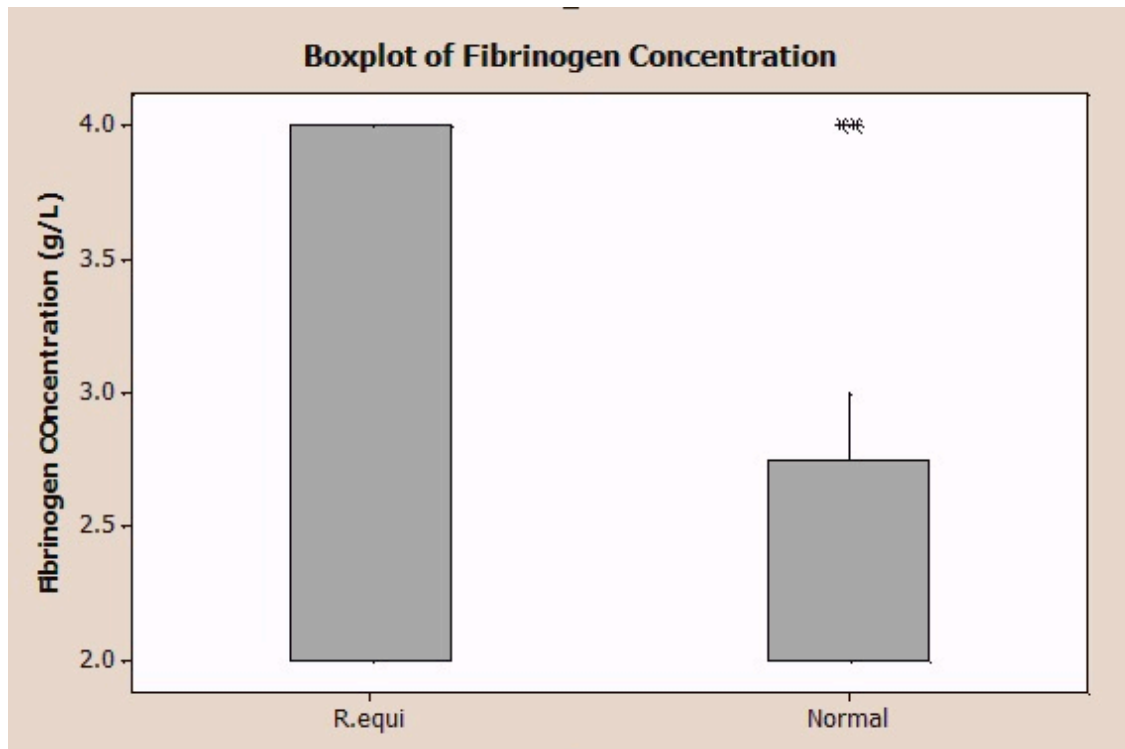
The Group 2 foals had a significantly higher concentration of SAA, than the normal foals ($p=0.0001$).

Figure 7.3 Box plot of Haptoglobin concentration for the 2 groups of foals



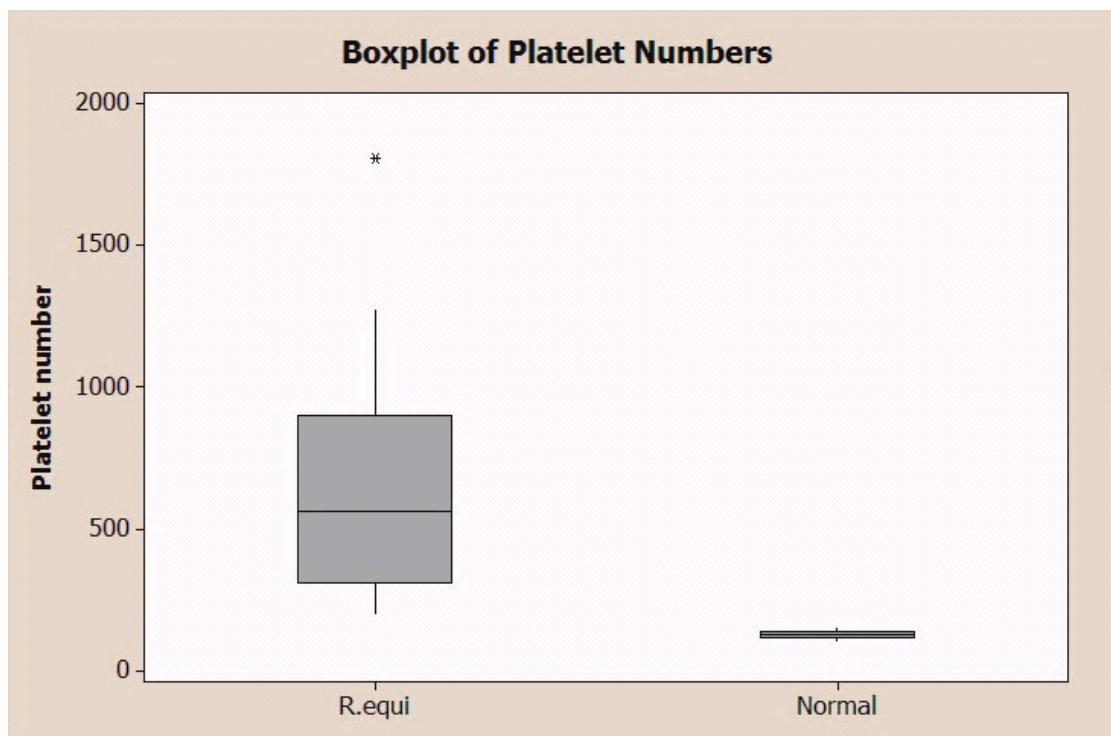
There were no significant differences in haptoglobin concentration between the two groups.

