The Structural Heterogeneity of the Acute Phase Proteins from Domestic Species

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ABSTRACT

The principal aims of the work presented in this thesis were to investigate the structural heterogeneity of the acute phase proteins from domestic animal species and to document the changes within the carbohydrate moiety during disease.

Alpha-1-acid glycoprotein (AGP) is a positive acute phase protein in many species, with its concentration in the plasma increasing 2 to 5 fold in a number of pathophysiological states. Human AGP is extensively glycosylated (45%) due to 5 N linked glycan chains. In normal serum, AGP does not exist in a single form but as a heterogeneous population of glycoforms. Heterogeneity arises through subtle structural differences in monosaccharide sequence and linkages, degree of branching and extent of fucosylation and sialylation. The existence of structurally distinct glycoforms implies a functional diversity since the properties of a glycoprotein are influenced by the structures of its oligosaccharide chains. During pathological conditions in humans, not only may the total concentration of AGP be altered but the relative proportions of the normal AGP glycoforms can change and abnormal glycoforms are expressed.

A method for the isolation of feline AGP from serum and ascetic fluid was developed which maintained the structural integrity of the glycan chains. Feline AGP was isolated from serum or ascitic fluid from 17 individual cats that were diagnosed to have feline infectious peritonitis (FIP), a disease known to cause an increase in AGP concentrations, and also isolated from pooled serum from animals without inflammatory disease. Following isolation of fAGP the monosaccharide composition and oligosaccharide profile was analysed by high pH anion exchange chromatography and pulsed amperometric detection. It was found that AGP from cats with FIP expressed significantly more ($p < 0.05$) N-acetylglucosamine and galactose residues than AGP isolated from pools of serum from cats without FIP or other inflammatory disease. Fucose residues were present on the fAGP glycan chains of cats 5 and 9. In the presence of FIP, fAGP carried both disialylated and trisialylated glycan chains while fAGP from cats without FIP only expressed disialylated glycan chains.

The structural heterogeneity of bovine AGP in bovine mastitis was examined by lectin binding with *Sambucus Nigra*, *Concanavalin A*, *Maackia Amurensis* or *Aleuria aurantia* lectins that bind α 2-6 linked sialic acid, diantennary chains, α 2-3 sialic acid and fucose residues respectively. Bovine AGP from cows suffering from mastitis was hypersialylated with more diantennary chains compared to AGP from control cows. It was also found that bovine AGP had no detectable α2-3 sialic acid or fucose residues.

A novel isoform of bovine β-haptoglobin was detected in milk during bovine mastitis. Investigation using PNGase F and lectins showed that the difference between the novel isoform and liver derived form was due to glycosylation changes. The novel isoforms of βHp was 3kDa heavier than serum Hp, however following removal of N-linked glycan chains by PNGase F both forms of Hp had a molecular weight of 30kDa. Fucose was expressed on the glycan chains of the novel isoforms of βHp while no fucose residues were detectable on the serum form of βHp.

Finally a comparative study looking at the glycan chain of AGP from cat, cow, sheep and man revealed distinct differences between the different species although AGP from the ruminants was more similar than AGP from man or cat. Fucose was expressed within the glycan chains of human AGP only, while only AGP from sheep expressed N-acetylgalactosamine residues. Alpha-1-acid glycoprotein from cat, cow and sheep expressed N-acetyl neuraminic acid as well as N-glycolyl neuraminic acid, while human AGP expressed N-acetylneuraminic acid only.

Overall the findings detailed in this thesis indicated that the structural heterogeneity of AGP from domestic species is influenced by the disease status of the host.

Authors Declaration

The work presented in this thesis was performed solely by the author except where the assistance of others has been acknowledged.

Kerry Rennie

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I am indebted to my mother and my mother in law. Without their support, encouragement and babysitting I could not have completed this study.

DEDICATION

This thesis is dedicated to my loving husband Allan and my beautiful daughters Taylor and Brooke who always provided a cuddle when I really needed it.

My beautiful daughters, I was blessed with two of you...

You will never know how proud I am of all the things you do.

You came into my world, so tiny and so small...

And I was in awe at the wonder of it all.

Contents

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%	Percent
µl	Microlitre
αLA	Alpha lactalbumin
° C	Degrees Celsius
AAL	Aleuria aurantia
ACTH	Adrenocorticotrophic hormone
ALD	Alcoholic liver disease
AGP	Alpha-1-acid glycoprotein
AMS	Automatic milking systems
Anti-Hp	Antiserum against haptoglobin
AP-1	Activating protein 1
ApoA-1	Apolipoprotein A-1
ApoSAA	Apolipoprotein serum amyloid A
APP	Acute phase protein
APPH	Animal production and public health
APR	Acute phase response
APS	Ammonium persulphate
Asn	Asparagine
βHCG	Beta human chorionic gonadotropin
βHp	Beta chain of haptoglobin
bAGP	Bovine alpha-1-acid glycoprotein
bHp	Bovine haptoglobin
BMSCC	Bulk milk somatic cell count
BSA	Bovine serum albumin
BTSCC	Bulk tank somatic cell count
C	Cytosine
Ca^{2+}	Calcium ion
Cl^-	Chloride ion
CMT	California mastitis test
CNS	Central nervous system
Con A	Concanavalin A
CRP	C-reactive protein
CSF	Colony stimulating factor
CV	Column volume
CZE	Capillary zone electrophoresis
Dol	Dolichol
Dol-P-P	Dolichol diphosphate
E. coli	*Escherichia coli*
EC	European Community
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum

E-selectin	Epithelium selectin
ESI	Electrospray ionisation
FCoV	Feline coronavirus
FECV	Feline enteric virus
FeLV	Feline leukaemia virus
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus
FIV	Feline immunodeficiency virus
FPLC	Fast protein liquid chromatography
g	Gram
Gal	Galactose
GalNAc	N-acetyl galactosamine
GlcNAC	N-acetyl glucosamine
GlcNAcT	N-acetyl glucosamine transferase
Glu	Glucose
Glucase	Glucosidase
GM-CSF	Granulocyte macrophage colony stimulating factor
GnHCl	Guanadinium hydrochlorine
H	Hour
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HbSR	Haemoglobin scavenger receptor protein
HCl	Hydrochloric acid
HDL3	High density lipoprotein
HGF	Hepatocyte growth factor
Hp	Haptoglobin
HPAEC	High pH anion exchange chromatography
HPLC	High pressure liquid chromatograph
HRP	Horseradish peroxidase
ICSCC	Individual cow somatic cell count
IEF	Isoelectric focusing
IL	Interleukin
IL-1R	Interleukin 1 receptor
IL-1Ra	Interleukin 1 receptor antagonist
INF-γ	Interferon gamma
IQSCC	Individual quarter cow somatic cell count
IS	Internal standard
JAK	Janus kinase
KDa	Kilo Dalton
K.pneumoniae	*Kleibsiella pneumoniae*
LPO	Lactoperoxidase
LPS	Lipopolysaccharide
M	Molar
MAA	Maackia Amurensis

MALDI	Matrix-assisted laser desorption/ionization
Man	Mannose
MAPK	Mitogen activated protein kinase
Mg_2Cl	Magnesium chloride
MHC	Major histocompatibility complex
MPD	Mean pixel density
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
M-SAA3	Mammary-associated serum amyloid A 3
Mwt	Molecular weight
Na^{2+}	Sodium ion
NA	Not available
NAGase	N-acetyl beta-D-glucosamide
NGNA	N glycoly neuraminic acid
$NaHCO_3$	Sodium bicarbonate
NANA	N acetyl neuraminic acid
NAOAc	Sodium acetate
NF	Nuclear factor
NF-κB	Nuclear factor kappa B
$(NH_4)_2SO_4$	Ammonium sulphate
NP	Normal pool
OD	Optical density
ORM	Orsomucoid
OST	Oligosaccaryl transferase
P	Phosphate
PAD	Pulsed amperometric detection
PBS	Phosphate buffered saline
PC	Phosphorylcholine
PCh	Phosphocholine
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pI	Isoelectric point
pKa	Acid dissociation constant
PKC	Protein kinase C
PNGaseF	Peptide N-4 N acetylβ D glucosaminyl asparagines amidase
PO_4^{2-}	Phosphate ion
PTM	Post-translational modification
r^2	Correlation coefficient
RA	Rheumatoid arthritis
RBC	Red blood cells

RID	Radial immunodiffusion
RT-PCR	Real time polymerase chain reaction
S	Second
S. aureus	*Staphylococcus aureus*
Strep. uberis	*Streptococcus uberis*
Strep.pneumoniae	*Streptococcus pneumoniae*
SA	Sialic acid
SAA	Serum amyloid A
SCC	Somatic cell count
SCN	Thiocyanate
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SELDI	Surface-enhanced laser desorption/ionization
Ser	Serine
SLeX	Sialyl lewis X
SNA	Sambucus Nigra
SRID	Single radial immunodiffusion
STAT	Signal transducer and activator of transcription
TBC	Total bacteria count
TBS	Tris buffered saline
TFA	Trifluoroacetic acid
TGF-β	Transforming growth factor beta
Th2	Type 2 T helper cells
Thr	Tyrosine
TNF-α	Tumour necrosis factor alpha
TNF-αR	Tumour necrosis factor alpha receptor
TOF	Time-of-flight
UV	Ultraviolet
w/v	Weight/volume

CHAPTER 1

1. Introduction

The objective of the studies in this thesis was to investigate the glycosylation of serum proteins in domestic species and the effect of disease on their glycosylation patterns during the acute phase response. This introduction therefore describes the nature of protein glycosylation, the acute phase response and the serum proteins known to be affected by disease, namely the acute phase proteins.

1.1 Glycosylation

Glycosylation is a term used to describe the covalent incorporation of carbohydrate moieties in the form of oligosaccharide chains into macromolecules, resulting in the formation of glycoconjugates. Glycoconjugates are classified according to the macromolecule that they are attached to i.e. if the oligosaccharide binds to a protein then it is referred to as a glycoprotein and, when it is a lipid that the oligosaccharide chain binds, it is referred to as a glycolipid. The oligosaccharide chains of glycoconjugates are highly organized structures constructed from monosaccharide residues. Several studies into the function of glycans have shown that they profoundly

influence the biological activities of the macromolecule to which they are bound (Paulson & Colley 1989;Varki 1993).

1.1.1 Monosaccharide

Monosaccharides are the simplest of carbohydrates and are based upon the empirical formula (CH_2O) n, with n usually being between 3 and 9. Monosaccharides are made up of one reducing (carbonyl group) and multiple hydroxyl functional groups. The monosaccharide can be classified according to the location of their carbonyl group i.e. if the carbonyl group is located on C_1 then the monosaccharide is described as an aldose while on the other hand when the carbonyl group is found on C_2 then the monosaccharide is described as a ketose. Monosaccharides can exist as linear or cyclical structures however it is more common to find them in the ring form.

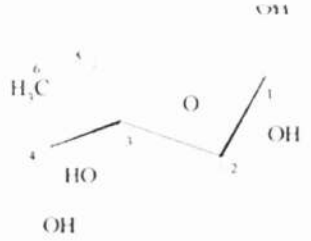
The favoured structure adopted by aldoses is a six membered ring which is referred to as a pyranose ring. Pyranose rings are formed by the reaction between the aldehyde group on C_1 and the hydroxyl group on C_5. On the other hand a ketose prefers to form a furanose ring which is a five membered ring resulting from the carbonyl group on C_2 reacting with the hydroxyl group of C_5.

Monosaccharide can also be classified according to the number of carbons they possess, such that monosaccharides which have four, five or six carbons are termed tetroses, pentoses and hexoses respectively. The most common constituents of glycans are monosaccharides comprising six carbons i.e. hexoses, hexosamines and deoxy hexoses. Hexoses are 6 carbon monosaccharides that have a neutral charge such as glucose,

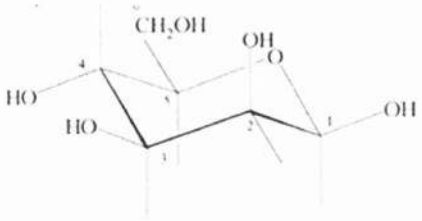
galactose and mannose (Figure 1.1). Hexosamines are hexoses with an amino group at position 2 which can be free or more commonly N acetylated e.g. N acetylglucosamine (GlcNAc). Finally deoxy hexoses are hexoses with the hydroxyl group on position 6 removed and replaced by a single hydrogen (H) atom. The carbonyl group of a monosaccharide is able to react with one of multiple hydroxyl groups of a second monosaccharide residue to produce a characteristic glycosidic bond. The glycosidic bond formed depends on the orientation of the carbonyl group, such that if the carbonyl group lies above the plane of the ring then a beta glycosidic bond will be formed whereas if the carbonyl lies below the plane of the molecule then an alpha glycosidic bond will be formed. The two different linkages confer markedly different structural properties and biological functions. Consequently sixteen isomers can be generated from the bond between 2 monosaccharides depending upon the location and orientation of the glycosidic bond and conformation of the ring structure. The continued addition of monosaccharides results in the formation of oligosaccharides (2-20 monosaccharides) and polysaccharides (>20 monosaccharides).

With the number of monosaccharides in existence and the number of individual linkages with which they can combine with other sugars it can be appreciated that oligosaccharides can offer great potential structural diversity. However studies carried out looking at the monosaccharide composition of glycoconjugates have shown that certain monosaccharides are more commonly found in glycoconjugates than others, and the composition of the monosaccharides is dependant on their source and that the linkages are dependant on the availability of the enzymes known as glycosyltransferases which are specific for the formation of certain types of glycosidic bond. The most commonly found monosaccharides seen in glycan chains of human glycoconjugates are

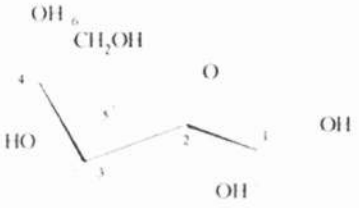
N-acetylglucosamine, mannose, galactose, N-acetyl galactosamine, fucose and sialic acid usually in the form of N-acetylneuraminic acid (Drickamer & Taylor 1998;Taylor & Drickamer 2003) (Figure 1.1).



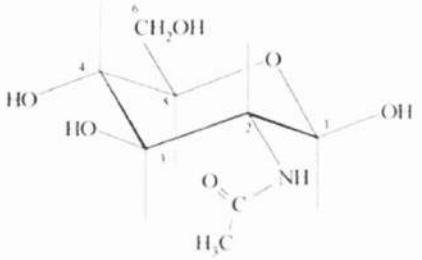
L-fucose



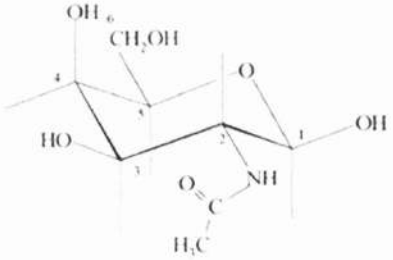
D-mannose



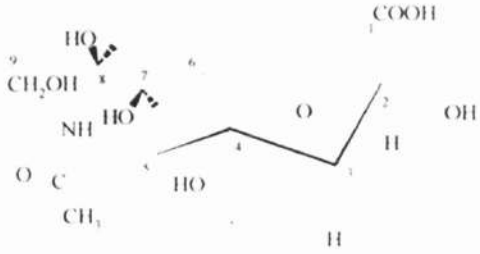
D-galactose



N-acetyl-D-glucosamine



N-acetyl-D-galactosamine



N-acetyl-neuraminic acid

Figure 1.1 Monosaccharides commonly found in N-linked glycan chains

1.1.2 Glycolipids

Glycolipids are formed when glycans are covalently attached to the head group of lipids, which are usually the phospholipids embedded in the membrane bilayer. The glycolipids fall into two structural categories depending on their lipid content. Those that are built on ceramide are known as the glycosphingolipids and are the most common grouping of glycolipids. On the other hand those glycolipids where the glycan is attached to phosphatidylglycerol are collectively known as glycophospholipids (Drickamer & Taylor 1998;Drickamer & Taylor 1998;Taylor & Drickamer 2003;Varki et al. 1999)

1.1.3 Glycoproteins

Glycoproteins are oligosaccharide chains covalently attached to the polypeptide backbone of proteins. Glycoproteins can be characterized according to the nature of the linkage between the amino acid chain and monosaccharide. When oligosaccharide chains are attached to the polypeptide backbone via the oxygen of a hydroxyl group on the side chain of a serine or threonine they are known as O-linked glycoproteins, alternatively when the oligosaccharide is attached to the polypeptide backbone via an amide group on the side chain of asparagines then the glycoprotein is referred to as an N-linked glycoprotein (Kornfeld & Kornfeld 1985). These two different types of glycosylation differ mainly in the core regions, whilst the monosaccharides composing the terminal elaborations are similar in both O and N-linked glycans.

1.1.3.1 N-Linked Glycoproteins

In animal cells the monosaccharide linked to an asparagine residue in the nascent polypeptide chain is almost inevitably N-acetylglucosamines and the linkage is always in the ß-configuration. This linkage can only occur when the asparagine residue is within the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline (Kasturi *et al.* 1995;Kornfeld & Kornfeld 1985).

All N-linked glycans contain a common pentasaccharide core region as shown in Figure 1.2. This common core arises because all N-linked glycans are derived form the same 14 residue oligosaccharide lipid linked precursor which is transferred to the nascent peptide chain and then processed to form this structure (Kornfeld & Kornfeld 1985).

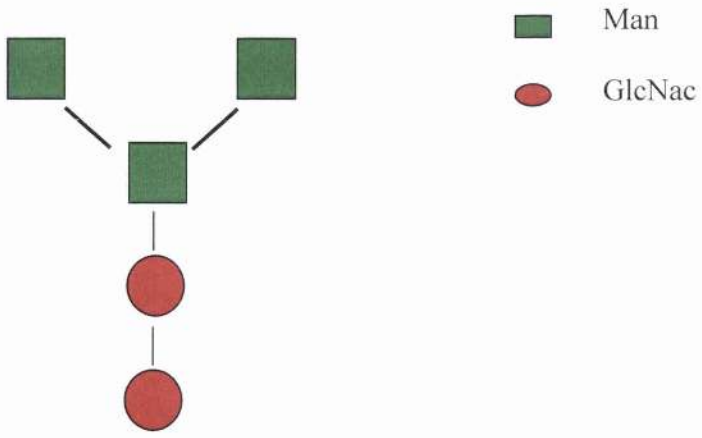


Figure 1.2 Schematic diagram of the common pentasaccharide core of N-linked glycan chains.

N-linked glycans can be classified into three main categories depending on the degree of processing required for the elongation of the pentasaccharide core.

The simplest category of N-linked glycosylation is termed high mannose glycan and as the name suggests the pentasaccharide core region is elongated with 2-6 Man residues (Figure 1.3A). The second category involves intermediate processing of the pentasaccharide core results in the formation of a hybrid glycan (see Figure 1.3B). The name is attributed to the fact that these glycans contain both high mannose (unsubstituted mannose residues attached to the core Man 1, 6 arm) and complex (substituted bisecting GlcNAc attached to the inner ß mannose) linkage. In the final category complete maturation of the glycan moiety results in the formation of complex glycan chains such that both Man α1, 3 and Man α1, 6 are substituted by GlcNAc residues. Complex glycans can be further elongated by the addition of Gal, Fuc and terminating SA residues. Complex glycans can be the source of great structural diversity for N-linked glycans as they can differ in chain length, composition and branching (see Figure 1.3C) (Taylor & Drickamer 2003;Varki, Cummings, Esko, Freeze, Hart, & Marth 1999). Figure 1.3 shows a diagrammatic representation of the three types of N-linked glycan chains.

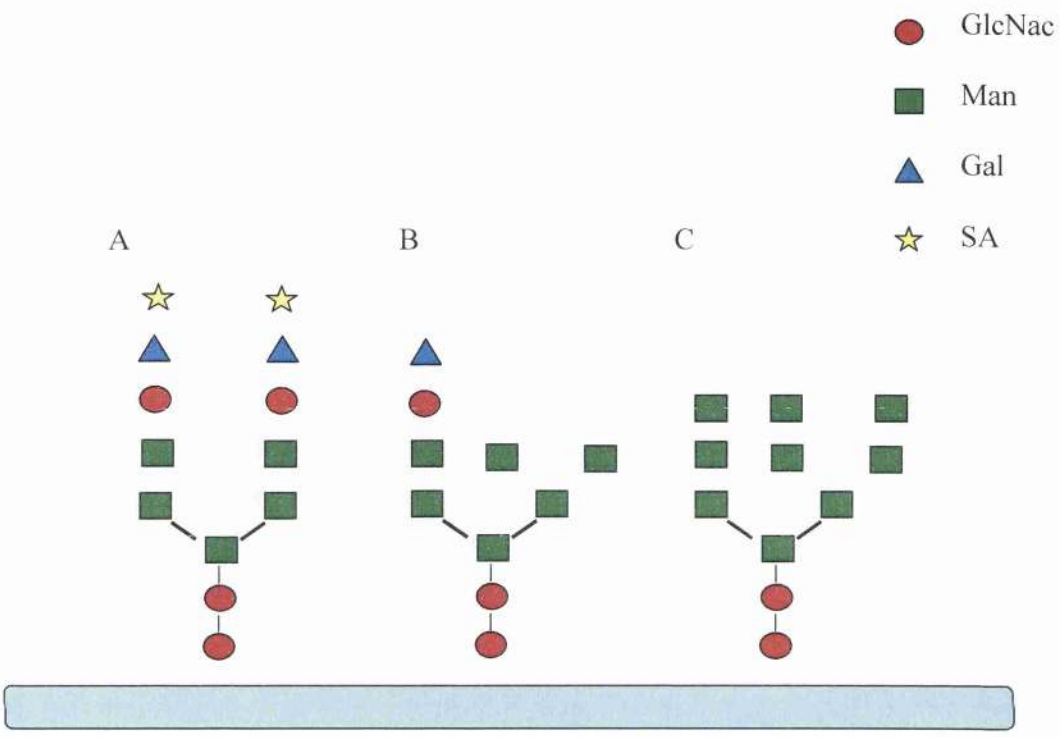


Figure 1.3 Representation of the three types of N linked glycan chains. A) complex type B) hybrid C) high mannose.

The biosynthetic pathway of protein N-glycosylation commences with the construction of a fourteen member, Man rich oligosaccharide on dolichol phosphate (Dol-P) (Kobata 1992) (Figure 1.4). Once all fourteen monosaccharide residues are attached to Dol-P the oligosaccharide moiety is transferred to an Asn residue located in the necessary consensus sequence (Marshall & Walter 1972) by oligosaccharyl transferase (OST) (Sharma, Lehle, & Tanner 1981;Silberstein & Gilmore 1996). Oligosaccharyl transferase attaches the oligosaccharide to the Asn residues by forming a complex with the precursor molecule, then through formation of a hydrogen bond using the hydroxyl

group of the serine or threonine residue the lipid linked precursor is transferred to the protein. This process results in the cleavage of the high energy GlcNAc-P bond with release of Dol-diphosphate.

Following the covalent attachment of the 14 residue oligosaccharide unit from the dolichol carrier to the appropriate Asn residue a series of processing reactions occur. The first few processing steps appear to be conserved among all eukaryotic cells and are known to play key roles in regulating protein folding.

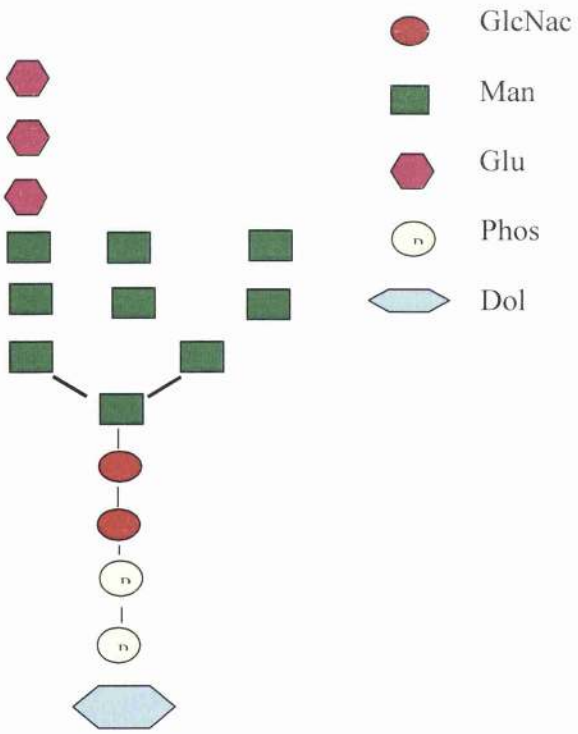


Figure 1.4 Diagrammatic representation of the 14 residue dolichol carrier.

The first processing steps involve trimming of the 3 glucose residues by glucosidase I and II. The outermost α1,2 linked glucose is removed by glucosidase I and the inner glucose residues are subsequently removed by glucosidase II. It has been proposed that the removal of the 3 glucose residues determines the retention time of the glycoprotein in the rough endoplasmic reticulum (Tokunaga, Hara, & Koide 2003). Following trimming of the glucose residue at least one exposed mannose residue is removed by a specific α mannosidase which is found in the membranes of the rough endoplasmic reticulum (RER). It is at this stage that high mannose structures can appear if the glycan processing is terminated (Figure 1.5). At this point the newly synthesized oligosaccharide is transported to the cis Golgi cisternae by means of a vesicle. When the glycoproteins arrive in the Golgi, they traverse the stacks from the cis through medial to trans cisternae by vesicular transport (Figure 1.5). The high mannose oligosaccharide which arrives in the Golgi can be further processed by the actions of a Golgi specific α1,2 mannosidase to yield Man5GlcNAc2 –Asn structure. It is when glucosaminyltransferase I (GlcNAcT 1) acts on this structure to add a ß1,2 GlcNAc that a hybrid structure is produced. The resulting N-glycan product GlcNAc Man3GlcNAc2-ASN is the specific substrate for GlcNAcT-II which catalyses the conversion of hybrid to complex N-glycans. At this stage in glycan processing fucosyltransferase may act to transfer a fucose to the innermost GlcNAc residue. The final steps of complex oligosaccharide synthesis occurs in the trans Golgi cisternae and consists of the addition of the outer chain galactose and sialic acid residues catalysed by galactotransferase and sialyltransferase respectively (Figure1.5). The newly synthesized glycoproteins then exit the Golgi and are transported to their final destination.

The final oligosaccharide structure assembled on a glycoprotein is dictated to a large extent by the order in which that glycoprotein encounters the processing glycosidases and glycosyltransferases and their specificity (Axford 1997).

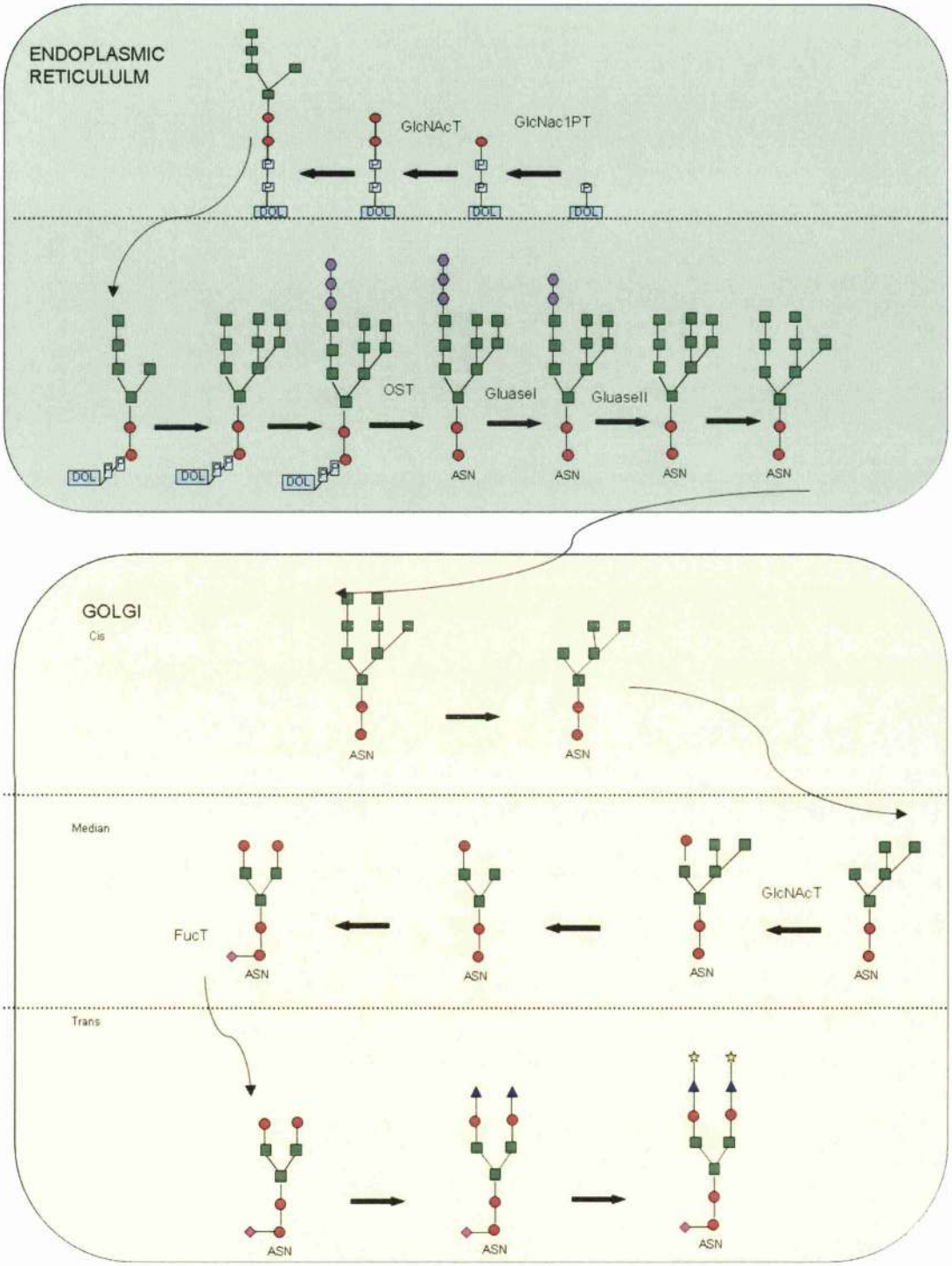


Figure 1.5 The biosynthetic pathway of N-linked glycan chains.

1.1.3.2 O-Linked Glycoprotein

The majority of O-linked oligosaccharides found in nature attach to the polypeptide backbone via a N-acetylgalactosamine residue (GalNAc). This type of linkage was first described in mucins and is often referred to as mucin type glycosylation. Other types of O linkage such as O-linked fucose (Fuc) and O-linked glucose (Glu) have been described however they are less commonly found (Taylor & Drickamer 2003).

O-linked glycans range in size from one monosaccharide residue to twenty and exhibit great structural diversity. The monosaccharides commonly found in O-linked glycans include GalNAc, GlcNAc, SA and Fuc.

O-glycan biosynthesis is simper than asparagine N-linked oligosaccharide generation in that a lipid-linked oligosaccharide precursor for transfer to the protein is not required. The initiating event in biosynthesis of O-glycans is simply the addition of a galactosamine (GalNAc) residue donated by UDP-GalNAc to serine/threonine. The transfer of the GalNAc to the serine /threonine residue is catalysed by a enzyme GalNAc transferase (Hanisch 2001). As yet no consensus sequence for this transfer has been elucidated, however a preponderance of adjacent proline residues has been associated with the site of O-linked glycosylation. The prolines appear to influence protein conformation by breaking helix formation and promoting the formation of ß-turns and ß-sheets.

O-glycans tend to be more variable in monosaccharide composition but less branched than N-linked glycans. O-linked glycoproteins are involved in a wide variety of cellular functions, with the oligosaccharide moiety playing an important role in biological activity (Varki 1993b;Varki, Cummings, Esko, Freeze, Hart, & Marth 1999)

Studies have shown that the oligosaccharide moiety of O-linked glycoproteins influence their secretion, function and interaction with other molecules. For example proper expression and activity of the IL-2 receptor, the antigenic protein of the Epstein Barr virus and the LDL receptor are all dependant upon O-linked glycosylation (Brockhausen *et al.* 1995;Hounsell, Davies, & Renouf 1996).

1.1.4 Functions of Glycoconjugates

Many bioactive molecules have been shown to be glycoconjugates. The glycan chains attached to these molecules have been shown to influence the biosynthesis, stability, action and turnover of these molecules in intact organisms. Many biological roles of oligosaccharides have been documented including masking of target structures, modulation of protein and cell-cell interactions and in maintaining structural integrity.

Glycans are located on the outer surface of cellular and secreted macromolecules and so are in the ideal position to influence structure as well as mediate a variety of events in cell-cell and cell-matrix interactions that are crucial to the development and function of complex multicellular organisms.

There is little doubt that some oligosaccharides such as those of the proteoglycans and the collagens are important in the physical maintenance of tissue structure, integrity and porosity. For example the heparin sulphated chains of proteoglycans interact with molecules such as fibronectin, laminin and collagen and are thus responsible for the organization of basement membranes and the extracellular matrix (Hook *et al.* 1984).

The role of glycans as masking structures on macromolecules has been well documented. Studies using testicular hyaluronidase (Lace, Olavesen, & Gacesa 1990b) and pancreatic ribonuclease β (Bernard, Newton, & Olden 1983b) have provided evidence that coating of proteins by glycans can protect the protein from recognition by proteases and antibodies. Studies carried out by Lace *et al.*,(1990) showed that when testicular hyaluronidase was deglycosylated there was improved recognition by antibodies. Furthermore removal of the oligosaccharide chains from pancreatic ribonuclease β made it more susceptible to proteases (Bernard, Newton, & Olden 1983).

Oligosaccharide involvement in protein folding in the endoplasmic reticulum (ER) and in subsequent maintenance of protein conformation is widely accepted as a function of the glycan chains attached to proteins. Many proteins that are glycosylated do not fold properly, fail to exit the ER and are consequently degraded. One such example is the CD4 protein. When this protein looses both glycosylation sites, it is retained in the ER and degradation occurs (Konig, Ashwell, & Hanover 1988;Tifft, Proia, & Camerini-Otero 1992).

Since all mammalian cells are covered with a dense coating of oligosaccharide structures, it has long been believed that oligosaccharides are vital determinants of cell-

cell interactions. One of the best documented examples is that of the selectin family of receptor proteins that mediate the adhesion of leukocytes to endothelial cells. The minimum carbohydrate moiety required is a sialylated, fucosylated glycan chain such as the tetrasaccharide Sialyl Lewis X (SLex). Lowe (1990) and Philips (1990) found that a member of the selectin family known as E-selectin, which is involved in the recognition of leukocytes by stimulated endothelium binds the glycan chains of a glycoconjugate on the surface of leukocyte it was subsequently discovered that the glycan that it bound was a sialylated, fucosylated polylactosamine chain known as SLeX. It was then revealed that the binding of this glycan to the E-selectin on the endothelium was responsible for primary adhesion of granulocytes, monocytes and certain lymphocytes to acutely or chronically inflamed tissue (Lowe *et al.* 1990). It has been reported that modifications of glycosylation may act as an on-off switch converting the molecule from an active state to an inactive state and vice versa. The best documented examples of changes in glycosylation affecting the biological activity of a molecule are haemopoietic growth factors such as GM-CSF and erythropoietin. Changes in their glycosylation alter their binding affinities and the biological activity of the glycoproteins (Oh-eda M 1990).

The hormone β-HCG is a glycoprotein which has N-linked glycans attached. It has been reported that these glycans are required for signal transduction and subsequently their removal results in the conversion to an antagonist which still binds to its receptor but fails to transmit the signal (Bielinska, Matzuk, & Boime 1989;Matzuk, Keene, & Boime 1989).

In conclusion, it is clear that although all the roles of glycoconjugates are not completely understood it is apparent that carbohydrate moieties profoundly influence biological functions. However most examples detailed above have been reported with reference to human medicine and there is little information on the comparative glycobiology of domestic animals.

1.2 Acute Phase Response

Following a wide variety of adverse stimuli including injury, trauma and infection a complex series of reactions are executed by the host in an effort to prevent ongoing tissue damage, isolate and destroy the infective organism and activate the repair processes that are necessary to return the host to normal function (Baumann & Gauldie 1994). The host organisms' first line of defence is non-specific and is generally characterised as the acute phase response. The acute phase response (APR) comprises a local and systemic reaction and is an integral part of the innate immune system. The local reaction manifests as acute inflammation at and around the site of injury whereas the systemic reaction consists of neurological, endocrine and metabolic alterations that are expressed as fever, leukocytosis, increased release of several hormones, activation of the clotting, complement and kinnin-forming pathways and dramatic rearrangement of plasma protein synthesis (acute phase proteins (APP) (Koj 1996). Most of the studies discussed in this section have been carried out with respect to the human disease and the human immune system or in experimental rodents and differences may arise in other species and this will be highlighted if and when appropriate.

1.2.1 Initiation and progression

The acute phase response is initiated at the site of injury by chemical patterns that are presented by invading microorganisms or revealed by tissue damage, (Coelho, Hogaboam, & Kunkel 2005) (Baumann & Gauldie 1994;Werling D 2004). These are recognised by the pattern recognition receptors such as toll-like receptors present on mononuclear cells such as tissue macrophages and blood monocytes, resulting in their activation and ultimately activation of the cascade of events that constitute the APR. Once activated the macrophages release a broad spectrum of mediators such as cytokines including interleukins 1 + 6 (IL-1, IL-6) and Tumour necrosis factor-α (TNFα). These are regarded as the most important as they initiate the next series of events. Locally IL-1 and TNFα act on stromal cells such as fibroblast and endothelial cells to initiate the release of secondary cytokines which activate the cellular and cytokine cascades that are involved in the complex process of the APR (Baumann & Gauldie 1994g;Gabay et al. 1994;Gabay & Kushner 1999). At the systemic level secreted cytokines exert actions on various tissues such as the brain and liver, however the liver is the primary target (Baumann & Gauldie 1994i). The liver's response to the systemic inflammatory mediators is characterized by changes in the transport of ions and metabolites in the activities of most metabolic pathways. There is also the co-ordinated stimulation of the acute phase plasma proteins (APP's). The inflammatory mediators that induce the hepatic APR can be divided in to four categories: IL-6 like cytokines, IL-1 like cytokines, glucocorticoids and growth factors. Il-6 like cytokines have been recognised as the principal regulator of most APP genes in hepatocytes. Glucocorticoids and growth factors mainly act as modulators of cytokine action rather

than directly inducing events of the hepatic APR (Baumann & Gauldie 1994a;Jensen & Whitehead 1998a;Moshage 1997e). APP genes can be divided into groups according to which cytokine is primarily responsible for inducing the cascade of events which lead to transcription(Moshage 1997d). Type I APP genes including SAA, CRP and AGP are induced by IL1-type cytokines however IL-6 type cytokine have been known to synergize with IL-1 type cytokines in the induction of type I APP genes. On the other hand Type II APP genes of which haptoglobin and alpha-2-macroglobulin are members are induced primarily by IL-6 type cytokines with IL-1 type cytokines having no affect on the induction of type II APP genes and in fact a possible inhibitory effect has even been suggested.