Figure 7.4 Box plot of Fibrinogen concentration for the 2 groups of foals



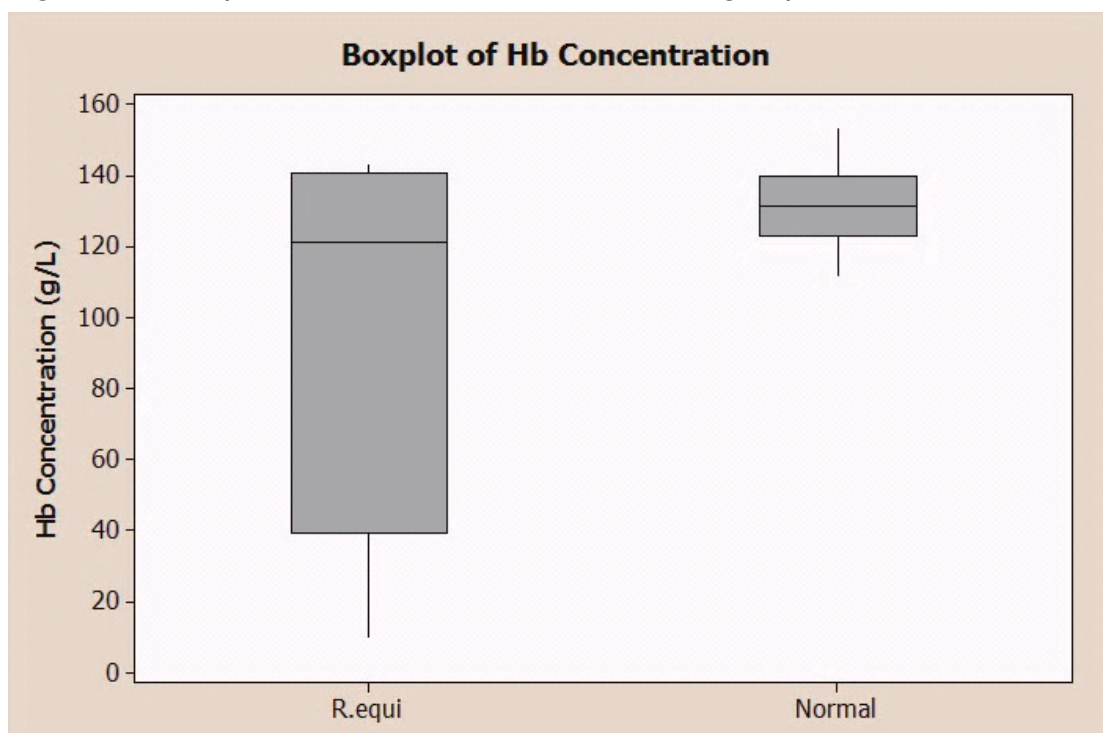
The Group 2 foals had a significantly higher concentration of Fibrinogen than the normal foals ($p=0.0087$).

Figure 7.5 Box plot of Platelet number for the 2 groups of foals



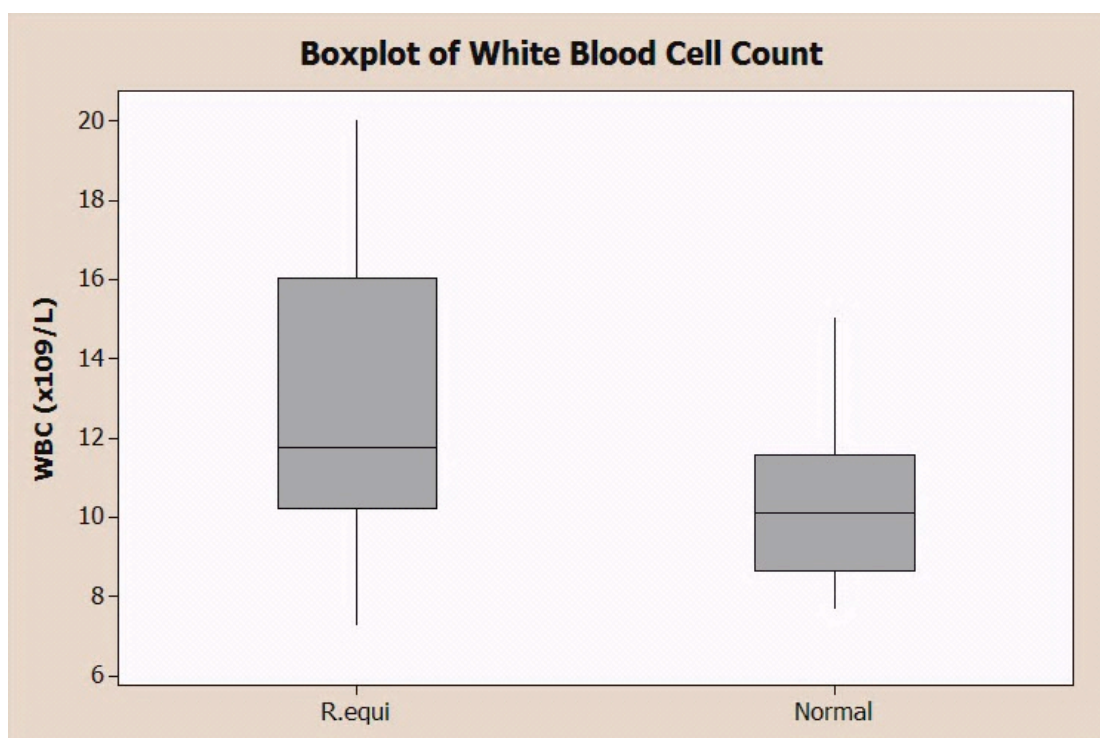
The foals in Group 2 had a significantly higher number of platelets than normal foals (Group 1), ($p=0.001$).

Figure 7.6 Box plot of Hb concentration for the 2 groups of foals



There were no significant differences in haemoglobin concentration between the two groups.

Figure 7.7 Box plot of WBC Count for the 2 groups of foals



There were no significant differences in white blood cell count between the two groups.

7.4 Discussion

The findings of this Chapter indicate that SAA concentration could be used to identify individual foals that ought to be screened for the presence of subclinical disease. Median SAA concentration in foals subclinically infected with *R. equi* was significantly greater than normal foals of the same breed and age reared under almost identical conditions. The same was true for fibrinogen concentration. As previously described, platelet count was also significantly associated with subclinical infection. Previous work on the use of acute phase proteins in relation to the diagnosis of *R. equi* showed that SAA concentration is increased in foals with fulminant signs of the disease (Chavatte *et al.*, 1991; Hulten *et al.*, 2002). In these studies, the foals also showed significant increases in fibrinogen concentration, WBC count, platelet numbers and Hb concentration (Giguere and Prescott 1997; Stoneham *et al.*, 2001; Hulten *et al.*, 2002 Giguère *et al.*, 2003). No previous studies have investigated the association between subclinical infection with *R. equi* and haptoglobin concentration, or compared changes in a number of acute phase proteins with the more traditional haematological markers of *R. equi* infection such as platelet count, WBC count or Hb concentration.

A number of serological assays have been described for the diagnosis of *R. equi* in foals (Martens *et al.*, 2002; Giguere *et al.*, 2003), and recently a PCR technique has been described (Bolton *et al.*, 2010). However, none of these has been used to detect subclinical disease.

Cohen *et al.*, (2005) investigated the use of SAA concentration for early diagnosis of *R. equi* in a large group of foals. In contrast to the current study, the concentration of SAA in foals between 7-14 and 21-28 days of age was not significantly different between infected and non-infected foals. Cohen *et al.*, (2005) concluded that SAA concentration could not be reliably used to detect subclinical *R. equi* infection. It is notable that the foals in the Cohen study were considerably younger than the foals described in this Chapter and sampling was carried out on three occasions, 2 weeks and 4 weeks after birth and at the onset of clinical signs of *R. equi*. The foals in the Cohen study were sampled on two separate farms where *R. equi* was endemic and as such represent a quite different population to the foals described here. In this study, blood samples

from similarly aged normal foals were obtained from the same farms as the infected animals. The median age for onset of clinical signs of *R. equi* in the Cohen study was 64 days (range 26-144). As a result, it may have been that some of the foals were not actually infected with *R. equi* at the time of their initial sampling. Furthermore, the difference in findings between the two studies may be related to the speed with which SAA concentration can return to normal after an inflammatory insult, as demonstrated in Chapter 4. In the study by Hulten *et al.* (2002), SAA concentration returned to normal between 3 and 8 days after hospitalisation in diseased foals. The study by Cohen *et al.*, (2005) also acknowledges that some of the foals, used as controls, assumed to be normal, may have in fact been subclinically infected with *R. equi*. There is some evidence that a proportion of foals that have been exposed to the *R. equi* organism, but are not affected with the disease, can yield positive cultures from tracheobronchial aspirates (Ardans *et al.*, 1986) and this may also partially account for the differences noted between the two studies. The foals in Group 2, presented in this Chapter, were between 3 and 6 months of age, the age group in which *R. equi* infection is most prevalent on Thoroughbred stud farms in Ireland (Muscatello *et al.*, 2012).

One might conclude that given the severe consequences of infection with *R. equi*, owners and stud farm managers might elect to treat foals considered to be at risk despite a lack of clinical signs. Treatment is classically based on the use of the synergistic bacteriostatic antimicrobials erythromycin, azithromycin or clarithromycin in combination with rifampin. While often highly effective, this therapy is not without risk to the foal in which adverse reactions are common. Complications of treatment include diarrhoea, idiosyncratic hyperthermia, tachypnoea, anorexia, bruxism, and salivation (Muscatello *et al.*, 2007). In addition acute and frequently fatal colitis has been reported as a complication of the accidental ingestion of foal faeces containing antimicrobials, by the mares of foals undergoing treatment. Finally, antimicrobial resistance of *R. equi* to erythromycin-rifampin combination has been reported (Giguere *et al.*, 2001). In conclusion, the need for treatment must be clear prior to instigation of therapy.