1.2.1.1 IL-1 type cytokine induced signalling

The liver is a principal target of inflammatory cytokines such as the IL-1 type cytokines. The hepatic APR is initiated by binding of cytokines to receptors on the membrane of the hepatocytes (Baumann & Gauldie 1994e). Two IL-1 receptors (IL-1R) have been characterised on the hepatocytes, type 1 is an 80kDA transmembrane glycoprotein and type 2 is a 60kDA glycoprotein. Both IL-1R belong to the immunoglobulin superfamily but represent different gene products (Moshage 1997c). Type 1 IL-1R is primarily responsible for transmission of the IL-1 signal including induction of the APP type 1 (Brint et al. 2002;Fitzgerald KA 2000;Moshage 1997b). In contrast IL-1R type 2 does not transmit a signal when IL-1 binds to it and for this reason type 2 is often thought of as an inhibitor of the IL-1 induced signalling as it competes with the type 1 IL-1R.

Type 1 and type 2 TNFα receptors (TNFαR) have also been described. Type 1 is 55kDA while type 2 is approximately 75kDa and both TNFαR's also belong to the immunoglobulin superfamily. However they are not homologous to IL-1 receptors. The basal levels of expression of both are low but increase during the acute phase response. Induction of APP's is mediated via the type 1 TNFαR which is also responsible for TNFα induced apoptosis (Moshage 1997).

In order for IL-1 type cytokines to induce APP genes they must activate a cascade, which transduces the signal from the outer cell membrane to the nucleus of the hepatocytes. However cytokine receptors have no intrinsic tyrosine kinase activity so a second receptor associated molecule is required for high affinity ligand binding and transmission of the cytoplasmic signal (Baumann & Gauldie 1994). It has been documented that IL-1 like cytokines induce expression of AP genes through activation of the transcription factors AP-1 and/or NFκB.

1.2.1.2 IL 6 and IL 6 type cytokines

The IL6 type family of cytokines are solely responsible for the induction of type II APP's and are capable of synergising with IL 1 type cytokines on type I APP's. IL-6 type cytokines such as, IL11, LIF and OSM are structurally distinct from IL-6 however produce a similar qualitative pattern of APP regulation (Baumann *et al.* 1983). Like other cytokine receptors IL-6 type cytokine receptors have no intrinsic tyrosine kinase activity and so in order to transmit the signal from the outer membrane to the nucleus a

receptor associated molecule is needed. Each member the IL-6 family of cytokines signals through a unique receptor complex however a common feature to all is the involvement of gp130 a signalling membrane glycoprotein. Following IL-6 association with the alpha subunit of the IL6 receptor a complex is formed with 2 gp130 molecules (Figure 1.6). It is the dimerisation of gp130 that is responsible for the cascade of events that lead to the induction of APP genes. Following dimerisation of the gp130 signalling molecules, cytoplasmic tyrosine kinases such as JAK are activated. Once activated, JAK phosphorylates the tyrosine residues within the cytoplasmic region of gp130 and subsequently the tyrosine residues of latent STAT proteins. Phosphorylation of the STAT proteins results in their dimerisation and consequently their translocation to the nucleus where they bind to IL 6 responsive elements and ultimately the induction of the type II APP genes.

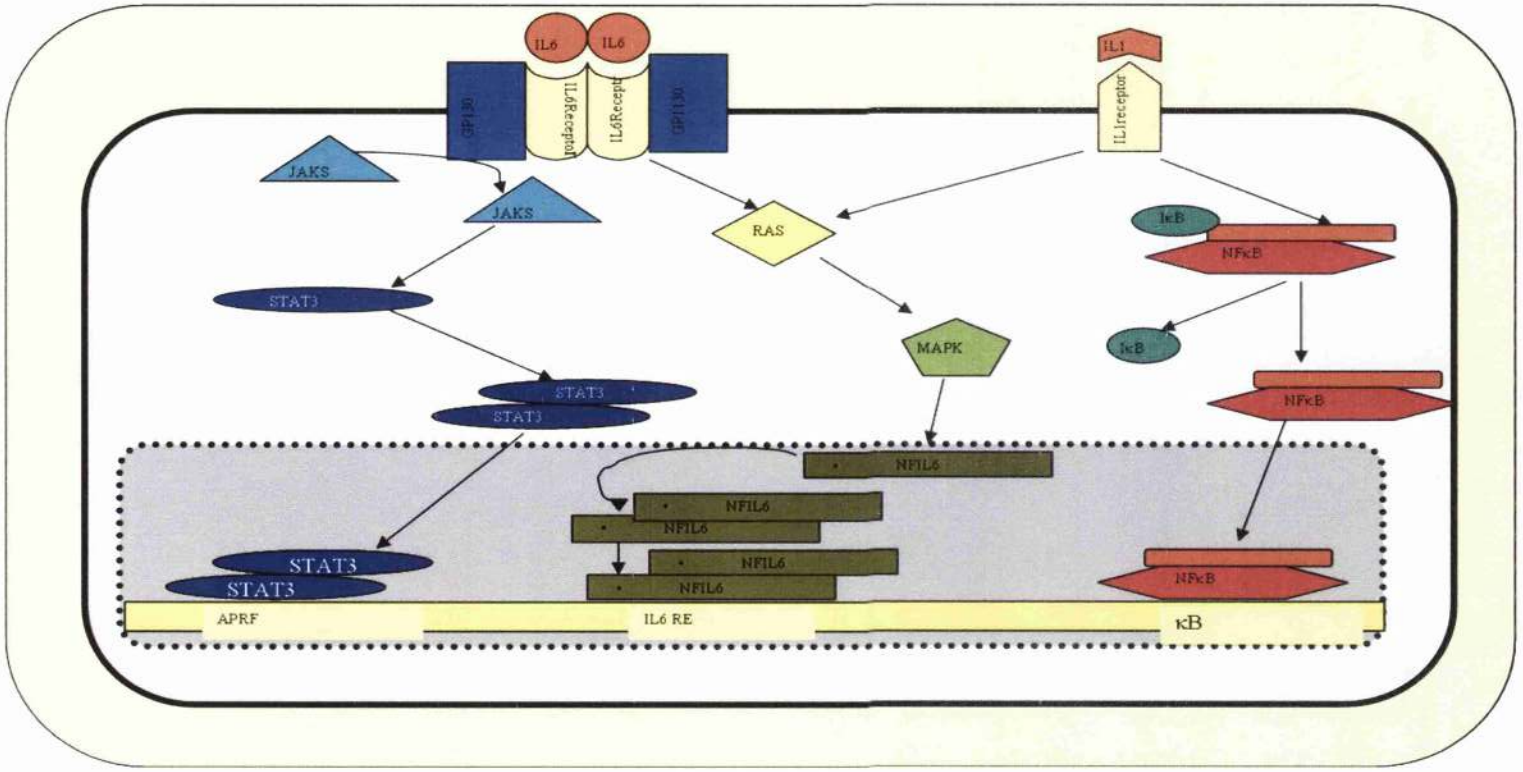


Figure 1.6 The cytokine signal transduction of the APR.

1.2.2 Resolution of the acute phase response

Most research on the APR has concentrated on the initiation and progression of the response however another aspect of the APR that is equally as intriguing and important is the termination of the response. Prolongation of the APR and the associated inflammation may have detrimental consequences for the host and so an effective inhibitory mechanism is desirable. It has been suggested that the APR is simply turned off due to degradation of the initiating cytokines (Baumann & Gauldie 1994). However several natural inhibitory mediators have been described. Some affect cytokine receptor interactions to prevent further signal transduction, whereas others interfere with the intracellular signalling cascade.

1.2.2.1 Soluble cytokine receptors and naturally occurring antagonists

In order for pro-inflammatory cytokines to elicit their response and induce the APR they must bind to their membrane bound receptors. Consequently inhibiting the binding of pro-inflammatory cytokines to their particular receptor would theoretically extinguish the APR. Soluble cytokine receptors for IL-1, IL-6 and TNFα have been described, which are thought to arise due to proteolytic cleavage of membrane bound receptors or by translation from an alternatively spliced mRNA (Koj 1998). Soluble cytokine receptors prevent further activation of the APR through posing as a decoy for the particular agonist binding which would otherwise prolong the response.

The IL-1 receptor antagonist (IL-1Ra) is the only natural antagonist to be described. IL-1Ra is a very powerful regulator of the APR as it competes with IL-1 for binding to cellular IL-1 receptors. This interaction can interfere directly with the ongoing cascade of inflammation, as binding of the IL-1Ra to IL-1R does not elicit a response and so dampens the IL-1 response.

1.2.2.2 Anti-inflammatory cytokines

Pro inflammatory cytokines are vital in the induction of the APR; however evidence suggests that many cytokines have the ability to exert both positive and negative effects depending on the cell types they interact with and the nature of the interaction. It is universally accepted that IL-6 plays a very important role in the initiation of the APR, however it has also been shown to exhibit some anti-inflammatory activities, including down regulating the gene expression and synthesis of other pro-inflammatory cytokines such as TNFα and IL-1 (Schindler *et al.* 1990). Furthermore it can stimulate the synthesis of naturally occurring antagonists such as soluble TNF receptors and IL-1Ra (Tilg *et al.* 1994). In addition specific anti-inflammatory cytokines do exist and in particular IL-4 and IL-10 have this activity.

IL-4 is mainly produced by type 2 T helper cells (Th_2) and its anti–inflammatory activities include inhibiting production of pro-inflammatory cytokines such as IL1, IL8, IL-6 and TNFα (Zurawski & de Vries 1994) as well as stimulating the release of IL-1Ra (Vannier, Miller, & Dinarello 1992). IL-10 is produced relatively late following

activation of the APR (de Vries 1995) by TH_2 cells, B lymphocytes, monocytes and macrophages and acts to inhibit the synthesis of pro-inflammatory cytokines including IL-1, IL-6, IL-8, TNFα and colony-stimulating factors (CSFs) as well as stimulating the release of IL-1Ra.

1.2.2.3 Glucocorticoids

Glucocorticoids are produced in the adrenal glands after induction by pro-inflammatory cytokines and can have positive and negative effects on the APR. They can either enhance the effects of the pro inflammatory cytokines in the liver or provide a natural feedback loop by inhibiting the production of many initiating and secondary cytokines by macrophages and stromal cells (Baumann & Gauldie 1994;Jensen & Whitehead 1998), which ultimately results in the termination of the APR.

Most of the studies discussed above on the action of cytokines, cytokine receptors and soluble receptors have been performed either in vitro or in laboratory animals. The presumption is that this also occurs in man and domestic animals however it remains to be seen if this is indeed the case.

1.3 Acute Phase Protein

The APPs are a diverse group of plasma proteins synthesised predominately in liver parenchymal cells whose concentration in the blood is increased (positive) or decreased (negative) by at least 25% during acute inflammation (Kushner 1982). Resolution of the APP response usually occurs within 4 to 7 days after stimulation if no further stimulation occurs.

The function of most APPs has not been totally elucidated. The positive APPs are regarded as having general functions in opsonisation and trapping of micro organisms and their products, in activating complement, neutralizing enzymes, scavenging free haemoglobin and radicals and in modulating the hosts' immune response.

The pattern of the APP response is species-specific with the exceptions of fibrinogen which increases by 50-100% and serum albumin concentrations which decrease by 10-30% in all mammalian species.

The circulating concentrations of the APP are related to the severity of the disorder and the extent of tissue damage in the affected animals. Quantification of their concentration can therefore provide valuable diagnostic and prognostic information.

1.3.1 Negative acute phase proteins

The negative APPs are proteins the concentration of which is decreased by at least 25% during an APR including albumin (all species) and transthyretin (human and pigs). It has been reported that the necessity for up-regulation of hepatic mRNA of the positive APPs results in decreased synthesis of normal blood proteins which result in the reduction in concentration of the negative APPs.

1.3.2 Positive acute phase proteins

Positive APPs can be subdivided based on their response pattern to inflammatory stimuli. Major positive APPs have very low or undetectable levels in serum of healthy animals but increase rapidly by more than 100 fold during an APR, whereas minor or moderate positive APPs are present in the serum of healthy animals and their concentrations increase during an APR but only up to 10 fold (moderate) and 1-2 fold (mild). However the moderate APP concentration remains elevated for a longer period after the challenge (Conner et al. 1988;Eckersall 1995;Gruys, Obwolo, & Toussaint 1994). In man CRP and SAA are the major APPs of use in diagnostic biochemistry while Hp and AGP are moderate APPs. However AGP and Hp are known glycoproteins and as such are described in detail below.

1.3.2.1 C-reactive protein

C reactive protein (CRP) a major APP, was first discovered in Oswald Avery's laboratory during the course of studies of patients with *Streptococcus pneumoniae* infection, and named for its capacity to bind C polysaccharide of *Strep.pneumoniae* (Tillett and Francis, 1930). C reactive protein is a positive APP featuring a homopentameric structure with Ca^{2+} dependant binding specificity for phosphocholine (PCh). The main biological function of CRP appears to be host defence against bacterial pathogens and clearance of apoptotic and necrotic cells. Though human CRP is not glycosylated it is known that canine CRP has both a glycosylated and a non-glycosylated subunit (Caspi et al. 1984).

1.3.2.2 Serum Amyloid A

Another major APP is Serum amyloid A (SAA) is the collective name given to a family of differentially expressed apolipoproteins encoded by multiple genes in a number of mammalian species. The human *SAA* gene family comprises a tightly linked cluster of three hepatically expressed genes (*SAA1 SAA2* and *SAA4*) and fourth locus (*SAA3*) which is a pseudogene. All four loci are clustered on the short arm of chromosome 11 (Sellar *et al.* 1994;Watson, See, & Woo 1994).

The exact functional role of SAA proteins remains obscure; however various effects and proposed functions have been reported such as cholesterol metabolism and transport (Liang & Sipe 1995).

1.3.2.3 Haptoglobin

Haptoglobin (Hp) is an alpha 2 acidic glycoprotein with haemoglobin (Hb) binding capabilities. In humans Hp is a normal constituent of plasma and is present at relatively high levels (0.9-1.3 mg/ml) (Langlois & Delanghe 1996). Some animals notably ruminants such as cattle do not constitutively express haptoglobin (Heegaard et al. 2000). Haptoglobin is a positive APP and in humans its concentration increases 2-10 folds (moderate APP) during an APR while in cattle it can raise 10-100 fold (major APP). Like other glycoproteins it is not just the concentration of haptoglobin that is under the influence of the APR as the oligosaccharide chains attached also undergo subtle changes.

1.3.2.3.1 Structure and Expression

Human Hp is a tetramer consisting of 2 α and 2 glycosylated β subunits covalently associated by inter-chain disulfide bonds (Turner 1995). The α 1 and β chains of Hp contain 83 and 245 residues respectively (Kurosky *et al.* 1980).

Variations in the α subunit of Hp exists in humans and gives rise to three major Hp phenotypes Hp1-1, Hp2-1 and Hp 2-2 (Yee & Brown 1999) . These phenotypes are genetically determined by two alleles: Hp1 and Hp2. Although Hp is found in serum of

all mammals this polymorphism has only been demonstrated in humans (Barnett, Bowman, & Lee 1972;Brune *et al.* 1984;Kurosky *et al.* 1975;Kurosky, Hay, & Bowman 1978)

Several functional differences between the phenotypes have been demonstrated and appear to have important biological and clinical consequences (Bamm et al. 2004;Delanghe et al. 1999;Langlois & Delanghe 1996). For example patients with phenotype 1-1 are less prone to the development of inflammation related cardiovascular diseases and diabetes (De Bacquer *et al.* 2001;Hochberg *et al.* 2002). These effects are explained by a phenotype dependant modulation of oxidative stress and prostaglandin synthesis.

The liver is the primary site of synthesis however extra hepatic expression has been reported. In addition to the liver haptoglobin has also been found to be synthesised in the lung (Yang *et al.* 1995), adipocytes (Friedrichs *et al.* 1995) and the uterus (Hoffman et al. 1996).

Inflammatory cytokines such as IL-6 significantly alter glycosylation of acute phase response proteins synthesised by hepatocytes and human hepatoma cell lines including Hp (van Dijk, Turner, & Mackiewicz 1994).

1.3.2.3.2 Carbohydrate moiety

The carbohydrate content of hepatic Hp is approximately 20% of the β chain mass (Black & Dixon 1970) and is found external to the protein, exclusively as N-linked, complex oligosaccharides chains (Kurosky et al. 1980;Turner 1992).

The β chains of human Hp each have four Asn-X-Ser/Thr sequences (Asn 23, Asn 46, Asn50, Asn 80) all of which may be occupied with glycan chains (Kurosky et al. 1980c).

Analysis of glycans derived from immunopurified human Hp by High Pressure Liquid Chromatography (HPLC) revealed the presence of diantennary complex glycans in a neutral, monosialylated and bisialylated forms and lower amounts of triantennary complex glycans that were present in disialylated and trisialylated forms. About 75% of charged Hp glycans were of diantennary complex structure some of which had one terminal sialic acid missing. Triantennary structures made up 25% of the glycome and highly branched glycan pools did not exceed 1% (Ferens-Sieczkowska & Olczak 2001b).

Microheterogeneity of the carbohydrate moiety Hp has been described (Katnik 1984).

1.3.2.3.3. Structural heterogeneity

Like other glycoproteins the heterogeneity of Hp is dependant on the pathological condition of the patient, several studies have revealed unique and characteristic changes in fucose content, sialic acid content and linkage going from α2-6 to α2-3 and glycan branching which are associated with different diseases as well as leading to novel

modifications in the Hp function (Baseler & Burrell 1981;Baseler & Burrell 1983;Oh et al. 1987;Oh, Pavlotsky, & Tauber 1990a) (Table1.1).

Increased fucosylation is a common finding and can be used to differentiate between different diseases or stage of disease. Fucosylated Hp is useful for discriminating between active and inactive rheumatoid arthritis (RA) with 94% of patients with active RA having high fucose content on Hp versus 5% of patients with inactive RA (Thompson *et al.* 1989). Further examples are given in Table 1.1

Fucosylated Hp has also shown potential as a prognostic marker as it can be used to monitor disease activity in cancer. Abnormally fucosylated forms of Hp have been measured in blood specimens from women with carcinoma of the ovary or breast who are undergoing chemotherapy. The proportion of fucosylated Hp increased if the women had progressive disease and decreased if they showed complete response to therapy (Thompson, Dargan, & Turner 1992).

Disease	Heterogeneity	reference
Rheumatoid arthritis	Increased fucosylation Increased sialylation Increased branching	(Thompson et al. 1989;Thompson et al. 1993)
Ovarian cancer	Increased fucosylation Increased branching	(Thompson, Dargan, & Turner 1992;Turner, Goodarzi, & Thompson 1995)
Crohns disease	Increased sialylation Increased branching Increased fucosylation	(Turner 1992;Turner 1995)
Alcoholic liver disease	Increased fucosylation	(Chambers et al. 1993;Mann et al. 1994)
Acute and chronic Inflammation	Expression of Sialyl Lewis X	(Brinkman-van der Linden *et al.* 1998)
Breast cancer	Increased fucosylation Increased Branching Increased sialylation	(Thompson & Turner 1987)

Table 1.1 Reports of structural heterogeneity of haptoglobin in various human disorders

1.3.2.3.4 Functions

The exact functional role of Hp remains elusive nonetheless it is generally accepted that haptoglobin plays an important part in the scavenging of free haemoglobin.

1.3.2.3.4.1 Binding of Haemoglobin (Hb)

The binding of Hb to Hp is the strongest non-covalent interaction known among the transport proteins in plasma and is not reversible. Hp binds Hb in a ratio of 1:1 with an association constant greater than 10^{-15} M (Dobryszycka 1997).

Efficient removal of free Hb following haemolysis of erythrocytes is essential since free Hb can accelerate lipid peroxidation, leading to production of toxic molecules. After destruction of erythrocytes, free Hb in the circulation passes through the glomerular filter and renal damage may occur. Haptoglobin effectively reduces the loss of Hb and iron because the Hp-Hb complex is not filtered through the glomeruli but is retained in the body. It was hypothesized that the complex is transported to the liver where the Hp-Hb complex can be broken down in the parenchymal cells (Kino *et al.* 1980;Kino *et al.* 1987), the mechanisms leading to the removal of the Hp-Hb complex from the blood remained elusive until the recent discovery of a novel Hb scavenger receptor protein known as CD163. This membrane protein found exclusively on monocytes/macrophages was identified as the receptor that engulfs Hb by mediating endocytosis of Hp-Hb complexes. CD163 will only bind Hp and Hb in complex suggesting that a neo-epitope is presented by this interaction (Kristiansen *et al.* 2001).