7.4.1 Developing the test using SAA and other acute phase proteins in clinical practice

The results of the study presented here suggest that SAA concentration would be a sensitive marker for the presence of disease if measured as part of a monthly sampling programme such as was in place on the farm in this study. In addition, the finding that fibrinogen concentration may also be increased suggests that these analytes could be combined in a panel for use in clinical practice. Many professional Thoroughbred breeding operations run a continuous monitoring programme with regular sampling. From a financial perspective, the cost of monitoring is considerably less than the long-term costs of the disease.

A number of specific questions arise with respect to the use of a group of biomarkers to detect subclinical cases of *R. equi* in foals, based on the findings of the study presented here.

1. Could analysis of SAA concentration in combination with measurement of fibrinogen and platelet count differentiate infected from non-infected foals across a range of management systems and at appropriate sensitivity and specificity to make the tests sufficiently robust for clinical practice?
2. Could the markers described be used to determine which foals should be subjected to therapy and are there specific concentration cut points at which therapy should be instigated?
3. Could the measurement of SAA concentration be further used to determine which foals are responding to treatment, and/or which foals may be subject to any of the long-term consequences of infection?

7.4.2 Limitations and further investigation

Although both groups of foals in this Chapter were drawn from large groups in a commercial Thoroughbred stud farm setting, the numbers of animals sampled in each group were relatively small. In clinical studies, welfare, commercial implications and time constraints are all factors limiting numbers. Furthermore, the ability to access animals of this quality is restricted to a small number of farms across the globe breeding large numbers of horses and subjecting those horses to a regular sampling regimen. For that reason, the results presented

must be regarded as a “snapshot” that could inform a larger study. In contrast to previous studies, the samples used in this Chapter were analysed either immediately or within 3 months of collection removing any potential effect of long-term storage on acute phase protein analysis. The foals in Group 1, normal animals, were not subjected to BAL, and therefore one could speculate that they could have been infected with *R. equi*. However, this is considered unlikely and the commercial and welfare implications of performing unnecessary and potentially damaging diagnostic procedures on apparently normal animals meant that this information is unlikely to ever be available to researchers. None of the normal foals subsequently developed *R. equi*.

The two groups of foals also differed temporally in terms of data collection with group 1 foals collected prospectively and group 2 collected and analysed retrospectively. This arose due to the opportunistic nature of the acquisition of the samples for Group 2. Given the value, and rarity, of samples from a group of animals which although clinically normal at the time of sampling, subsequently developed, and were confirmed with *R. equi*, it was considered worth working with this limitation in study design. The foals were managed in exactly the same way, on the same premises, but geographically separated, by the same group of people and this will have limited many of the potential confounding factors.

A wide range of clinical signs were reported for the foals in group 2 which developed *R. equi*, this is common when dealing with this disease and all of the foals had a confirmed diagnosis based on the positive identification of the organism from a BAL and a positive culture. No other disease was identified amongst these individuals.

With access to a larger group of foals, both normal and subclinically infected with *R. equi*, the goals would be to determine whether the results presented here were repeatable and further to determine what combination of markers and clinical parameters was most effective for identifying infected foals. Furthermore, to determine if there was a specific acute phase signature or foot print for *R. equi* and perhaps utilising ROC curves to determine cut points for the marker or markers.

At the time the work described in this Chapter was conducted, the PCR (Sellon *et al.*, 1997; Vivrette *et al.*, 2000; Selon *et al.*, 2001; Arriaga *et al.*, 2002) for

detection of *R. equi* was at an experimental stage and not readily available; if the study was repeated the use of this technique would be an obvious and useful addition.

To date there has been no investigation into the effect of animals carrying, but not infected with a pathogen, on the acute phase response in horses. In cattle, experimental work with *Salmonella* sp. infection and the viral disease Foot and Mouth, suggests that this may be an area worth investigating (Oikawa, *et al.*, 1997; Stenfeldt *et al.*, 2011).

It is true to say that there are many potential causes for an increase in SAA concentration, indeed a number of these are presented in this thesis. The strength of the measurement of the acute phase response is not in its ability to diagnose a specific disease, but rather in the ability to identify individuals that are abnormal and therefore require clinical scrutiny or more specific diagnostic testing just as is commonplace in human medicine (Pepys and Hirschfield, 2003).

7.4.3 Conclusions and way forward

Rapid diagnosis and the instigation of treatment are fundamental to the effective treatment of diseased foals. However, successful early diagnosis is perhaps one of the most challenging scenarios facing the equine clinician (Muscatello *et al.*, 2007). Failure to identify infected individuals early in the course of any disease, including infection with *R. equi*, has major implications for wellbeing, welfare, finance and future performance (Ainsworth *et al.*, 1998). The work presented in this Chapter suggests that monitoring SAA concentration, particularly when coupled with traditional haematological markers of infection, could play an important role in better identifying foals in need of specific diagnostic testing prior to the onset of debilitating clinical signs. This description of the use of SAA concentration in this study mirrors exactly the routine use of CRP in human medicine in order to identify individuals with potentially serious disease but with few or no clinical signs in the early stages of infection.

Chapter 8

General Discussion and Conclusions

8.1 Introduction and Summary of Findings

The assessment of health remains one of the great challenges in veterinary and human medicine. Indeed, the acceptance that health is more than just the absence of disease is a key starting point in the quest to improve the welfare of all veterinary species (Gunnarsson, 2006). Methods used to diagnose, prognosticate and monitor the treatment of disease were described in antiquity by Imhotep of Egypt and Hippocrates of Greece and in ancient China where the four diagnostic methods; inspection, auscultation-olfaction, interrogation, and palpation were employed (Ralston, 1977; Savel and Munro, 2014). These methods are based upon the identification of abnormalities noted during clinical examination and are still employed today. More recently, the findings of haematological and clinical biochemical examination and the use of advanced imaging techniques have been employed to the same end. Such techniques rely upon recognition of a pattern whereby a set of clinical signs is associated with a particular disease or syndrome. The success or failure of this method is somewhat dependent upon the experience of the diagnostician and is therefore potentially imperfect.

The development of acute phase markers that can be used to identify disease that is fulminant and overt, latent or subclinical has become commonplace in human medicine (van der Linden *et al.*, 2010; Tilemann *et al.*, 2011; Kiefer *et al.*, 2012) and a small number of veterinary species; however, to date, with the exception of fibrinogen, this approach has not gained widespread acceptance in horses. While a number of studies have described changes in the concentration of serum acute phase proteins subsequent to the development and diagnosis of disease (Jacobsen and Andersen, 2007; Hobo *et al.*, 2007; Christoffersen *et al.*, 2010; Lavoie-Lamoureux *et al.*, 2012; Copas *et al.*, 2013; Andersen *et al.*, 2014;), few have used specific markers to identify the disease or syndrome in the first instance (Cohen *et al.*, 2005, Pihl *et al.*, 2015)

The objective of the studies presented in this thesis was to evaluate whether changes in the concentrations of acute phase proteins in horses, specifically SAA and haptoglobin, could be used to detect clinical disease, subclinical infection and to determine which individuals might have a good prognosis for recovery from major inflammatory diseases such as equine colic. To date, studies of acute

phase proteins in horses have principally described the effect of a disease or inflammatory state on the concentration of the protein; the studies presented here attempted to bridge the gap between observation of the effect of a known disease on the protein to that of a diagnostic and prognostic tool. Ultimately, the aim must be that these markers could be used as a measure of the fitness of individuals for surgical intervention and as a general measure of health. The findings, albeit in not necessarily representative populations of horses, presented suggest that SAA and haptoglobin are easy to measure, have a tight normal range and return to normal concentrations rapidly following recovery. Serum Amyloid A concentration increases in response to surgical trauma in both clinically normal horses and those with pre-existing disease. In horses with surgical colic, SAA and haptoglobin concentration, when modeled with other routinely measured clinical parameters, were associated with outcome following surgical intervention. The study also suggested that increasing SAA concentration was associated with the presence of gastric ulceration in Thoroughbred race horses, such that SAA concentration might be used, along with other clinical parameters, as a tool to select which animals should be subjected to more invasive and specific investigative techniques such as gastric endoscopy. The final section of the study suggested that the measurement of SAA concentration in foals in a commercial stud farm setting could be an effective method for determining which individuals could be subclinically infected with disease, in this case, the respiratory pathogen *Rhodococcus equi*.

The studies on the kinetics of acute phase proteins, most notably SAA are a contribution to disease and welfare management in horses. They can be used to identify acute and chronic inflammation, and therefore be of value in the diagnosis and prognostication of disease as well as determining response to treatment. However, the non-specific nature of the acute phase response, as exemplified in the studies presented here, might lead one to speculate that their value as diagnostic and prognostic markers lies primarily in their use alongside other ancillary diagnostic methods to confirm the presence of a disease of which there is a strong suspicion. It would be fair then, to suggest that the horse with a low serum concentration of SAA could be assumed to be normal, in the same manner that a human with a low CRP is unlikely to be suffering from significant pathology (Pepys and Hirschfield, 2003).

8.2 The relationship between these studies and previous work

Previous work in this field demonstrated the normal ranges for SAA and haptoglobin in horses and showed that SAA increases in response to surgical intervention and to surgery of different intensities (Hulten *et al.*, 2002, Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2009, Pihl *et al.*, 2015). To date no one has compared elective and non-elective surgery. The results presented here agree with previous work indicating that surgical trauma produces an acute phase response but that the response is variable amongst individuals and depends on the markers used (Allen *et al.*, 1988, Pepys *et al.*, 1989; Ellis and Humphreys, 1992; Hulten *et al.*, 1999; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2009).

Despite a myriad of studies investigating methods used to predict the outcome of horses affected by colic (Orsini *et al.*, 1989; Pascoe *et al.*, 1990; Reeves *et al.*, 1990; Furr, *et al.*, 1995; Blikslager and Roberts, 1995; Thoenes *et al.*, 2000; Proudman *et al.*, 2002a, 2002b; Mair *et al.*, 2007) there is little specific work on the use of acute phase proteins (Sandholm *et al.*, 1995); however, one study suggests that non survivors had statistically greater concentrations of SAA than survivors in a large group of horses (Vandeplas *et al.*, 2005). Recently Pihl *et al.*, (2015) suggested that SAA and other clinical parameters could be used to differentiate between horses that could be treated medically from those which should be subjected to surgical intervention. These findings are broadly similar to those presented in Chapter six in that they both use the concentration of acute phase proteins to inform decision making in the management of horses with clinical signs of colic. However, the Pihl *et al.*, (2015) study also focused on comparisons between paired serum and peritoneal fluid acute phase protein concentration and on establishing reference intervals for peritoneal concentrations of acute phase proteins.