The receptor ligand interaction is calcium dependant and of high affinity. Internalisation of Hp-Hb in macrophages is followed by lysosomal proteolysis of globulin and conversion of haem to iron and bilirubin.

During inflammatory conditions the Hp-Hb and CD163 system might be co-ordinately induced, thereby enhancing the Hb clearance capacity in response to injury (Gordon 2001).

The carbohydrate moiety of haptoglobin has been shown to be important in the capture of haemoglobin as total removal of sialic acid diminished the haptoglobin-haemoglobin complex formation by 15% while further removal of 25% of galactose residues totally inhibited the ability of haptoglobin to bind haemoglobin (Kaartinen & Mononen 1988).

1.3.2.3.4.2 Other functions of haptoglobin

Although the capture of haemoglobin is the most documented function of haptoglobin a variety of other putative biological functions have been ascribed to Hp. However some of the roles outlined in the Table may be a direct consequence of haemoglobin binding (Table 1.2).

Role	Reference
bacterostatic	(Barclay, Clark, & Thomas 1985)
Inhibits Neutrophil respiratory burst	(Oh, Pavlotsky, & Tauber 1990)
Inhibits cathepsin B	(Snellman & Sylven 1967;Snellman & Sylven 1974)
angiogeneis	(Cid *et al.* 1993;Cockerill, Gamble, & Vadas 1995)

Table 1.2 Functions of Hp apart from haemoglobin binding.

1.3.2.4 Alpha-1-acid glycoprotein

Alpha-1-acid glycoprotein (AGP) formerly known as orosomucoid is a plasma glycoprotein. Alpha-1-acid glycoprotein was initially characterised in 1950 by two research groups headed by Schmid and Weimer respectively (Schmid 1950;Weimer, Mehl, & Winzler 1950). Since its discovery AGP has been the subject of many studies, which have highlighted unique physio-chemical properties such as its high carbohydrate content, its low pI value and its high solubility.

The concentration of AGP found in normal human plasma usually falls within the range of 0.36-1.46 mg/ml (Blain *et al.* 1985). However AGP has been found to be an acute phase glycoprotein and falls in to the class II positive acute phase protein group (Routledge 1989). The levels of AGP are increased two to five fold in both acute and chronic inflammatory conditions.

1.3.2.4.1 Amino Acid sequence

Using protein-sequencing Schmid *et al.*,(1973) reported that the polypeptide moiety of human AGP was a single chain composed of 181 amino acids (Schmid *et al.* 1973). However studies carried out by Dente (Dente et al. 1987;Dente, Ciliberto, & Cortese 1985;Tomei et al. 1989) established that AGP actually had 183 (Figure1.7) amino acids with two disulphide bonds that link residues cys5 + cys147 and cys72+cys164. Twenty-four possible amino acid substitutions exist within the AGP amino acid sequence, with each only involving two residues (Dente, Ciliberto, & Cortese 1985).

1.3.2.4.2 Genetic variants

Since the initial discovery of the structure of AGP many variant forms have been detected, with differences identified in the amino acid sequence and heterogeneity of the oligosaccharide chains described.

Human AGP is the product of a cluster of 3 adjacent genes termed *AGPA*, *AGPB* and *AGPB'* which are all located on chromosome 9. These three genes have been shown to encode two polypeptide variants of AGP known as ORM1 and ORM2. ORM1 is the major component of serum AGP it is encoded by *AGPA* and actively expressed in human liver. *AGPB* and *AGPB'* are identical, probably as the result of a recent gene duplication. *AGPB* and *AGPB'* genes differ from the *AGPA* gene by 22 base substitutions and code for ORM2. *AGPA* can be induced by acute phase stimuli whilst

AGPB and *B'* cannot. Of the twenty four known possible amino acid substitutions twenty-two are due to the difference between *AGP-A* and *AGP-B/B'* (Dente, Pizza, Metspalu, & Cortese 1987;Dente et al. 1988;Fournier, Medjoubi, & Porquet 2000b;Schmid, Emura, Nimberg, Ikenaka, & Schmid 1973;van Dijk, Havenaar, & Brinkman-van der Linden EC 1995).

Although some degree of variability is produced by these amino acid substitutions alterations in the glycosylation profile induce far greater structural heterogeneity.

Ile-Pro-Leu-Cys-Ala-Asn-Leu-Val-Pro-Val-Pro-Ile-Thr-Asn*-Ala-Thr-Leu-Asp-Arg/Gln-Ile-Thr-Gly-Lys-Trp-Phe-Tyr-Ile-Ala-Ser-Ala-Phe/Ala-Arg-Asn-Glu-Glu-Tyr-Asn*-Lys-Ser-Val-Glu-Glu-Ile-Gln-Ala-Thr/Ala-Phe-Phe-Tyr-Phe-Thr-Pro-Asn*-Lys-Thr-Glu-Asp-Thr-Ile-Phe-Leu-Arg-Glu-Tyr-Gln-Thr-Arg-Gln-Asp/Asn-Gln-Cys-Ile/Phe-Tyr-Asn*-Thr/Ser-Thr/Ser-Tyr-Leu-Asn-Val-Gln-Arg-Glu-Asn*-Gly-Thr-Ile/Val-Ser-Arg-Tyr-Val/Glu-Gly-Gly-Gln/Arg-Glu-His-Val/Phe-Ala-His-Leu/Asn-Leu-Ile/Phe-Leu-Arg-Asp-Thr-Lys-Thr-Leu/Tyr-Met-Phe/Leu-Gly/Ala-Ser/Phe-Tyr/Asp-Leu/Val-Asp/Asn-Asp-Glu-Lys-Asn-Trp-Gly-Leu-Ser-Phe/Val-Tyr-Ala-Asp-Lys-Pro-Glu-Thr-Thr-Lys-Glu-Gln-Leu-Gly-Glu-Phe-Tyr-Glu-Ala-Leu-Asp-Cys-Leu-Cys/Arg-Ile-Pro-Arg/Lys-Ser-Asp-Val-Met/Val-Tyr-Thr-Asp-Trp-Lys-Lys-Asp-Lys-Cys-Glu-Pro-Leu-Glu-Lys-Gln-His-Glu-Lys-Glu-Arg-Lys-Gln-Glu-Glu-Gly-Glu-Ser-COOH

Asn* = N-linked glycosylation site

Xxx/xxx = possible amino acid substitutions.

Figure 1.7 The amino acid sequence of human AGP

1.3.2.4.3 Carbohydrate Moiety

Human AGP has a molecular weight of 41-44kDa, 45 % which is made up of 5 complex type N-linked glycan chains. These are located on asparagines 15, 38, 54, 75 and 85 (Figure 1.7).

Differences in the glycosylation profile of AGP can arise in the complexity of oligosaccharide chain branching as the oligosaccharides chain can contain 2, 3 or 4 branches. Differences may also arise in the degree and type of outer chain substitutions thus they may have different degrees of sialylation and fucosylation, as well as through attachment of different oligosaccharides to each of the sites. Theoretically the glycosylation profile of AGP offers the potential of 10^6 different glycoforms of AGP each bearing a unique combination of glycans at the 5-glycosylation sites. However in nature only 12-20 glycoforms have been found in normal human serum. This is due to the fact that glycosylation site 1 never carries a tetra-antennary glycan, glycosylation site 2 never carries glycans containing fucose, glycosylation site 4 never carries a bi-antennary glycan and only glycosylation sites 4 and 5 carry tetra-antennary glycans with more than 1 fucose. However this micro-heterogeneity has been shown to be dependant on the pathophysiological conditions. (Elliott et al. 1997;Fournier, Medjoubi, & Porquet 2000;Mackiewicz & Mackiewicz 1995;Ryden et al. 1997;Smith et al. 1994;van Dijk, Havenaar, & Brinkman-van der Linden EC 1995). The glycosylation pattern of AGP seen at any time point is dependant on the pathophysiological condition of the tissue (Ryden et al. 1997;Smith et al. 1994).

1.3.2.4.4 Hepatic and extra-hepatic synthesis

Alpha-1-acid glycoprotein is one of many plasma proteins that are synthesised by the liver. The cells primarily responsible for its synthesis are the hepatocytes. Once AGP has been synthesised by these cells it is distributed in body fluids such as plasma, mucus, gastric juice, cerebrospinal fluid, ascitic fluid and peritoneal effusions. Although AGP is mainly synthesised in the hepatocytes, extra-hepatic synthesis has been reported.

Alpha-1-acid glycoprotein was initially reported extra-hepatically in 1977 in normal breast and colon tissues (Twining & Brecher 1977). In 1982 extra hepatic synthesis of AGP was reported in human breast epithelial cells (Gendler *et al.* 1982). Since then AGP synthesis has been reported in a variety of cells and tissues that include granulocytes, monocytes, endothelial cells, lung breast and alveolar type II epithelial cells as well as alveolar macrophages. (Fournier, Medjoubi, & Porquet 2000;Gahmberg & Andersson 1978).

1.3.2.4.5 Structural Heterogeneity in inflammation

Despite the extensive variation possible from the glycosylation profile of AGP only 12-20 glycoforms of AGP are observed in normal human plasma. However this can change due to the pathophysiological condition of the subject. It has been reported that the glycosylation profile of AGP in human sera is subject to marked changes during acute inflammation as a result of the cytokine-induced acute phase response. In situations of acute inflammation such as bacterial infection, surgery and trauma, there is an increase in AGP glycoforms containing bi-antennary chains (De Graaf et al. 1993;Mackiewicz et al. 1987;Pawlowski, Mackiewicz, & Mackiewicz 1989;Pos et al. 1990;van Dijk, Havenaar, & Brinkman-van der Linden EC 1995). On the other hand in situations of chronic inflammation such as alcoholic liver cirrhosis and rheumatoid arthritis, the opposite is seen, and there is an increased appearance of tri and tetra-antennary structures (Biou et al. 1989;Jezequel et al. 1987;Serbource-Goguel et al. 1986;Seta et al. 1997;Seta et al. 1986).

In rheumatoid arthritis (RA) the variation in glycan branching of AGP is indicative of both the severity and the progression of the disease (Hrycaj et al. 1993;Hrycaj et al. 1993;Mackiewicz et al. 1987;Pawlowski, Mackiewicz, & Mackiewicz 1989). Differences have been detected between the glycoform population seen in the early and late stages of the disease. Patients with early RA, displayed an increase in bi-antennary glycoforms, which persisted over the first year however as the disease progressed to the chronic stage an associated decrease in bi-antennary and hence an increase in tri and tetra-antennary glycoforms was observed.

In addition to the heterogeneity in the branching pattern of AGP, variations also occur

in the minor heterogeneity of the oligosaccharide chains during both acute and chronic inflammation. Increased fucosylation has been observed in acute inflammation including burns, liver cirrhosis and RA (De Graaf et al. 1993;Elliott et al. 1997;Poland et al. 2001;Ryden et al. 2002;Turner 1992). This increase in fucosylation is in part due to an increase in the expression of the antigenic structure Sialyl Lewis X (SLex) (De Graaf et al. 1993;Poland et al. 2001). The expression of this antigen on AGP is very low in control serum and upregulation in inflammation is associated with the presence of tri and tetraantennary structures. The SLex antigen is composed of the sequence NeuAcα2-3Galβ1-4(Fucα1-3) GlcNAc-, and thus contains both α1-3 fucose and α2-3 linked sialic acid. Consequently, if there is an increase in the appearance of SLex on AGP in acute inflammation then it is likely that there would be an increase in sialic acid and fucose levels.

The effect of inflammation on the extent of sialylation of AGP is somewhat confused as while some reports describe a decrease in level of sialylation in liver disease (Serbource-Goguel *et al.* 1983;Serbource-Goguel *et al.* 1986) and rheumatoid arthritis , whilst others have reported an increase. The inconsistency in these findings may be due to different methods of purification and assessment of sialic acid content as harsher methods of purification can lead to the degradation of the terminal sialic acid. Further examples of disease related glycosylation modifications are given in Table 1.3

Disease	Effect on AGP	Reference
Cancer	Increased expression of diantennary chains	(Rudman *et al.* 1974)
	Increase bi-antennary: decrease sialic acid: increase fucose	Tamura *et al.*, 1988
Lung cancer	Increased expression of diantennary chains	(Hansen, Larsen, & Boghansen 1984)
	hypersialylation	(Moule *et al.* 1987)
Chronic leukemia	Increased expression of tri and tetraantennary chains	(Treuheit & Halsall 1992)
Diabetes	Increased fucose expression	(Poland et al. 2001)
Pregnancy	Increased expression of tri and tetraantennary chains	(Raynes 1982)
Pneumonia	Increased expression of diantennary chains	(Treuheit & Halsall 1992b)
Inflammation	Increased expression of tri and tetraantennary chains in lung inflammation	(Hansen, Larsen, & Boghansen 1984)
acute inflammation	Increased expression of diantennary chains with increased fucose content	(Higai *et al.* 2005)
RA	Increase expression of tri and tetraantennary chains	(Elliott et al. 1997f;Pawlowski, Mackiewicz, & Mackiewicz 1989;Smith et al. 2002)

Table 1.3 Reports of glycosylation changes on human AGP in disease.

1.3.2.4.6 AGP Functions

Despite extensive investigation, the precise functional role of AGP remains to be elucidated. Several parameters can influence the function of AGP, which makes it difficult to interpret the way AGP functions *in vivo*, as AGP has been shown to exert different activities depending on the cell type it interacts with and it can display several activities on one cell type, with the activity it display on a certain cell dependant on the stimulus. The glycosylation profile can influence its activity while some effects of AGP can be concentration dependant.

1.3.2.4.6.1 Drug Binding

For many years it has been known that AGP has drug binding capabilities and is thought to be one of the major drug binding molecules in plasma along with albumin. Due to a high proportion of sialic acid found in AGP, it carries a large negative charge. Consequently, it preferentially binds basic drugs with pKa values of 8 or higher as these will then have a positive charge at physiological pH. However AGP has also been shown to bind certain steroids and acidic drugs (Kremer, Wilting, & Janssen 1988;Piafsky *et al.* 1978;Sadler *et al.* 2001).

It was originally thought that AGP only had one binding site but further studies have characterised seven binding sites each with varying affinities and capacities. Drugs that are known to bind AGP preferentially include amitiptylin, cocaine, erythromycin, imipramine, methadone, rifampicin, thalidomide, verapamil. Changes in the plasma concentration of AGP seen in the acute phase response can therefore significantly

influence the free plasma concentration and consequently both the pharmacokinetics and pharmacodynamics of the drug (Israili and Dayton, 2003)

1.3.2.4.6.2 Immunomodulatory Effects

1.3.2.4.6.2.1 Effects of AGP on lymphocytes

Human AGP has been shown to influence lymphocyte proliferation as AGP at physiological concentrations inhibited lymphocyte proliferation in vitro (Cheresh, Haynes, & Distasio 1984). The inhibitory effect of AGP was found to be at the level of both the mitogen and the lymphocyte. Alpha-1-acid glycoprotein has been shown to interact directly with lymphoid membrane (Cheresh, Haynes, & Distasio 1984). The degree of inhibition exerted by AGP was found to be dependant on its glycosylation profile (Bennett & Schmid 1980) as it was shown that non-reactive Con A and fully sialylated forms of AGP were more effective at inhibiting the anti-CD3-induced proliferation of human peripheral blood mononuclear lymphocytes than Con A reactive forms and disialylated forms of AGP (Pos et al. 1990). The importance of the carbohydrate moiety of AGP on its ability to inhibit lymphocyte proliferation was confirmed by Shiian and co-workers (1994). They showed that when the glycan chains of AGP where transferred to a synthetic matrix which mimicked the polypeptide moiety of AGP the same degree of inhibition was observed (Shiian et al. 1994).

1.3.2.4.6.2.2 Effect of AGP on neutrophils

Neutrophils are among the first leukocytes to respond to infection and injury and accumulate at the pathological site. Once neutrophils are activated they are responsible for destroying invading organisms by phagocytosis. They can also release cytotoxic compounds such as proteases and reactive oxygen species into the environment. This action can be detrimental to the host as well as advantageous, because if the neutrophils are activated at the wrong location or are excessive then they can then damage healthy tissue, whereas if released at the right location they can act to clear the organism. Alpha-1-acid glycoprotein has been shown to inhibit several activities of neutrophils such as the chemotactic response, superoxide generation and at doses above 0.5mg/ml it inhibits neutrophil aggregations (Hochepied *et al.* 2003). All these activities imply that AGP may act to switch off or downregulate the immune response.

1.3.2.4.6.2.3 Effects of AGP on Platelets

Alpha-1-acid glycoprotein has been shown to inhibit platelet aggregation in a dose-dependant manner. Initial studies that revealed AGP as an inhibitor of platelet aggregation used concentrations greater than the physiological concentration range (Snyder & Coodley 1976). However further studies carried out by Costello *et al.*,(1979) showed that AGP could inhibit platelet aggregation at concentrations seen in inflamed serum (Costello et al. 1979). Costello also reported that desialylation of the AGP increased its inhibitory effect. The inhibitory effect exerted on platelet aggregation by AGP was found to be directed against the secondary wave of platelet aggregation, which suggests that AGP may contribute to the down regulation of the further

recruitment of platelets and so in some way may exert an anti-thrombic function.

1.3.2.4.6.2.4 Effects *in vivo*

In 1983 Friedman proposed that AGP exerted non-specific anti-infectious activities (Friedman 1983). This arose from the finding that AGP was able to inhibit the ability of *Plasmodium falciparum* to infect red blood cells. This inhibitory effect was shown to be glycosylation-dependant as desialylation removed the inhibitory activities of AGP. It was suggested that inhibition resulted from the interaction of AGP with a sialic acid receptor on the parasite, preventing uptake into the host erythrocytes and reduced infection (Friedman 1983).

Support for Friedmans proposal of AGP being a non-specific anti-infectious agent came when Hochepied *et al.*, (2000) reported that AGP was involved in the induction of non-specific resistance to infection when *Kleibsiella pneumonia* was used as a model of bacterial shock in mice. These studies revealed that mice that had received AGP prior to a lethal challenge by *K pneumonia* were protected. This protection was shown to be at the level of bacterial spread and clearance as mice pre-treated with AGP showed less bacteria in their blood and different tissues. The protective role exerted by AGP in this study may be due to AGPs ability to alter capillary selectivity and hence control the transport of molecules through the endothelium (Hochepied *et al.* 2000).

In vivo, AGP infusion was shown to protect mice against lethal shock induced by TNF α (Libert, Brouckaert, & Fiers 1994) but not against LPS induced lethality (Muchitsch, Auer, & Pichler 1998). Muchitsch *et al.*, 1998 suggested that the partial protective effect of AGP in different rodent models of shock may be explained by enhancing the

capillary barrier function and thereby maintaining the perfusion of vital organs. However the protective activities exerted by AGP may also be due to its inhibitory activities on platelet aggregation, chemotaxis and oxidative metabolism of neutrophils.

Therefore although many studies support that AGP may act *in vivo* as a non-specific anti-infectious agent, the exact mechanism behind this ability has not been elucidated.