To date there are no markers of gastric ulceration routinely used in equine practice; this is in contrast to the situation in human medicine in which serum alpha-1-antitrypsin is used as a biomarker of ulceration and SAA is used to detect gastric neoplasia (Chan *et al.*, 2007; Hsu *et al.*, 2007). Currently, definitive diagnosis of gastric ulceration in equidae requires gastric endoscopy, a relatively invasive procedure.

Previous work on the use of acute phase proteins in relation to the diagnosis of *R. equi* suggested that SAA concentration is increased in foals with fulminant signs of the disease (Chavatte *et al.*, 1991; Hulten *et al.*, 2002). In these studies, infected foals also showed significant increases in fibrinogen concentration, WBC count, platelet numbers and Hb concentration (Giguere and Prescott, 1997; Stoneham *et al.*, 2001; Hulten *et al.*, 2002; Giguère *et al.*, 2003). No previous studies have investigated the association between subclinical infection with *R. equi* and haptoglobin concentration, or compared changes in a panel of acute phase proteins with the more traditional haematological markers of *R. equi* infection. Cohen *et al.*, (2005) investigated the use of SAA concentration for early diagnosis of *R. equi* in a large group of foals and concluded that SAA concentration alone was too variable to be of benefit during diagnosis; nevertheless, the sampling regimen and population of foals used by Cohen *et al.*, are sufficiently different to explain the differences in findings compared to the work presented here. Given that the long-term consequences of infection with *R. equi* can be devastating with regard to potential future performance (Giguere *et al.*, 1997; Giguere 2001), a simple and reliable initial test to identify abnormal individuals, applicable in the field, is potentially very welcome.

In light of the findings presented here and the significant body of literature on the topic of acute phase proteins in horses, it is perplexing that routine use of the measurement of acute phase proteins concentration in horses is not more widespread. Reasons for the lack of uptake amongst equine veterinary surgeons may be related to lack of availability of the tests in commercial veterinary laboratories, or cost, but are more likely to be due to a lack of large evidence based studies of the type which have informed the use of acute phase proteins in human medicine (Tilemann *et al.*, 2011; Kiefer *et al.*, 2012).

8.3 Factors that may have affected the outcomes and conclusions presented

There is no doubt that undertaking studies utilising populations of non-experimental animals, that is “real” animals, living and working within a normal or typical environment, is extremely challenging. Modern experimental study design parameters can be difficult to accommodate and owners, keepers, trainers, and indeed the horses themselves, are apt to introduce variables that

the researcher had not considered prior to undertaking the projects described. The majority of horses that were included in these studies (221 of 246) were Thoroughbreds (either racehorses, youngsters destined to race, or former racehorses that had become brood mares). These animals are often subject to major changes in management related to commercial pressures, injury or disease and environmental conditions. Many of the factors which may have affected the results, and therefore conclusions, have been discussed in the preceding chapters; however, as is the case in many clinical studies a greater number of animals would have been of benefit, particularly in relation to the animals affected by surgical colic and *R. equi*. In addition, the opportunity to apply some of the models that were developed to a different population of horses would have added another level of validation, and this is something that could be considered in any future work. As a consequence of some of the management conditions for the horses included in these studies, there were some clustering effects due to relationships between individual horses, groups of horses or premises; where possible, an attempt was made to account for these during the analysis.

8.4 Future and ongoing work - the way ahead

The potential for future work in the area of acute phase proteins in horses is enormous and it is this author's view that within a decade, in-clinic, "horse-side" acute phase protein testing will be a reality. As alluded to throughout this thesis, it is likely that there is an acute phase "signature" or "footprint" for many of the commonly encountered pathologies in horses and other veterinary species. These "footprints" may utilise existing and yet to be discovered acute phase markers and, in all likelihood, many disease states may be most appropriately monitored by a panel of markers, perhaps in concert with clinical observations.

The use of proteomic and metabolomic technologies is likely to "open a window" on many existing and new biomarkers (Bouwman *et al.*, 2010). In addition, it is likely that further site specific analogues of existing markers will be used, allowing better localisation of pathology (Jacobsen *et al.*, 2006). Perhaps these might include specific markers that could be measured in diagnostic samples of body site specific fluid, such as material recovered from a bronchoalveolar lavage, an arthrocentesis or cerebrospinal fluid puncture. The use of SAA to

monitor the response to treatment in septic arthritis is already being trialed in a number of clinics in the UK and Ireland.

Perhaps the most important conclusion of the work presented in this thesis is the need to use SAA and other acute phase protein results in conjunction with commonly measured clinical parameters that can then be incorporated into a model. The most obvious next step is to test the models generated with an unknown and new group of horses to determine how effectively, if at all, the model would perform in the clinical setting.

8.5 Mapping the results of the work presented onto the aims and general hypothesis as stated in Chapter One

The specific objectives of this project, laid out in Chapter one, were to answer the following questions;

- (1) Are there age, sex and breed differences in the plasma concentrations of the acute phase proteins, serum amyloid A and haptoglobin in clinically normal horses within the normal horse population?

Although based on small numbers of horses, the concentration of the acute phase proteins studied in this project were tightly conserved across a range of horses of mixed age and sex.

- (2) Are the results of the serum amyloid A and haptoglobin tests consistent across the various groups of animals studied, and do the results fall within a tight range?

The behaviour of the acute phase proteins measured during this project were similar across all of the groups of horses studied, that is that their concentrations increased rapidly in response to the presence of disease and inflammation and began to reduce in concentration in the postoperative period.

- (3) Is surgical stress a significant factor in the plasma concentration of serum amyloid A and haptoglobin in the horse?

The findings presented in this project suggest that surgery results in a measurable acute phase response in horses, and that the response is similar following elective and non-elective intervention.

- (4) Is the intensity of training a significant factor in the plasma concentrations of serum amyloid A and haptoglobin within a group of Thoroughbred racehorses?

Although there was an apparent weak association between the level of exercise and SAA concentration in Thoroughbred racehorses, the methodology for assessing work level and the similarities between the level of work between groups was such that further investigation is required to confirm these findings.

- (5) Does gastric ulceration have a significant association with the plasma concentrations of serum amyloid A and haptoglobin, and are these acute phase proteins useful markers for identification of different grades of ulceration within a group of Thoroughbred racehorses in training?

The results of the work presented here suggest there is an association between the concentration of SAA and the presence of gastric ulceration. Although the association was in some cases weak, the results suggest that measurement of SAA could be used as a broad screening tool for the identification of horses that should be considered for gastroscopic examination.

- (6) Are the plasma concentrations of the acute phase proteins serum amyloid A and haptoglobin associated with the severity and eventual outcome in horses with acute gastrointestinal compromise

Studies investigating the association between acute phase protein concentration and the outcome in horses presented for surgical investigation of colic suggest that both SAA and haptoglobin concentration could be used to help assess whether surgical intervention is likely to be effective.

- (7) Are the concentrations of serum amyloid A and haptoglobin significantly increased in foals subclinically infected with *Rodococcus equi*, but lacking clinical signs.

Increases in the concentration of SAA, and haptoglobin were associated with disease caused by *R. equi* in the foals studied and presented in Chapter seven

and as such SAA concentration could be used to identify individuals worthy of further investigation for the presence of subclinical disease.

- (8) Do serum amyloid A and other acute phase proteins have a role to play as a general measure of health in horses?

This project sought to investigate the association between a variety of different factors that initiate inflammation, both acute and chronic, and acute phase protein concentration. The findings broadly support the general hypothesis that the acute phase proteins serum amyloid A and haptoglobin can be used to test accurately for the presence of inflammation, tissue injury and training stress in horses and, as such, can be used to detect the presence of subclinical pathology, and for the diagnosis and prognostication of disease in horses. Ultimately these markers could be used as a general measure of health.

8.6 Conclusion

The work presented in this thesis represents a series of field based observational studies of populations of horses revealing the potential of acute phase proteins to identify overt, latent and subclinical disease and to inform the prognosis for surgical intervention in horses. A number of the associations between the disease and inflammatory states identified here were relatively weak and, as such, they should not be over interpreted. There is no doubt that further confirmatory studies, in other populations of horses, are needed. However, if the lessons from human medicine are to be learned, the results presented here set the tone for an overhaul of current thinking on the use of acute phase proteins in order to allow these ideas to be incorporated into everyday clinical veterinary medicine. This is something that has not happened to date, and that has the potential to contribute an evidence-based approach to the management of many significant equine disease and syndromes with the long-term aim of improving equine welfare.

Chapter 9

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Appendices

1. Signalment and details for the normal horses used in the study.....242
2. Signalment and details for the 100 Thoroughbred horses in training
.....244
3. Pollock PJ et al., 2005. Effects of surgery on the acute phase response
in clinically normal and diseased horses. Vet Rec 156:538-542

Appendix 1 - Signalment, haematology and biochemistry results for the normal horses discussed in Chapter 3

	Sex	Age	Use of Horse
1	F	6 months	Youngster bred for sale
2	F	5 months	Youngster bred for sale
3	M	7 months	Youngster bred for sale
4	M	8 months	Youngster bred for sale
5	M	8 months	Youngster bred for sale
6	F	5months	Youngster bred for sale
7	M	6 months	Youngster bred for sale
8	F	6 months	Youngster bred for sale
9	F	7 months	Youngster bred for sale
10	F	7 months	Youngster bred for sale
11	M	6 months	Youngster bred for sale
12	M	5 months	Youngster bred for sale
13	F	11 yrs	Brood mare
14	F	5 months	Youngster bred for sale
15	M	5 months	Youngster bred for sale
16	M	5 months	Youngster bred for sale
17	F	5 months	Youngster bred for sale
18	F	7 yrs	Brood mare
19	F	10 yrs	Brood mare
20	F	16 yrs	Brood mare
21	F	14 yrs	Brood mare
22	F	10 yrs	Brood mare
23	F	2 yrs	In training
24	F	2 yrs	In training
25	F	2 yrs	In training
26	F	2 yrs	In training
27	F	2 yrs	In training
28	F	2 yr	In training
29	F	2 yrs	In training
30	F	2 yrs	In training
31	M	2 yrs	In training
32	M	2 yrs	In training
33	M	2 yrs	In training
34	M	2 yrs	In training
35	M	2 yrs	In training
36	M	2 yrs	In training
37	M	2 yrs	In training
38	M	2 yrs	In training
39	M	2.5 yrs	In training
40	M	3 yrs	In training
41	M	3 yrs	In training
42	M	3 yrs	In training
43	F	3 yrs	In training
44	M	3 yrs	In training
45	M	3 yrs	In training
46	M	3 yrs	In training
47	M	3 yrs	In training
48	M	2.5 yrs	In training
49	F	3 yrs	In training
50	F	3 yrs	In training

Appendix 2 -

Signalment and Yard Number for the 100 Thoroughbred Horses in Race Training.