1.3.2 APP response in various species

Since the discovery of CRP as an acute phase protein in humans many studies have been carried out to discover other acute phase proteins and the role they play in the innate immune response. Studies however have not just been aimed at human acute phase proteins but have moved into the veterinary research. Although most species express the same acute phase proteins as humans the response of each APP is dependant on the species. For example CRP which is a major positive acute phase protein in humans, although expressed by cows it is only a minor APR in this species. There have been a number of excellent reviews in the recent past on the APR in companion animals (Ceron, Eckersall, & Martinez-Subiela 2005;Jacobsen et al. 2005) and production animals. Table 1.4 gives an outline of the APP response in various animal. In addition the introduction to the experimental chapters of this thesis was considered the more appropriate location for information on the APR and APP in the species and condition being investigated,

	Human	Cat	Cow	Sheep	Horse	Dog	Pig	Rat
α-1 acid glycoprotein	++	+++	++	++	++	++	NA	++
Haptoglobin	++	++	+++	+++	++	++	++	
C-Reactive protein	+++	NA	con			+++	+++	
Serum Amyloid A	+++	+++	+++		+++	+++	+++	
Albumin	-	-	-	-	-	-	-	-
Transthyretin	-	NA					-	
A-2 macroglobulin		NA	+					++
ceruloplasmin		NA				++	++	

Table 1.4 The acute phase protein response in various animal species. +++= major APP ++=moderate APP, += minor APP, NA= not available, con= constitutively expressed based on (Ceron, Eckersall, & Martinez-Subiela 2005;Jacobsen et al. 2005)

1.3.3 APP's in veterinary medicine

The significance of APP's as non-specific variables to monitor inflammation activity has been extensively reviewed.

Acute phase proteins can be used as non specific markers of clinical and subclinical infections to discriminate between acute and chronic disease and for prognostic purposes, since the duration and magnitude of the response reflect the severity of the disease (Hulten *et al.*,1997; Saini and Werbert, 1991; Peltola, 1982) and the effect of treatment (Hulten *et al.* 2002).

1.3.3.1 Haptoglobin in veterinary medicine

Numerous studies have highlighted the significance of Hp as a clinically useful parameter for monitoring the occurrence and severity of inflammation responses in cattle with mastitis (Hirvonen, Pyorala, & Jousimies-Somer 1996), pasteurellosis (Conner et al. 1989;Horadagoda et al. 1999), bovine respiratory syncytial virus (Heegaard et al. 2000) and pneumonia (Heegaard *et al.* 1998;Wittum *et al.* 1996). Bovine haptoglobin levels have been correlated to the severity of experimental infections with foot and mouth disease (Hofner *et al.* 1994).

In pigs haptoglobin measurements can be used to differentiate between normal pigs and pigs that will have low rates of weight gain due to subclinical infection (Eurell *et al.* 1992;Pineiro *et al.* 2007;Sorensen *et al.* 2006).

1.3.3.2 SAA in veterinary medicine

In horses measurement of SAA levels is shown to be useful in the diagnosis of viral infections or surgical trauma (Pepys *et al.* 1989) and in equine influenza infection (Hulten *et al.* 1999). Elevated SAA levels have also been found in cows with experimentally induced (Hirvonen, Pyorala, & Jousimies-Somer 1996c) and naturally occurring (Eckersall et al. 2001) mastitis and found to be related to the severity of the disease (Hirvonen, Pyorala, & Jousimies-Somer 1996).

1.3.3.3 APP in bovine mastitis

There has been particular interest in the use of APPs in diagnosis of bovine mastitis. In dairy herds mastitis is a production disease of major importance. Cows with clinical signs of mastitis are easily identified by experienced dairymen, however subclinical infection may not be detected and remain untreated. Serum levels of Hp and SAA are elevated in bovine mastitis however it is non specific as the levels of bHp and SAA levels are increased in various experimental and natural occurring inflammatory conditions. However Hp and SAA have been detected in milk during mastitis. Higher Hp concentrations have been found in infected quarters compared to opposite non-infected quarters and to milk from cows without mastitis (Eckersall et al. 2001).
Similarly cows suffering from mastitis caused by field infections had significantly higher SAA concentration in their milk from infected quarters compared to non-infected

quarters (Eckersall et al. 2001). The concentration of SAA in milk samples increased prior to the increase in somatic cell counts (SCC) in cows experimentally infected with *Streptococcus uberis* (Pedersen et al. 2003).

Thus there is growing evidence that measuring Hp and SAA in milk can be used to identify clinical and subclinical mastitis.

1.3.3.4 AGP in veterinary medicine

Feline infectious peritonitis (FIP) is the leading infectious cause of cat mortality and early identification of the disease is very difficult as clinical signs are very general. However it has been shown that cats suffering from FIP had elevated AGP levels (Duthie et al. 1997), and that cats with FIP had significantly higher levels of AGP than cats suffering from other inflammatory conditions which can cause raised AGP. Since the publication of this study the feline laboratory at the University of Glasgow has incorporated the measuring of AGP levels into the panel of tests performed for diagnosing FIP (personal communication Dr D Addie).

Feline infectious peritonitis and bovine mastitis are clinically important in their respective species. Feline infectious peritonitis is a clinically important disease due to the debilitating symptoms and the difficult diagnosis. Mastitis is not only clinically important but is also economically important as a financial penalty is paid for milk from cows suffering from mastitis.

Several studies have shown AGP to be a moderate APP in both cats and cows with concentrations rising 2-5 fold during times of inflammation. Further information on the APPs from these species is contained in chapter 2 and 4 respectively. Human studies have shown that the glycosylation fingerprint of AGP is dependant on the physiological condition of the patient and a potential for diagnostic and prognostic markers has been suggested. However there have been few investigations of the glycosylation patterns of the APPs during disease in the domestic species.

The objective of the work presented in this thesis was to examine the structure of the glycan moieties attached to AGP from domestic animals with an ultimate aim being the use of its structural heterogeneity in clinically important disease as a method of diagnosis. We set out to investigate this possibility firstly by looking at differences within the glycan chains of feline AGP (fAGP) isolated from the peritoneal fluid of cats with confirmed FIP and comparing it to the glycan chains attached fAGP isolated from the serum from animals that have no signs of FIP or other inflammatory process. To achieve this objective a method had to be developed for the isolation of fAGP. Once isolated the carbohydrate moiety of fAGP was determined using High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Subsequently the structural heterogeneity of bovine AGP in mastitis was examined using lectin based methods which allowed initial assessment of glycan structure for this species and the effect of disease. A novel isoform of bovine Hp was detected in milk during mastitis and shown to be caused by modification of the carbohydrate moiety. Finally a comparison was made between the glycan moiety and pI of AGP from cat, cow, sheep and man by lectin blotting and isoelectric focusing (IEF).

CHAPTER 2

2. Isolation of feline alpha-1-acid glycoprotein

2.1 Introduction

Glycosylation is a functionally and clinically significant post translational modification. The majority of proteins in plasma are glycoproteins and it is now recognized that the oligosaccharide chains attached to these proteins are important factors in the biological functions exerted by the protein (Bernard, Newton, & Olden 1983;Lace, Olavesen, & Gacesa 1990). Alpha-1-acid glycoprotein is a positive acute phase protein in cats (Ceron, Eckersall, & Martinez-Subiela 2005;Duthie et al. 1997;Giordano et al. 2004). Many studies looking at AGP in human disease have reported changes within the 5 N-linked glycan chains attached to AGP (Anderson et al. 2002;Elliott et al. 1997;Smith et al. 1994;Smith et al. 2002). This has lead to the hypothesis that oligosaccharide composition of AGP is dependant on the health status of the host. Although numerous studies have looked at the importance of the glycosylation pattern of AGP in human disease, the veterinary field of research is trailing in knowledge as far as this subject is concerned. Therefore it was the aim of this project was to venture into the field of

veterinary glycobiology by looking at the glycosylation pattern of AGP in clinically important diseases such as feline infectious peritonitis (FIP).

2.1.1 Feline Infectious Peritonitis

2.1.1.1 Introduction

Feline infectious peritonitis (FIP) is a fatal immune mediated viral disease of domestic and wild cats, characterized by protein rich effusions in body cavities and granulomatous inflammatory lesions in several organs. The causative agent is part of the coronavirus family (Ward 1970) and is often referred to as FIP virus (FIPV) however feline coronavirus (FCoV) is a more accurate definition.

International studies have shown that more than 50% of cats that are frequently in a multiple cat environment test positive for the presence of FCoVs (Horzinek & Osterhaus 1979;Sparkes et al. 1992). Susceptibility to FIP depends on individual factors, such as age, sex, breed and genetic disposition. A cat of any age can develop FIP; however disease occurs most often in cats from 6 months to 5 years of age with the majority of cases occurring in cats of 1 year of age or less.

Feline infectious peritonitis is now considered to be the most important viral disease of cats taking over this position from the feline leukemia virus. Approximately 1 in 200 new cases presenting to American teaching hospitals represent a cat with FIP (Rohrbach *et al.* 2001). FIP is a major problem for veterinary clinicians due to difficulties in

definitive diagnosis, which arise from non-specific clinical signs, lack of haematological, and biochemical abnormalities as well as low sensitivity and specificity of diagnostic tests.

2.1.1.2 Coronaviruses

Members of the coronavirus family occur in many animal species (e.g. mouse hepatitis virus, canine coronavirus) including humans (e.g. severe acute respiratory syndrome (SARS)), causing respiratory, gastrointestinal, cardiovascular and neurological diseases, though many coronavirus induced disease are restricted to a single organ system (Hoskins 1993).

The family coronaviridae represents a group of enveloped, positive stranded RNA viruses with prominent surface projections known as spikes or peplomers (Pedersen *et al.* 1981); which give the coronavirus its distinctive appearance when examined under an electron microscope. The RNA genome of coronaviruses is approximately 30 kilobases in length which is the largest of all RNA genomes described to date.

The organization of the genome of all coronaviruses are similar such that the gene order from the 5' end to the 3' end is polymerases, spike protein (S protein), membrane protein (M protein) and nucleocapsid protein (N protein) with nonstructural protein genes variably dispersed (Lai 1990). Some coronaviral genomes also encode a fourth structural protein known as HE however this has not yet been described in the feline coronavirus.

2.1.1.3 Feline Coronaviruses

Two coronaviruses have been described in cats, feline enteric coronavirus (FECV) and Feline infectious peritonitis virus (FIPV). FECV infections are generally subclinical however in immunosuppressed individuals such as kittens a mild or moderate enteritis may develop. In contrast FIPV infection causes a chronic and usually fatal vasculitis. Although FECV infections and FIPV infections differ greatly in their pathogenic potential more than 98% of their genomes are indistinguishable (Vennema et al. 1998) which suggests a common ancestry between the two coronaviruses. It is now believed that FIPV is merely a virulent variant of FECV caused by sporadic mutations within a specific individual rather than a separate virus. As a consequence of these reports it is more accurate to refer to the previously known FECV and FIPV as FCoVs. The exact location of the mutations that gave rise to the virulent form of FCoV that causes FIP has not been established however deletions in the non-structural genes 3C and 7B have been suggested (Vennema et al. 1998).

Until recently it was believed that the main difference between the avirulent non-mutated FCoV and the virulent mutated FCoV is the invasive nature of the mutated FCoV (Hoskins 1992;Pedersen 1987;Pedersen 1987;Vennema et al. 1998). Avirulent FCoV replicates primarily in the enterocytes of the intestines whereas the virulent FIP causing FCoV also infects blood monocytes and spreads and infects various organ systems. However a recent study has outlined the ability of avirulent FCoV to infect macrophages with the spike protein being the determinant for efficient macrophage/monocyte infection (Rottier *et al.* 2005).

2.1.1.4 Transmission

FCoVs are distributed worldwide in domestic and wild cats. The virus is endemic especially in environments such as catteries, shelters and multicat homes where cats live in close proximity to each other. In the UK 82% of show cats, 53% of cats in breeding institutes, and 15% in single cat homes have antibodies to FCoV (Hartmann 2005;Horzinek & Osterhaus 1979;Sparkes et al. 1992). FCoV infected cats shed the virus in their faeces, which can consequently infect other cats that come in contact with the faeces. Naturally infected healthy carrier cats can shed the virus in their faeces for up to 10 months (Herrewegh et al. 1995). However some cats may become chronic shedders for several years or even life long (Gut *et al.*,2002). The ingestion and inhalation of virus particles in faeces are believed to be the primary routes of infection (Evermann *et al.* 1981;Fiscus, Rivoire, & Teramoto 1987). Transplacental transmission has been suggested as FIP has been found in a 4 day old kitten and in stillborns and weak newborns born to a queen that had FIP during the later stages of pregnancy (Addie & Jarrett 1990).

All cats are usually infected with the avirulent non-mutated FCoV, through FCoV containing faeces shed by a cat with FCoV infection. The manifestation of the infection after a cat becomes infected with FCoV is dependant on the specific host. Most cats infected with the virulent FCoV that developed FIP also shed FCoV (Addie *et al.* 1996) however the virus load in faeces seems to decrease after a cat has developed FIP (Gut *et al.*,2002). So far there have been no reports of the mutated form of FCoV that brings

about FIP being detected in the secretions or excretions of FIP infected cats other than faeces.

2.1.1.5 Disease Development

Although the exact mechanism of disease development remains unknown it is generally accepted that FCoV is mutated within a specific region of its genome (Herrewegh et al. 1995;Herrewegh et al. 1995). The mutation leads to changes in the surface structures of the virus that allow the virus once phagocytosed by macrophages to bind to the ribosomes in these macrophages (Hartmann 2005). Hence the mutated virus can survive and replicate within the macrophages; this is considered to be the key event in the pathogenesis of FIP as pathogenic FCoV can then be transported within the monocytes/macrophages to various tissues throughout the body.

There are two common hypotheses concerning the events that occur after viral dissemination from the intestines. The first proposed mechanism is that FCoV infected monocytes leave the bloodstream enabling the virus to enter the tissues. The virus then attracts antibodies, complement is fixed, and more macrophages and neutrophils are attracted to the lesion (Hartmann 2005) and as a consequence typical granulomatous changes develop. The other hypothesis is that FIP occurs as a result of circulating immune complexes exiting from the circulation into the blood vessel walls, fixing complement and leading to the development of the granulomatous changes (Hartmann 2005). Complement fixation leads to the release of vasoactive amines, which cause endothelial cell retraction and thus increased vascular permeability. Retraction of capillary endothelial cells allows exudation of plasma proteins, hence the development

of characteristic protein-rich exudates (Mochizuki *et al*, 1997) seen in effusive FIP. The immune mediated vasculitis leads to activation of the coagulatory system and to disseminated intravascular coagulation (DIC). It is not the virus itself that causes major damage but the cats own immune reaction that leads to the fatal consequences that accompany FIP.

The immune response to the mutated FCoV appears to be largely cell mediated. The type and strength of the immune response also determines the disease form. Feline infectious peritonitis can manifest in three forms effusive, noneffusive and a mixed form. It has been suggested that effusive FIP occurs in cats that mount a humoral immune response but fail to develop concurrent protective cell mediated immunity and noneffusive FIP is thought to occur in cats that produce partial cellular immunity sufficient to limit the spread and degree of virus (August 1989;Barlough 1985;Hoskins 1993).

2.1.1.6 Diagnosis

2.1.1.6.1 Clinical Signs

A definitive diagnosis of FIP antemortem can be extremely difficult in many clinical cases because clinical signs of FIP are non-specific (e.g. weight loss, chronic fever and anorexia) and variable, as many organs, including the liver, kidneys, pancreas and eyes as well as the CNS can be involved. The clinical signs that occur in FIP are a direct

result of the vasculitis and organ failure that result from damage to the local blood vessels.

As mentioned previously three different forms of FIP have been identified; an effusive (wet) form, a noneffusive (dry) form and a mixed form. The effusive form is characterized by a fibrinous peritonitis, pleurisies or pericarditis. The non-effusive form is characterized by granulomatous changes in different organs including the eyes and CNS. In cats without effusions, signs are often vague and include fever, weight loss, lethargy and decreased appetite. Abdominal palpitation may reveal enlarged mesenteric lymph nodes and irregular kidneys.

The effusive (wet) form of the infection is commonly associated with swelling of the abdomen; however a study carried out on cat effusions revealed that less than 50% of cats presenting with swollen abdomens were diagnosed with FIP.

Laboratory findings provide some help in diagnosis of FIP but these are also general and may be seen in other clinically similar diseases.

2.1.1.6.2 Laboratory Findings

There are a number of laboratory tests which yield abnormal results in cats with FIP however FIP cannot be diagnosed on these findings, as they are not pathognomic. In many cases diagnosis of FIP can only be established with histopathological testing of biopsy or necropsy samples, which would usually reveal granulomatous lesions in several organs.

The most accurate laboratory test for diagnosis of FIP involves staining for the presence of coronavirus in the macrophages in effusions or tissues; in the event of a positive result it has a sensitivity of 100%. However recent evidence suggests that the avirulent FCoV may also be able to infect macrophages depending on the host (Dewerchin, Cornelissen, & Nauwynck 2005) which may lead to false positives and a specificity of less than 100%.

The most consistent laboratory finding in cats with FIP is an increase in total serum protein concentration. The increase in total protein is due to increased globulins, mainly γ globulins and also leads to a decrease in the albumin-to-globulin ratio. However these changes can be seen in most inflammatory based diseases so has a low specificity.

Blood cell counts are often analyzed when FIP is suspected; lymphopenia and neutrophila are typical results for cats with FIP. Measuring antibody titers is extensively used in the diagnosis of FIP however a large percentage of cats that have high antibody titers to FCoV never develop FIP so the results must be interpreted with caution. It has been suggested that, if effusions are present then it is more important to measure antibody titers in the effusive fluid rather than in blood as it has a much higher diagnostic value. However the presence of effusion itself is not diagnostic.

Measurement of acute phase proteins and in particular AGP is considered helpful in the diagnosis of FIP as discussed in section 2.1.2. However APPs are increased in many other diseases and are not specific to FIP.

Due to the lack of a single diagnostic test to differentiate FIP from other clinically similar disease, clinicians must consider a variety of different factors in order to diagnose FIP such as antibody titers, clinical signs at presentation, history of FIP in cats in the patient's environment, albumin-globulin ratio and AGP levels. It is only with all the relevant information that a diagnosis can be considered.

2.1.1.7 Treatment

In the majority of cases FIP is fatal as there are no effective cures. Most treatments for FIP are symptomatic and are aimed at suppressing the immune system because FIP is an immune mediated disease (Hartmann et al. 2003;Hartmann 2005). Human interferon is being used more often in the treatment of FIP however it has shown no significant decrease in mortality although in some cases survival times were significantly increased (Hartmann et al. 2003;Hartmann 2005)

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2.1.2 Feline Acute Phase Proteins

Serum amyloid A, Hp and AGP have been identified as positive acute phase proteins in cats (Duthie et al. 1997;Giordano et al. 2004;Kajikawa et al. 1999). Kajikawa *et al.*, (1999) measured SAA, Hp, AGP and CRP in the sera of clinically normal cats; cats post surgery and cats following induction of inflammation by turpentine oil injection. It was reported that SAA, Hp and AGP concentrations rose 7-11 times in cats following

induction of inflammation and in post surgery cats, SAA concentration were the first to rise. C reactive protein concentrations were not significantly altered in cats post surgery or following inflammation, induction when compared to control cats.

As mentioned above AGP, is a positive acute phase protein in cats and is considered diagnostically important in FIP being commonly used in the panel of tests used to differentiate FIP from other clinically similar diseases (Duthie *et al.*, 1997). Duthie *et al.*,1997 measured the levels of AGP in cats with naturally occurring FIP, Feline immunodeficiency disorder (FIV) and clinically health cats. It was found that AGP concentrations were significantly raised in both FIP and FIV compared to healthy cats. Alpha-1-acid glycoprotein concentrations in blood and effusions are generally greater than 1.5g/L (Duthie et al. 1997) in cats with FIP compared to 0.02-1.48 g/L for cats with FIP like diseases and an AGP assay was suggested as being useful in distinguishing FIP from other clinically similar conditions.

In agreement with Duthie *et al.*,1997, Giordano *et al.*,2004 reported that cats exposed to FCoV had increased concentrations of AGP and that cats that developed the clinical form of FIP had the higher concentrations of AGP than animals with clinically similar diseases. The increased APP response in cats exposed to FCoV was transient whereas the high AGP response persisted in cats that developed the clinical form of FIP (Giordano et al. 2004a)

Correa *et al.*,2001 showed that concentration of AGP was raised in cats suffering from lymphoma however the levels did not return to normal when in remission, which led to

the author concluding that AGP levels could not be used to monitor progression of the disease (Correa *et al.* 2001).