Horse name	ID No.	YOB	Age	Sex	Yard No.
Clonmel Commercial	1	1991	9	M	1
Feelin' Looser	2	1993	7	F	1
Galaxy Desine	3	1997	3	F	1
Faateq	4	1993	7	M	1
Hebron Rose	5	1993	7	F	1
Lady Monilousha	6	1996	4	F	1
Princess Pearl	7	1993	7	F	1
Crimson Tirol	8	1994	6	M	1
Stradbally Hall	9	1990	10	M	1
Etak	10	1996	4	F	1
Ace Conqueror	11	1996	4	F	1
Bayyanna	12	1996	4	F	1
UN by Camden Town	13	1996	4	F	1
Its Time For A Win	14	1992	8	M	2
Joe Cullen	15	1995	5	M	2
Ikdam Melody	16	1996	4	M	2
Tryphaena	17	1995	5	F	2
Peggy's Lad	18	1993	7	M	2
Punters' Friend	19	1996	4	M	2
Prince Kay	20	1995	5	M	2
Ammericanconnection	21	1996	4	M	2
UN by Warcraft	22	1996	4	M	2
Mossy Green	23	1994	6	M	2
Micko's Dream	24	1992	8	M	2
Summer in Siberia	25	1996	4	M	2
Killultagh Storm	26	1994	6	M	2
Bassett Tiger	27	1996	4	M	2
Ballyamber	28	1995	5	M	2
Be My Royal	29	1994	6	M	2
The Bunny Boiler	30	1994	6	M	3

Hill Society	31	1992	8	M	3
Miss Emer	32	1995	5	F	3
River Pilot	33	1994	6	M	3
Royal Jake	34	1994	6	M	3
Saddlers' Mark	35	1995	5	M	3
Hirapour	36	1996	4	M	4
Creux Noir	37	1996	4	M	4
Beautiful Drifter	38	1998	2	F	4
Among Equals	39	1997	3	M	4
Worldly Treasure	40	1997	3	M	4
Medkhan	41	1997	3	M	4
Tarry Flynn	42	1994	6	M	4
Moving On Up	43	1994	6	M	4
Amplified	44	1997	3	M	4
Jammaal	45	1997	3	M	4
Bust Out	46	1996	4	M	5
Stormy	47	1994	6	F	5
Super Gale	48	1996	4	M	5
Summer Soldier	49	1994	6	M	5
Lisabrack Native	50	1996	4	F	5
Eurobus	51	1995	5	M	5
Spirit Leader	52	1996	4	F	5
Captain Stewart	53	1994	6	M	5
Shadowing	54	1995	5	F	5
UN by Dancing Dissident	55	1997	3	F	5
Buffallo Bill	56	1996	4	M	6
Kilcash Castle	57	1995	5	M	6
Kaldan Khan	58	1991	9	M	6
UN by Danehill	59	1998	2	F	6
UN by Indian Ridge	60	1998	2	F	6
Hurmuzan	61	1993	7	M	7
Buck Leader	62	1994	6	M	7
Southern County	63	1995	5	M	7

Impeccable Dante	64	1995	5	M	7
Shaiydari	65	1995	5	M	7
Lady Marlan	66	1996	4	F	7
Samapour	67	1994	6	M	7
Native Hall	68	1996	4	M	7
Aungier Gale	69	1994	6	M	7
World Leader	70	1996	4	M	7
Cronin's Boy	71	1994	6	M	8
La Colina	72	1995	5	M	8
Earlshill Song	73	1994	6	M	8
Roca Maderia	74	1994	6	F	8
Louises Glory	75	1995	5	M	8
Yash Can Step	76	1994	6	M	8
Camden West	77	1994	6	M	8
Magical Fun	78	1992	8	M	8
Supreme Chanter	79	1992	8	M	8
Satco Express	80	1996	4	M	8
UN Louis	81	1997	3	F	9
UN John N	82	1997	3	M	9
UN Billy G	83	1995	5	M	9
Oriental Buck	84	1993	7	M	9
Gallaghers Walk	85	1993	7	M	9
Persian Tiger	86	1993	7	M	9
Inagh Road	87	1995	5	M	9
Native Scout	88	1996	4	M	9
UN Garvey	89	1997	3	M	9
UN Eamon	90	1996	4	M	9
Mac An Ri	91	1994	6	M	9
Legal Crack	92	1996	4	M	9
Largy Line	93	1995	5	M	9
Abbadore	94	1996	4	F	9
Work Away Horse	95	1995	5	M	9
UN by Phardante	96	1996	4	M	9

Dustin	97	1994	7	M	9
UN Slattery	98	1995	5	M	9
Aye Surely	99	1994	6	M	9
Killimor Castle	100	1992	8	M	9