2.1.3 Isolation of Feline AGP

In order to study the glycosylation pattern of fAGP a method was required for the isolation of this protein from feline serum and effusions. Many studies have reported methods for the isolation of human AGP however only a few studies have describe the isolation of fAGP. Most of the studies that describe the isolation of fAGP required the isolation for antibody production or standards for assays (Bence, Addie, & Eckersall 2005) and for these purposes maintaining the structural integrity of the carbohydrate moiety was not essential and such methods have included precipitation at low pH. The exception, to this were Ceciliani *et al.*,(2004) and Pocacqua *et al.*, (2005) who isolated AGP from the 0.5-1ml of serum by initially precipitating non-soluble protein at pH4 by dialysing against a pH 4 citrate-phosphate buffer. The remaining serum proteins were then continually kept at pH4 throughout the remainder of the isolation procedure, which involved anion exchange followed by cation exchange chromatography (Ceciliani et al. 2004;Pocacqua et al. 2005).

Bence *et al.*, (2005) described the isolation of fAGP to be used as a standard in an immunoturbidimetric assay for the measurement of fAGP. In this instance fAGP was isolated by initial precipitation with 1.2M perchloric acid followed by anion exchange using a salt gradient to separate out the proteins (Bence, Addie, & Eckersall 2005).

As discussed in section 1.3.2.4.5 the glycan residue structure on AGP is known to be altered in a number of diseases in humans. There is also evidence from lectin blot studies that glycosylation on fAGP may be altered in FIP (Ceciliani et al. 2004).

The aim of this study was to analyse the carbohydrate moiety of AGP from cats with FIP and compare it to the AGP from cats without FIP in order to characterise the oligosaccharide chains attached to fAGP and identify any differences induced by FIP that could be potential diagnostic markers or influence the functions of fAGP. It was therefore necessary to isolate AGP from the serum or effusions of cats with and without FIP prior to glycosylation analysis. For this purpose a fractionation scheme was devised which enabled sufficient AGP for glycan analysis to be isolated from the ascitic fluid or serum of individual cats with increased concentrations of AGP (> 1 g/l). A problem for the investigation was in the acquisition of AGP from normal cats to act as a control as these have a relatively low AGP concentration. Therefore a higher volume of starting material was required. To avoid unnecessary bleeding of healthy cats, normal pools were prepared combining the serum and effusions of residual sample submitted to the Diagnostic service of the Faculty of Veterinary Medicine, University of Glasgow in which the concentration of AGP was in the reference range for healthy cats i.e. less than 0.45g/L. These pooled samples were used as the starting material for the isolation of normal AGP.

2.2: Materials and methods

2.2.1 Materials

Reagents were obtained from Sigma (Poole, UK) unless otherwise stated.

2.2.1.1 Samples

Ascitic fluid collected from 17 cats (cats 1-17), 11 of which were confirmed as having FIP, were kindly gifted by Dr D Addie of the Feline Virus Laboratory, Faculty of Veterinary Medicine, University of Glasgow. Serum samples from non-FIP infected cats (n=49) with normal AGP concentrations $\leq$ 0.45g/L were provided by the Clinical Pathology laboratory of the Faculty of Veterinary Medicine, University of Glasgow as well as Dr D Addie. The serum from the non-FIP cases were combined into four pooled samples (NP1-NP4). These samples had been submitted from a variety of cases with suspected hyperthyroidism or with suspected FIP in which all criteria for this disease was not present and AGP was in the normal range. The ascitic fluid and serum was not taken for experimental purposes but for clinical investigation with the residue after analysis being used for this investigation.

2.2.1.2 AGP isolation

Polyethylene glycol (PEG) Mwt 3350 and 8000, Trizma base, sodium chloride (NaCl), sodium acetate were used in the purification of AGP. Pre-packed Hitrap Blue 5ml column Hitrap Mono Q and Mono S 5ml columns were used on the AKTA FPLC system and were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK) and controlled by a Compaq deskpro EN, which ran Unicorn version 4 software. Acetic acid was obtained from BDH (Poole, UK). Centricon, centrifugal filter units were supplied by Millipore Corporation (Massachusetts, USA).

Dialysis tubing used for buffer exchange was purchased from Medicell.

2.2.1.3 Gel electrophoresis and Western blotting

Sodium dodecyl sulphate (SDS), ammonium persulphate (APS), Trizma base, bromophenol blue, mercaptoethanol, N,N,N',N'-Tetramethylethylenediamine (TEMED), coomassie blue, NaCl and Tween 20. Hydrochloric acid (HCl), methanol and acetic acid were supplied by BDH. Glycerol was purchased from Fisher scientific (Loughborough, UK).

30% Acrylamide mix, Standard marker, nitrocellulose and the 4-chloro-1-naphthol (4CN) substrate were all purchased from BioRad (Hercules, USA). The sheep anti AGP and horseradish peroxidase labelled sheep AGP were supplied by Dr Laura Bence, division of APPH, Faculty of Veterinary Medicine, and University of Glasgow.

Gel electrophoresis was carried in a Miniprotean II electrophoretic tank and protein transfer to nitrocellulose was carried out following the manufacturers' instruction in mini trans-blot electrophoretic transfer tank, both were supplied by BioRad (Hemel-Hempstead, UK).

2.2.2 Methods

2.2.2.1 Optimisation of PEG precipitation

In order to optimise the initial precipitation step in the isolation of fAGP, 1ml aliquots of peritoneal fluid from cat No 17 had 300, 400 and 500 μg of PEG 8000 or 300, 400, 450 and 500 μg of PEG 3500 added before vortexing and incubation at 4°C overnight. The supernatant was then collected following centrifugation at 3000g for 30 mins. An aliquot of the supernatant (10μl) was added to 10μl of SDS-PAGE sample buffer containing mercaptoethanol and run on an SDS-PAGE gel (section 2.2.2.3)

2.2.2.2 Feline AGP Isolation

Optimisation of PEG precipitation showed that the addition of 400μg of PEG 8000 caused the maximum precipitation of non-AGP protein with least loss of AGP (see section 2.3.2). Therefore in the first step of fAGP isolation low solubility protein in samples (2-5 ml) were precipitated by addition of 400μg per 1ml of sample (40%w/v) of PEG Mwt 8000 followed by incubation overnight at 4°C before centrifugation at 3000g for 30 mins with the supernatant, containing AGP, removed for further purification.

The isolation of AGP from feline ascitic fluid or serum was performed with the following procedure.

As cities (2-5ml)

PEG Precipitation

40%w/v PEG Mwt 8000

Supernant

Affinity chromatography

5ml Cibacron blue column (50mMTris HCl pH 7.4)

Unbound fraction

Anion exchange chromatography

5ml Mono Q column (50mMTris HCl pH 7.4)

First eluted Bound fraction

Cation exchange chromatography

5ml Mono S column (0.1M Acetate pH5)

Unbound fraction

Isolation confirmed

SDS-PAGE/Western blot

In the chromatography steps, peaks containing AGP identified by SDS-PAGE, were retained and either frozen (-80°) and lyophilised or concentrated with Centricon centrifugal filter devices (3000 Da cut off) to a volume of approximately 1ml then dialysed overnight against the equilibration buffer for the next step. In all of the chromatography runs the eluate was monitored at 280nm and protein containing peaks collected

The supernatant retained from the PEG precipitation step was dialysed against buffer 1A (Table 2.1) overnight at 4°C and then applied to a 5ml prepacked HiTrap blue column that was equilibrated with 2-3 column volumes (CVs) of buffer 1A at a flow rate of 5 ml/min. This initial chromatography step removed albumin from samples. The column was washed with buffer 1A and the initial breakthrough peak was eluted and collected. After the absorbance at 280nm returned to baseline the albumin bound to the column was eluted with 4-5 CVs of buffer 1B. The AGP containing breakthrough peak was then frozen and lyophilised, re-dissolved and applied to a 5ml Mono Q column equilibrated with 2 CVs of buffer 2A (Table 2.1) at a flow rate 5 ml/min. After the sample was injected, the column was washed with 2 column volumes of buffer 2A before a continuous gradient with buffer 2B was applied. The gradient used was 0-50% buffer 2B over 15 CVs and protein containing peaks were retained. The column was regenerated using 2-5 CVs of 100% buffer 2B (Table 2.1). Gel electrophoresis of an aliquot of each protein peak was carried out to determine which peak contained AGP. Fractions containing AGP were lyophilised, re-dissolved and dialysed against buffer 3A overnight at 4°C and then applied to a 5 ml High trap SP column. AGP was not retained by the SP column and eluted with the equilibration buffer (3A) (Table 2.1).

Proteins bound to the column were eluted using 100% of buffer 3B (Table 2.1). Occasionally samples had to be reapplied to the SP column. Purity was checked by SDS-PAGE and the purified AGP was then desalted using centricon centrifugal filter devices.

Column used	Buffer used	Sample Buffer
5ml HiTrap blue	1A 50mMTrisHCl pH 7.4 1B 1M NaCl, 50mMTris HCl pH 7.4	50mMTrisHCl pH7.4
5ml Mono Q (anion exchange)	2A 50mMTrisHCl pH 7.4 2B 1M NaCl, 50mM Tris HCl pH7.4	50mMTris HCl pH7.4
5ml Hitrap SP (cation exchange)	3A 10mMNaOAc, acetic acid pH5 3B 1MNaCl,10mMNaOAc, acetic acid pH5	10mMNaOAc/acetic acid pH5

Table 2.1 Sequence of columns and buffers used for AGP isolation AGP .

2.2.2.3 Gel electrophoresis and Western Blotting

SDS –PAGE was carried out according the method of Laemmli, (1970) using a 4% stacking gel and a 10% separating gel. Gels were stained with Coomassie blue 0.2% (w/v) protein stain made up in 20%(v/v) methanol, 8% (v/v) acetic acid or electroblotted onto nitrocellulose for antibody based detection. After staining the proteins were visualized by destaining in 20% (v/v) methanol, 8% (v/v) acetic acid solution. Western blotting was performed by transfer of separate protein onto a nitrocellulose membrane according to the method of Towbin (1974). Following electrotransfer of the proteins from the gel to the nitrocellulose membrane, the membrane was blocked using 10% (w/v) dried milk reconstituted in TBS tween (buffer composition in appendix). The

AGP on the western blot was detected with a 1:1000 dilution in TBS Tween of a polyclonal antibody to fAGP raised in sheep (primary antibody). The membrane was incubated with the primary antibody for 1 hour at room temperature. A 1:1000 dilution in 0.5M Tris buffered saline pH 7.4 + 0.1% Tween 20 (TTBS) of a horseradish peroxidase (HRP) conjugated secondary antibody to sheep IgG was used to detect the binding of the primary antibody to fAGP (gift from Dr L. Bence). The nitrocellulose was incubated with the secondary antibody for 1 hour at room temperature. The membrane was washed 3 times with TTBS between each step. A 4CN (Biorad, UK) substrate was then used to visualize the electroblotted AGP.

2.2.2.4 Radial immunodiffusion

A radial immunodiffusion (RID) technique (Mancini, 1965) was used to determine the quantity of AGP in each sample. A standard 2% (w/v) agarose type IIIA (supplied by sigma) solution in 0.2M phosphate buffered saline pH 7.4 was heated in the microwave until the solution turn clear, allowed to cool to 37°C then 8% v/v sheep anti AGP (gift from Dr L. Bence) was added and the mixture was allowed to set at room temperature on horizontal plates. Wells (5µl) were punched in the gel and samples (5µl) were applied. Standards of AGP (kindly supplied by Dr L. Bence) at concentrations of 2, 1, 0.5, 0.25 and 0.125 mg/ml were also applied to the RID gel. The gel was then incubated overnight at 37°C in a humidity chamber and the diameter of the precipitate rings measured. A calibration curve from the results of the standards (diameter (mm) vs concentration (mg/ml)) was drawn to determine the AGP concentrations in the samples.

2.3 Results

2.3.1 Samples

Effusions (ascites) from 11 cats (cats 1-11) with histopathologically confirmed FIP were analysed. In addition cats 12 and 13 had high AGP levels and from the clinical examination FIP was suspected but not confirmed by histopathology examination. Cats 14, 15 and 16 also presented with suspected FIP. These animals were included in the FIP group despite having AGP concentrations within the normal range as they had positive antibody titres; FIP was not confirmed by histopathology examination due to the absence of tissue. The AGP concentration in each sample was determined by a RID method and the results are listed in Table (2.2). Cats with confirmed FIP had a mean of 1.65±0.28 g/L as compared to a mean of 0.18±0.05 g/L for the cats pooled to represent a normal population.

Four pools of feline serum samples with 4-20 samples were pooled with a total volume of (2-5ml) and cat 17 were used to represent a non-FIP population. The samples in the pools were classified as non acute phase by measuring their AGP concentration. An AGP concentration of less than 0.45g/L, which is the upper level of the normal range (Duthie *et al.*, 1997), was used to confirm that the samples were not from cats during an acute phase reaction.

Cat Number	AGP level (g/L)	FIP/Non
1	1.35	confirmed
2	0.6	confirmed
3	1.3	confirmed
4	2.5	confirmed
5	NA	confirmed
6	NA	confirmed
7	2.2	confirmed
8	1.7	confirmed
9	2	confirmed
10	2.86	confirmed
11	3	confirmed
12	3.5	suspected
13	1.1	suspected
14	0.45	suspected
15	0.3	suspected
16	NA	confirmed
17	NA	suspected
18	0.25	fibrosarcoma
NP 1 (n=6)	0.1±0	Non FIP
NP 2 (n=8)	0.16±0.02	Non FIP
NP3 (n=15)	0.32±0.03	Non-FIP
NP 4 (n= 20)	0.14±0.02	Non-FIP

Table 2.2 The clinical data from samples used throughout the experiments reported within this thesis. NA= not available NP=normal pool

2.3.2 PEG Precipitation

The high solubility of AGP was exploited in the first step of the isolation procedure in which proteins of low solubility were precipitated by PEG. Polyethylene glycol Mwt 8000 was more effective at precipitation of non–AGP serum proteins. At concentrations of 30, 40 and 50% w/v of PEG 8000 the main band on SDS-PAGE of supernatant was AGP (lanes 6-8 Figure 2.1). There was contamination with a protein at 60kDa which was reduced at 50% PEG 8000 but at this concentration the yield of AGP was considerably reduced. With PEG 3500 considerable non-AGP protein remained in the supernatant at PEG concentrations of 30, 40, 45 and 50% w/v (lanes 2-5 Figure 2.1). Even at 50% w/v of PEG 3500 contaminating protein at 60kDa was apparent in the SDS-PAGE of the supernatant. Thereafter 40% (w/v) PEG 8000 was chosen to precipitate non-AGP serum proteins.

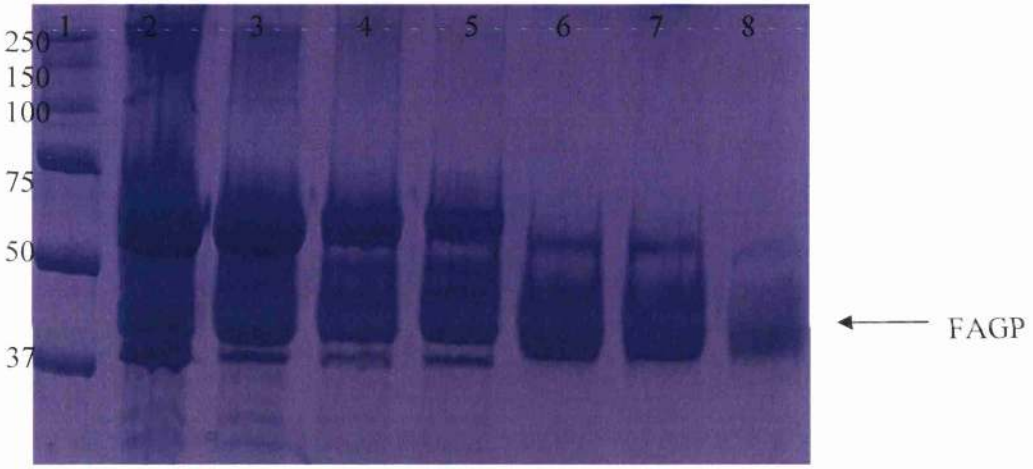

Figure 2.1 SDS-PAGE of 10µls supernatant obtained following precipitation with various concentrations of PEG Mwt 3350 and PEG Mwt 8000 Lanes 1 shows precision plus protein molecular weight markers Lanes 2-5: 30, 40, 45, and 50% PEG Mwt 3350 respectively. Lanes 6-8: 30, 40 and 50% PEG Mwt 8000 respectively.

2.3.3 Purification of feline AGP

As examples, the purification of AGP from cats 14 and 9 through the FPLC chromatographic steps is shown in Figure 2.2 A. B.C and D.E.F respectively. When the supernatant from the PEG precipitation was applied to the Cibacron blue column (Figure 2.2A) the AGP was found by SDS-PAGE in the break through peak with a small peak eluting with high salt buffer B. The AGP containing fraction from the Cibacron blue column was concentrated and applied to a mono Q column. AGP was retained but eluted using a NaCl gradient (Figure 2.2 B+E). AGP was eluted when the gradient reached approximately 20% of buffer 2B that is approximately 0.2M NaCl. Two peaks were obtained the first was found to the AGP containing peak whilst the second peak was found to contain mostly albumin. The final step in the isolation method was cation exchange on a Mono S column. The AGP containing fractions collected from the anion exchange was dialysed against buffer 3B and applied to the mono S column in a more acidic buffer (pH 5) in order to take advantage of AGPs highly acidic nature. The AGP was collected in the break through peak (Figure 2.2 C+F) while other contaminants such as final traces of albumin where retained by the column. Purification of AGP was confirmed using SDS-PAGE and western blotting using a polyclonal antibody raised in sheep against fAGP (Figure 2.3). The SDS-PAGE gel showed three main bands in the supernatant after precipitation with PEG 8000, this was reduced to 2 bands after Cibacron blue and a final band after mono Q and mono S columns. The western blot with anti fAGP revealed that the single protein band at approximately 50kDa in the break through peak after mono S is fAGP (Figure2.3B). Cat 9 had two breakthrough peaks with the SP column both where AGP containing peaks

however only the second peak had no contaminating proteins. The whole isolation procedure had a yield of AGP of approximately 26% (Table 2.3). Of this 22% was due to PEG precipitation. 30% lost after Cibacron blue column, 29% lost to mono Q column and 3% loss on the SP column. This procedure was applied to all samples (Table 2.2) yielding the purified AGP necessary for the investigation of Chapter 3.

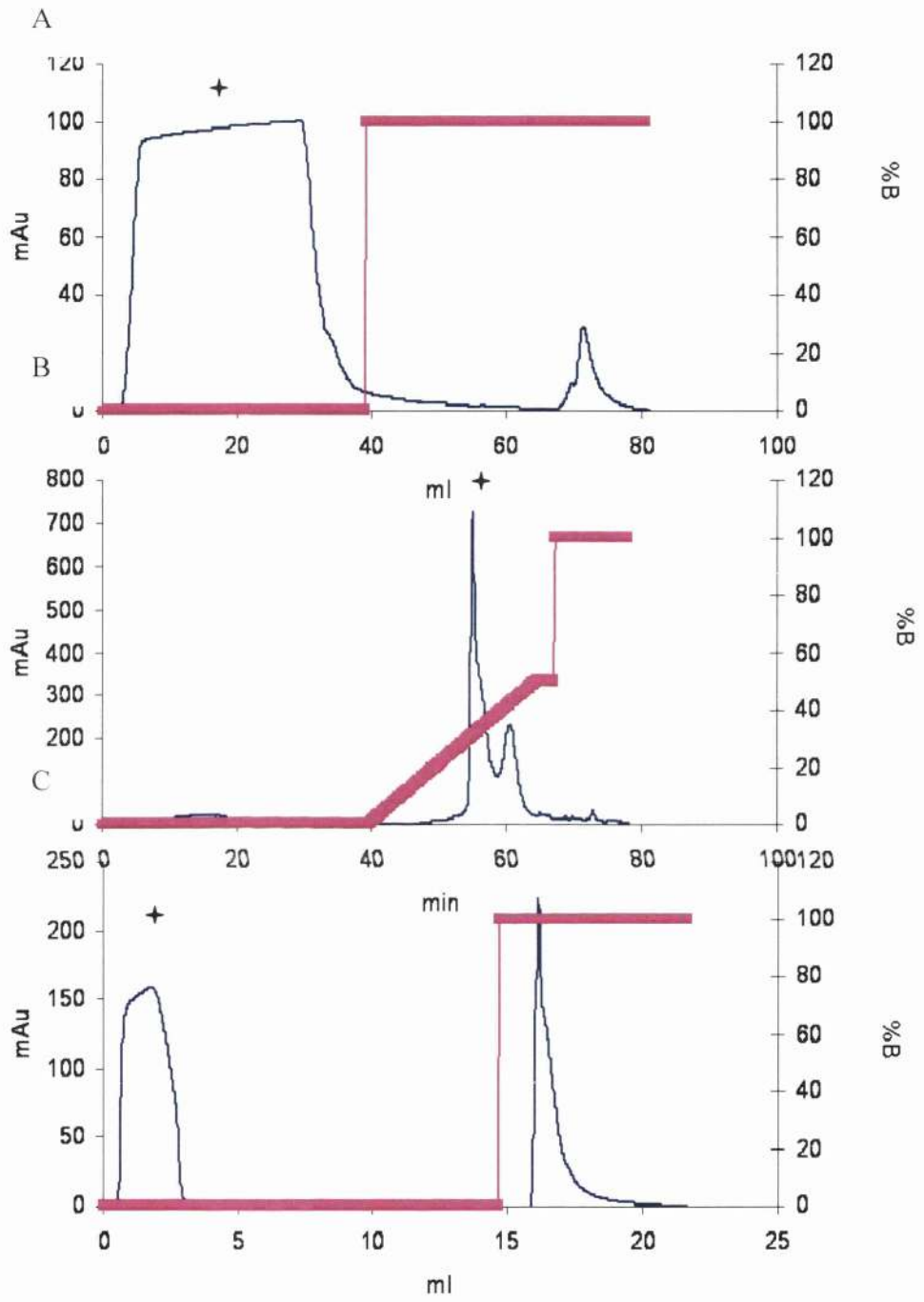

Figure 2.2 1 Chromatography for the isolation of feline AGP ascites from cat No 14 A) Cibacron blue affinity column B) MonoQ anion exchange column C) Mono S cation exchange column. mAU=milli absorbance unit. The data series shown in blue is the OD at 280nM and the data series in pink is the percentage of elution buffer running at that time.

✦ **Indicates the AGP containing peak**

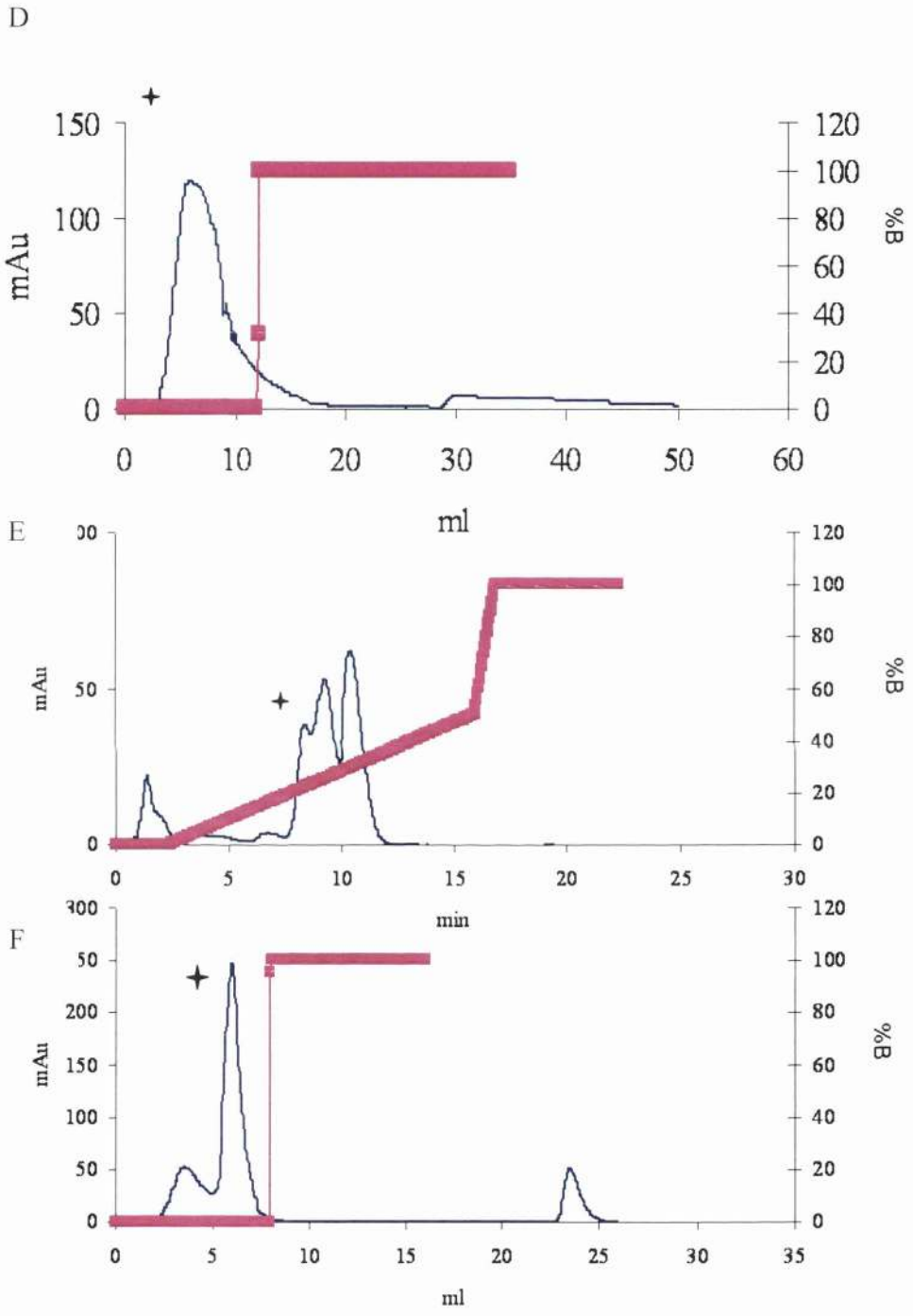

Figure 2..2 2 Chromatography for the isolation of feline AGP from ascites of cat No 9 D) Cibacron blue affinity column E) MonoQ anion exchange column F) Mono S cation exchange column. mAU=milli absorbance units

✦ Indicates the AGP containing peak

Step	AGP mg/ml	ml	AGP mg	% recovery
Start	0.45	10	4.5	100
PEG	0.13	28	3.64	80
Blue	0.15	14	2.1	47
Q	0.56	2.3	1.3	29
SP	3.9	0.3	1.2	26

Table 2.3 The recovery of AGP from each step of the isolation procedure. Data from purification of AGP ascites of cat 14.

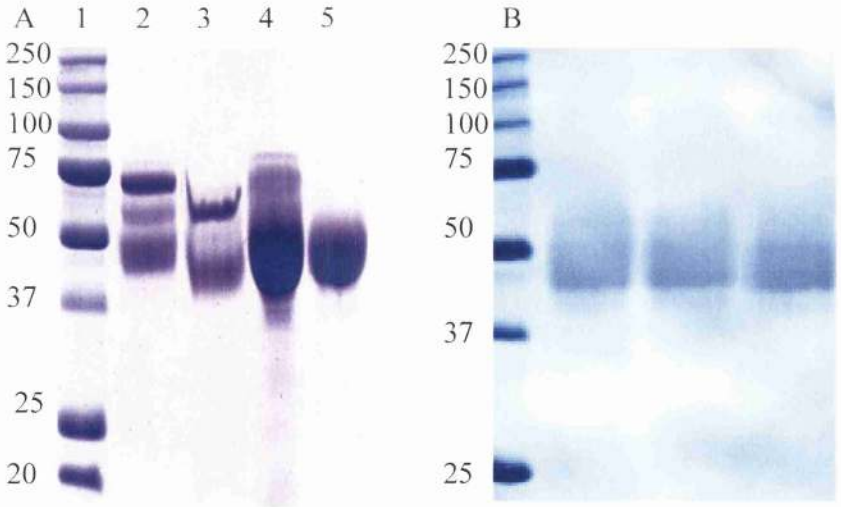

Figure 2.2 A) Shows the SDS-PAGE of the AGP containing fraction collected from each step of the isolation procedure from cat 14 Lane 1 Mwt markers, Lane 2 supernatant after 40% PEG precipitation Mwt 8000 lane 3 Throughput collected from Cibacron blue column lane 4 AGP fraction collected from anion exchange column. Lane 5 isolated AGP following cation exchange column. B) Shows the western blot of isolated feline AGP from cats 10, 11 and 14.

2.4 Discussion

In order to comprehensively analyse the monosaccharide and oligosaccharide composition of fAGP during FIP it was necessary to isolate the AGP from effusions.

Effusions from FIP confirmed patients (cats) had an average concentration of 1.65±0.28 g/L with a range of 3.5-0.25 g/L which was significantly higher compared with 0.18±0.05 g/L for the non-FIP samples which ranged from 0.1-0.32 g/L and which were combined in 4 pools to represent the normal population. Effusion in FIP is exudative in nature, so the protein content of the effusion should be representative of the blood levels. It was for this reason that effusions from cats with FIP instead of serum or plasma were used. It has been shown that effusions are a result of leakage from the blood and can be considered a good representation of the serum (Hartmann 2005).

In healthy cats the reference range for AGP concentration has been reported to be 0.1-0.48 g/L. Levels of AGP greater than 1.5 g/L in serum plasma or effusions are reported to be of greater value than the albumin:globulin ratio in distinguishing field cases of FIP and clinical similar diseases (Duthie et al. 1997). Measuring of AGP levels has now been introduced to the panel of tests involved in diagnosing FIP at the Feline virus laboratories of Glasgow University.

Pools of non-FIP samples had to be used to represent a control population instead of true normals as this was not a study with a convenient cohort of healthy control animals and only samples submitted to the diagnostic labs for testing were used. It was difficult to obtain samples from healthy cats and indeed it was deemed unethical to obtain samples from healthy cats in the necessary volume. The volume of sample required to

isolate sufficient AGP for analysis meant that using single samples from cats without an acute phase response was not possible.

Many techniques for isolation of human AGP from plasma have been reported however very little data is available regarding the isolation of fAGP. Previous techniques to isolate human AGP utilize acid precipitation followed by the application of chromatographic techniques using diethylaminoethyl and or carboxymethyl-cellulose columns to successfully purify the glycoprotein (Kishino & Miyazaki 1997). More recently Azzimonti *et al.*,(2003) reported a one step method using Cibacron blue F3 – GA and various buffers in an FPLC system to isolate AGP and separate AGP into its genetic variants through the change in pH (Azzimonti *et al.* 2003).

Although these previous investigators report higher yields of AGP with recovery than the technique used in the present study it was desirable to avoid the use of highly acidic buffers which could result in the destruction of the sialic acid residues present on the termini of the glycan chains and hence hinder complete analysis of the carbohydrate moiety. For this reason the previously described techniques were considered unsuitable for the purpose of this study.

Bence *et al.*, (2004) described a method recently used for the isolation of fAGP. This involved a harsh acidic precipitation with perchloric acid which was followed by an application to a mono Q column. This method was ruled out as a possible means of isolating fAGP for the purpose of this study as these harsh acidic conditions could result in removal of the terminal sialic acid residues (Bence *et al.*, 2004).

Ceciliani *et al.,* (2004) also reported a method for the isolation of fAGP which exploited the solubility and acidity of AGP. The method involved initial precipitation with pH4 which was followed by anion exchange chromatography and subsequently cation exchange chromatography, however no attempt was made to monitor the integrity of the glycan chains was reported (Ceciliani et al. 2004). In contrast the lowest pH in the present method was pH5 used in the mono S column and it was considered that the glycan structure would be minimally affected by this method.

For this research a modification of the low pressure chromatography technique developed by Smith *et al.,*(1994) was selected for the purification of AGP (Smith, Davies, & Hounsell 1994). The procedure reported by Smith *et al.,*(1994) has since been shown to purify AGP without denaturation, desialylation and without detrimental effect to the oligosaccharide moiety as measured by HPAEC-PAD (Elliott *et al.* 1995). Since the buffers used by Smith *et al.,* (1994) have not been altered it has been assumed that this method would also be without detrimental effects to the oligosaccharide chains of fAGP. The successful purification of AGP was confirmed using SDS-PAGE and western blotting.

Fractional precipitation using polyethylene glycol (PEG) 8000 was the first step in the isolation of fAGP and the use of this procedure for human plasma was reviewed by (Ingham 1990). Polyethylene glycol is a water soluble polymer which forms micelle-like structures around soluble proteins rendering them insoluble thus removing them from solution, while highly soluble proteins such as AGP remain in the supernatant. PEG does not tend to denature or interact with proteins even at high concentrations (Ingram1990) as it is chemically inert. Ingram (1990) reported that PEG polymers with

molecular weights greater than 6000 offered no advantage over smaller molecular weight preparations when used to precipitate insoluble constituents of human plasma. However it was found that in the case of feline effusions, PEG 8000 was more effective as an initial fractionation step than PEG 3350 as more contaminating proteins remained even with 50% of PEG 3350 compared to PEG 8000. All samples where therefore subjected to precipitation with PEG 8000 with the supernatant retained for further purification.

Albumin is the most abundant protein in plasma and is also highly soluble meaning it was not completely removed by PEG 8000 precipitation and therefore it had to be removed by chromatography. Hence the supernatant from the PEG precipitation was applied to a Cibacron blue column. Cibacron blue is a sulphonated polyaromatic dye that has been shown to remove up to 98% of human serum albumin (Travis *et al.* 1976). It has been reported that the Cibacron blue dye binds albumin through the bilirubin binding site although little or no binding to the bilirubin binding sites of rabbit, sheep, cow or horse serum albumins was reported. However interaction with the hydrophobic fatty acid anion binding domains of the serum albumins from these species has been described (Leatherbarrow & Dean 1980). As with these animal species Cibacron blue did not completely remove the feline albumin present in the samples but the majority of the albumin was retained by the column while all other proteins including AGP present in the supernatant from the PEG precipitation were eluted in the breakthrough peak. This fraction was therefore collected and further purified. Albumin constitutes the majority of the protein concentration in feline effusions and was obviously present in the supernatant from PEG treatment. For this reason the Cibacron blue column was a necessary step as in preliminary experiments it was found that the Mono Q and Mono S

columns were rendered ineffective for AGP purification in the presence of an abundance of albumin.

Q Sepharose is a strong anion exchanger with quaternary ammonium functional groups attached to a 6% cross linked agarose beads. Proteins are separated on the basis of charge with the quaternary ammonium functional groups with positive charge. Therefore the negative charge on the sialic acids of AGP is the cause of the binding of AGP to this column. The application of an increased sodium chloride concentration results in differential release of the proteins bound to the column on the basis of charge. This was found to divide the fraction eluted from the Cibacron blue column into three separate peaks, two where eluted in quick succession at approximately 20-25% of 1M NaCl and a third was eluted with 100% of 1M NaCl. AGP was found in the first peak eluted at approximately 20% of 1M NaCl, while the second peak which followed quickly afterward was found to contain predominantly albumin. The AGP containing fraction was assessed by SDS-PAGE and it was found to be still contaminated with trace amounts of albumin and at least 2 other unidentified proteins. Therefore a strong cation exchanger column (Hitrap SP) was employed to exploit the highly acidic nature of AGP in an effort to isolate fAGP from minor contaminants still present. An acetate buffer at pH 5 was used. Alpha-1-acid glycoprotein is known to have a pI below 5 so it did not bind to the column at the pH used while the other contaminants with higher pI bound to the column and were eluted using a 1M NaCl solution. Isolation of fAGP was confirmed via SDS-PAGE and western blotting (see Figure).

The combination of PEG precipitation and chromatographic technique used here have been shown to be successful due the lack of degradation to the glycan chains however

the procedure is long and requires a lot of steps and only 26% of intact AGP is recovered from the procedure. However when characterising the monosaccharide and oligosaccharide structures of glycoproteins as in the following chapter it is essential that the isolation procedure does not interfere with structure and composition of the carbohydrate moiety.

CHAPTER 3

3. Analysis of the glycan chains of feline AGP

3.1 Introduction

3.1.1 Carbohydrate influence on the biological functions of AGP

The exact functional role of AGP remains elusive, though many *in vitro* roles have been reported. The most documented of these is AGPs immunomodulatory properties. This activity predominantly resides in the glycan moiety (Bennett & Schmid 1980;Routledge 1989) and therefore glycosylation changes seen in pathological diseases may result in an alteration of the function of AGP.

It has been reported that the glycan chains attached to AGP are responsible for AGPs ability to suppress the proliferative response of lymphocytes (Bennett & Schmid 1980;Pos et al. 1990). Furthermore a study performed by Pos *et al.*,(1990) on anti CD3 stimulated lymphocytes concluded that Con A non reactive forms and sialylated variants of AGP exerted a stronger inhibitory effect on the proliferation of lymphocytes than Con A reactive forms (Pos et al. 1990;Pukhalsky et al. 1998).

The structure of the glycan chains attached to human AGP has been shown to influence AGPs ability to modulate cytokine production in vitro (Shiyan and Bovin 1997). This study described the synthesis of a pseudo AGP were the glycan chains were cleaved from AGP and attached to an inert polyacrylamide carrier. The pseudo AGP exerted the same effect on cytokine production as normal AGP which lead to the conclusion that the glycan chains and not the polypeptide backbone of AGP was responsible for this function (Shiyan & Bovin 1997). The effect of various AGP glycoforms separated on the basis of branching was then investigated revealing that all glycoforms of AGP can stimulate TNF production but that glycoforms carrying only tri and tetra antennary oligosaccharide chains suppressed the production of IL1 by monocytes while lower diantennary branched glycoforms stimulated IL1 production (Vasson *et al.* 1994).

AGP has been reported to inhibit human neutrophil aggregation and superoxide anion generation induced by a variety of stimuli. However it has been shown that the inhibition is dependant on the glycoforms present as inhibition was reversed when the terminal sialic acid and penultimate galactose residue were removed (Costello et al. 1979;Costello, Gewurz, & Siegel 1982)

Hyperfucosylation of AGP has been reported in rheumatoid arthritis (RA). The fucose residues are part of the sialyl Lewis X (SLeX) moiety. As described in chapter 1 SLeX is the minimum antigen required to bind E selectin and facilitate the movement of leukocytes across the capillary epithelium. Thus AGP carrying the tetrasaccharide antigen (SLeX) may influence the binding of blood cells to E-selectin. A study aimed at testing this hypothesis was conducted by Jorgensen *et al.*, (1998), which found that hyperfucosylated AGP isolated from plasma of patients suffering from RA inhibited

Sialyl Lewis X binding to E-selectin and could reduce the transport of WBC across the capillary epithelium (Jorgensen *et al.* 1998).

Friedman (1983) demonstrated the ability of AGP *in vitro* to inhibit the multiplication of the malarial parasite *Plasmodium Falciparum* by 80%. The activity depends on AGP blocking parasite-erythrocyte interaction and therefore parasite multiplication. The inhibitory activity of AGP was found to be reliant on high serum concentration and sialylation. Friedman has hypothesized that "by binding virus-cell interactions and attenuated viraemias, AGP could modulate infection by any virus that recognises sialic acid containing cell surface components, including influenza, measles and herpes" (Friedman 1983).

It is widely accepted that the structural heterogeneity of plasma glycoproteins is dependant on the pathological condition of the individual. Many studies have been carried out to examine at the glycan chains of different plasma glycoproteins in a variety of human diseases to investigate the possibility of their value as diagnostic or prognostic markers. To date there is limited information on the composition of the carbohydrate moiety of glycoproteins in animals.

3.1.2 Structural heterogeneity of serum glycoproteins in disease

3.1.2.1 Liver disease

One of the most widely studied diseases in which the structure of the carbohydrate moieties of glycoproteins is altered is liver disease (Table 3.1). Studies looking at glycoproteins in liver diseases have examined various proteins and numerous hepatic diseases including alcoholic liver disease (ALD). Transferrin, a glycoprotein involved in the transport of iron in the circulation, has been investigated as a marker of alcohol abuse, and is regularly monitored in diagnosis. Transferrin isolated from alcoholics has a high isoelectric point suggesting a lack of sialic acid residues. A reduction in the levels of galactose and N-acetylglucosamine has also been reported. This form of transferrin is termed carbohydrate deficient transferrin (Landberg et al. 1995;Turner 1992). The glycans of Hp have also been investigated in ALD and it was reported that glycan chains attached to Hp from patients with this disease express more fucose and N-acetylglucosamine residues than normal Hp (Mann *et al.* 1994).

Studies analysing the glycan chains of AGP in ALD revealed an increase in the proportion of tri-tetra-antennary oligosaccharides (Anderson et al. 2002;Jezequel et al. 1988). Increased branching of the oligosaccharide of AGP was also observed in patients with cirrhosis and hepatitis along with elevated levels of fucosylation of the glycoprotein in some hepatitis patients and all cirrhosis patients studied (Biou et al. 1989). A study of a range of liver diseases, including ALD, hepatitis B and C and cirrhosis was conducted by Anderson *et al.,* (2002). In cirrhosis patients, AGP had an

increased proportion of tri-tetra antennary chains compared to normal subjects. The most significant increase in tri and tetra antennary chains was observed in hepatitis C. Monosaccharide analysis of AGP from various liver diseases revealed the presence of N acetylgalactosamine on the AGP isolated from the plasma of patients suffering from Hepatitis C. The fucose content of AGP from all liver disease was increased however the greatest increase was seen in hepatitis B and C. In hepatocellular disease, decreased sialylation of AGP was observed by Bordas *et al.,* (1982) (Bordas *et al.* 1982)

Yamashita *et al.,* 1989 found that the glycans chains attached to transferrin were also modified in patients with hepatocellular cancer. It was reported that there was an increased proportion of tri and tetra antennary chains with the addition of fucose residues on both the peripheral N-acetylglucosamine and the pentasaccharide core. Sialic acid was also noted to be higher than that seen in the normal population (Yamashita *et al.* 1989).

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Disease	Protein	Altered glycosylation
Alcoholic liver disease	Transferrin Haptoglobin AGP	↓ sialic acid ↑ fucose ↑GlcNAc ↑ branching
Cirrhosis	AGP	↑ fucose ↑ branching
Hepatitis	AGP	↑ fucose ↑ branching
Hepatocellular cancer	Transferrin	↑ fucose ↑ branching ↑ sialic acid

Table 3.1 The glycosylation changes of serum proteins reported in specific liver diseases.

3.1.2.2 Cancer

Plasma glycoproteins expressed in patients with cancer also undergo disease specific changes in glycosylation. For example increased levels of fucose have been noted in Hp in ovarian and breast cancer patients, as well as in the α 1 proteinase inhibitor in ovarian cancer (Turner 1992).

An earlier study comparing fucose and sialic acid levels in AGP oligosaccharides chains in cancer revealed that fucose levels were raised more often in advanced disease than in restricted tumours whereas sialic acid concentrations were found to be similar in both groups. The report stated that the combined use of serum fucose and sialic acid levels may have value in monitoring patients with cancer: the sialic acid providing an index of the acute phase response and the fucose a measure of tumour spread (Turner et al. 1985). A study conducted by Hashimoto *et al.*, (2004) also recorded hyperfucosylated glycoforms of AGP agreeing with the finding of (Turner et al. 1985). As well as a

difference between the diseased group and the normal groups the recent study found that fucosylation was dependant on the clinical status of the host. It was concluded that patients with highly branched and fucosylated glycoforms of AGP had a poor prognosis (Hashimoto et al. 2004).

3.1.2.3 *Rheumatoid arthritis*

In RA the alterations in the oligosaccharide branching of AGP are indicative of both the severity and the progression of the disease (Pawlowski, Mackiewicz, & Mackiewicz 1989). AGP in patients with rheumatoid arthritis displayed a decrease in di-antennary glycoforms, however differences have been detected between the glycoform population seen in the early and late stages of the disease. Patients with early RA actually displayed an increase in di-antennary glycoforms, which persisted over the first year of the condition. As the disease progressed to the chronic stage an associated decrease in di-antennary and hence an increase in tri and tetra-antennary glycoforms was observed.

Clearly the heterogeneity of AGP due to variation in glycoforms is considerably altered in many disease states, especially inflammatory diseases and has also been observed in physiological changes such as pregnancy. These alterations in heterogeneity may serve as useful markers for disease progression. However it is useful to remember that factors such as concurrent infection can alter the expected glycosylation profiles of AGP in particular disease.

3.1.3 Structural heterogeneity of AGP in veterinary species

There have been few reports on glycosylation of AGP in animals but recently Ceciliani *et al.*, (2004) reported that AGP purified from cats diagnosed with FIP displayed decreased levels of sialic acid when compared to AGP from non-diseased cats. This study probed the carbohydrate chains of fAGP from cats with and without FIP using lectins to look at the branching, fucosylation and sialylation. A similar method was used by Pocaque *et al.* (2005) who reported an increased expression of sialic acid residues on fAGP from cats infected with Feline leukaemia virus. Studies using lectin binding gave valuable information on the glycosylation of AGP but was limited to specific motifs. In order to fully characterise the glycan residues of glycoproteins it is necessary to remove the glycan and chemically identify their composition.

3.1.4 Methods of glycomics

Oligosaccharide profiling of glycoproteins has become a common tool in both academic laboratories, as well as in the commercial environment. Most techniques employ enzymatic digestion to release the N-linked oligosaccharides; however, methods have been employed to investigate intact glycoproteins such as 1D and 2D gel electrophoresis (Schulenberg, Beechem, & Patton 2003) followed by lectin binding studies and lectin affinity chromatography.

There are various methods used for the detection of enzymatically released oligosaccharides. These include analysis of fluorescently labelled oligosaccharides by capillary electrophoresis (Lacunza *et al.* 2006) or high-performance liquid chromatography (HPLC), along with analyses of the unlabeled oligosaccharides by high-performance anion-exchange chromatography (HPAEC) and mass spectrometry.

The resolution of monosaccharides by HPAEC is achieved by exploiting the weakly acidic nature of the hydroxyl groups of the monosaccharides to form oxyanions in the basic environment created by a NaOH gradient. Individual monosaccharides can be separated due to slight differences in pKa arising from positioning of the individual hydroxyl groups. Following elution from the column the oxyanions can be detected using pulsed amperometric detection which allows for detection in the pmolar range.

HPAEC-PAD is also capable of determining differences in oligosaccharide profiles between individual samples (McGuire, Douglas, & Smith 1996). Oligosaccharide chains are primarily separated according to sialic acid content. Thereafter the resolution of peaks within each charge band was on the basis of many subtle differences such as fucosylation, branching type of linkage between sialic acid and the galactose residue.

The aim of this study was analysis the carbohydrate moieties of AGP isolated from cats with FIP and compare it to the AGP from cats without FIP in order to characterise the carbohydrate motif and analyse the alteration, if any within the oligosaccharide chains in disease. In order to examine the biological function of a glycoprotein it is necessary to fully understand the carbohydrate moiety and changes that arise in disease.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Samples

Sufficient isolated AGP from the serum or ascetic fluid of Cats 2,5,6,7,8,9,10,13,16,17,18 (as described in 2.2.1) and NP1 and NP2 was available for monosaccharide composition analysis and from cats 3,4,6,7,8,10,12,15,18 and NP3 and NP4 for oligosaccharide analysis. Sample 18 was from a non-FIP cat all other individuals had FIP.

3.2.1.2 Monosaccharide analysis

HPLC grade trifluoroacetic acid and hydrochloric acid were purchased from Pierce (Illinois, USA). Dowex – 50 W hydrogen form strongly acidic cation exchanger (4% cross-linked, dry mesh 200-400) was obtained from Sigma (Poole UK).

Galactose, mannose, 2 deoxygalactosamine, glucosamine, mannose, were supplied by Sigma (Poole, UK). 50% sodium hydroxide (NaOH) was purchased from BDH (Poole, UK) and the HPLC grade water was obtained from Rathburn (Walkerburn, UK).

3.2.1.3 Oligosaccharide Analysis

PNGase F (EC number 3.5.1.52), 10x G7 buffer, 10% (v/v) NP-40 were purchased as a kit from New England Biolabs (Herts, UK). HPLC grade water was obtained from Rathburn (Walkerburn, UK).

3.2.2 Methods

3.2.2.1 Isolation of AGP

The monosaccharide and oligosaccharide analysis used samples of fAGP isolated from serum or ascetic fluid as described in section 2.2.

3.2.2.2 Monosaccharide Analysis

Monosaccharide analysis was performed on Dionex DX-600 High pH anion exchange chromatography system (Dionex, California, USA) using pulsed amperometric detection. This system utilizes a GP50 gradient pump and an ED40 electrochemical detector controlled by a Dell desktop computer running Peaknet software.

After the last steps of the AGP purification procedure, 66µg of each sample was desalted in centricon centrifugal tubes, dried under vacuum and then resuspended in a solution consisting of 50µls of 4M HCl and 100µls of 2M TFA. Samples were then placed in a reacti vial and heated at 100°C for 4 hours. The hydrolysed sample was then

passed through Dowex 50 H^+ cation-exchange column and eluted in a 2 ml fraction of HPLC grade water. This fraction was then dried under vacuum to dryness and resuspended in 20µl of HPLC grade water ready for analysis on the Dionex HPLC system.

Monosaccharide analysis was carried out by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Separation was carried out on a Carbopac-100 ™ column and guard column (250x4mm and 50x4mm respectively) using an isocratic gradient of 30mM NaOH over 35minutes. The column was then regenerated using 0.5M NaOH for 10mins before equilibration with 30mM NaOH. Monosaccharide concentration was determined using a calibration curve based on a standard monomix which consisted of 5 mg/ml mannose, fucose, galactose, N-acetyl glucosamine and 2-deoxygalactose (Sigma, Poole, UK).

Identification of individual monosaccharides peaks was achieved using an internal standard, 2-deoxy-D-galactose as although the retention time of particular monosaccharides may vary between runs the position of the peak is constant relative to that of the other peaks including the internal standard (IS). The identity of unknown peaks is calculated by dividing the retention time given for the unknown peak with the retention time of the IS which can then be compared with the ratios for monosaccharides in a standard mix.

The amount of each monosaccharide present in a sample mixture can also be calculated directly from the chromatogram, due to a linear relationship between the area under the

peak and the quantity of monosaccharide. The area under the peak is then compared to previously constructed standard curves (Figure 3.3).

3.2.2.3 Oligosaccharide analysis

Oligosaccharide analysis was also carried out on a Dionex DX-500 High pH anion exchange chromatography system using pulsed amperometric detection, which utilizes a GP40 gradient pump and an ED 40 electrochemical detector controlled by a desktop computer running Peaknet software.

After desalting 50µg of AGP was dried under vacuum and reconstituted in 100µl of HPLC grade water and incubated at 100°C for 30-60 mins. Thereafter 10 µls of 10% (v/v) NP 40, 10 µls of sodium phosphate buffer (supplied with enzyme) and 100 units of PNGase F were added and the mixture incubated at 37°C overnight. The cleaved oligosaccharides were separated from the protein by ethanol precipitation, in which 500µl of ice cold ethanol was added to the mixture and centrifuged at 3000g for 10 mins, this was then repeated and the supernatant containing the oligosaccharides was dried under vacuum and reconstituted in 20µl of HPLC grade water.

Oligosaccharide analysis was then performed on the HPAEC-PAD and the separation was carried on a carbopac-100 column and guard column. Solvents were degassed using helium. The column was equilibrated with a mixture of 10% of 1M NaOH (solvent A), 5% of 1M acetic acid (solvent B), 85% of HPLC grade water (solvent C). These conditions were continued for 10 minutes after sample injection and there after a

linear gradient of 10% A, 5%B, 85%C to 10%A, 20%B, 70%C was achieved after 40 mins and maintained for a further 5 minutes before the column was regenerated with 50% A 50% C for 10 minutes (Figure 3.1).

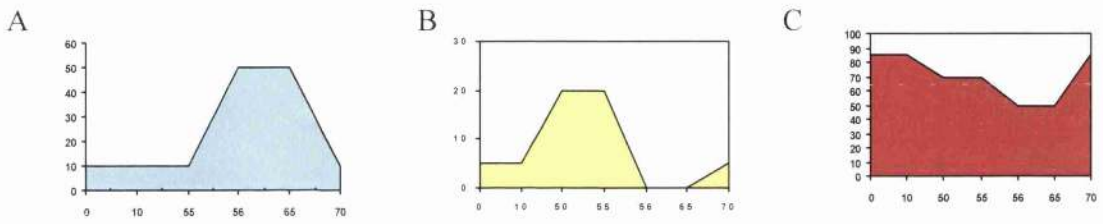


Figure 3.1 The buffers and percentage at each stage of the oligosaccharides separation. A= 1M NaOH. B=1M Acetic acid. C= HPLC grade water.

3.3 Results

3.3.1 Monosaccharide analysis

Prior to HPAEC aliquots of 66μg of isolated fAGP were hydrolysed using TFA and HCL to degrade the glycan chains into their component monosaccharides which were then analysed by HPAEC-PAD. A typical trace obtained by the separation of a standard monosaccharide mixture can be seen in Figure 3.2.

The monosaccharide composition of fAGP isolated from 11 individual cats and two pools was determined by measuring the area under each of the peaks in comparison to a previously established standard curve for each individual monosaccharide Figure 3.3 shows the standard curves used to calculate the number of moles of each monosaccharide present. The trace of a normal pool (NP2) and examples of 3 cats (Nos 5, 7 and 9) are shown in Figure 3.4, identification of peaks in these samples are in Table 3.2. The quantity of each monosaccharide from the examined samples is expressed as moles of monosaccharide per moles of AGP hydrolysed assuming a molecular weight of 47kDa for AGP is shown in Table 3.3. Due to a shortage of sample it was only possible to run each sample once.

Table 3.3 shows the individual cat monosaccharide composition from this we see that cats 5 and 9 had a detectable fucose concentration (the chromatograms for these cats can be seen in Figure 3.3) whereas the other eight cats which were included in the FIP group

had no detectable fucose residues. Cat number 18 and the two non-FIP pools which made up the non-FIP group had no detectable fucose present within their oligosaccharide chains. All cats in the included in the FIP group had increased numbers of GlcNAc and Gal residues with means of 18.8±1 mol/molAGP and 17.8+1.4 mol/molAGP respectively compared with a means of 7+2 mol/molAGP and 5.8+1.4 mol/molAGP in the non-FIP group respectively (Table 3.4). Two from ten cats in the FIP group (cats 6 and 9) had very low levels of mannose residues with 3 and 1 mol/molAGP respectively.

Using a Mann-Whitney statistical test it was calculated that cats with FIP had statistically significantly higher levels of GlcNAc and Gal residues present within the glycan chains attached to AGP compared to the non-FIP group ($p<0.05$ for both). No statistically significant differences were found for the mannose residues $p=0.13$ or for the fucose $p=0.67$.

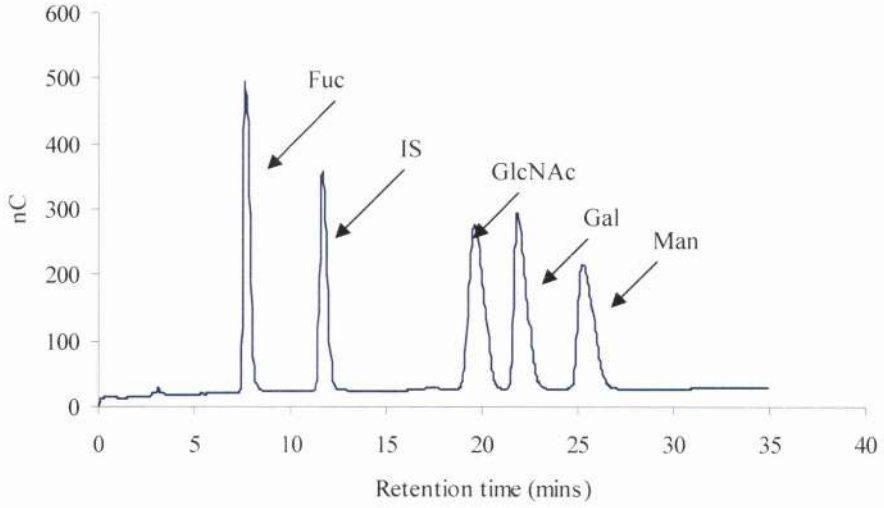
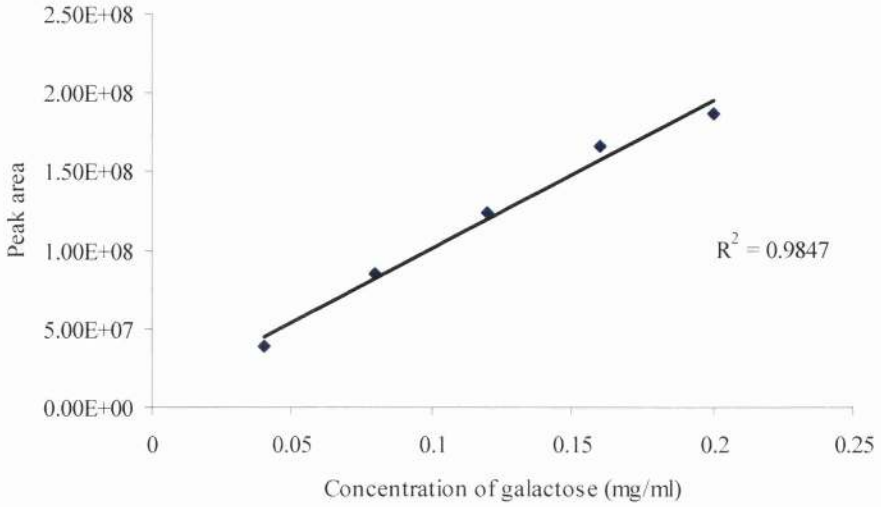


Figure 3.2 A representative HPAEC-PAD chromatogram of a standard monosaccharide mixture. Fuc=Fucose IS=Internal standard GlcNAc = N acetyl glucosamine Gal = Galactose Man = Mannose.

Cat No	Peak No	Monosaccharide
NP2	1	Solvent front
	2	Internal standard
	3	N-acetylglucosamine
	4	Galactose
	5	Glucose
	6	Mannose
Cat 7	1	Solvent front
	2	Internal standard
	3	N-acetylglucosamine
	4	Galactose
	5	Mannose
Cat 5	1	Solvent front
	2	Fucose
	3	Internal standard
	4	N-acetylglucosamine
	5	Galactose
	6	Mannose
Cat 9	1	Solvent front
	2	Fucose
	3	Internal standard
	4	N-acetylglucosamine
	5	Galactose
	6	Mannose

Table 3.2 Identification of the peaks present in the chromatograms shown in Figure 3.3



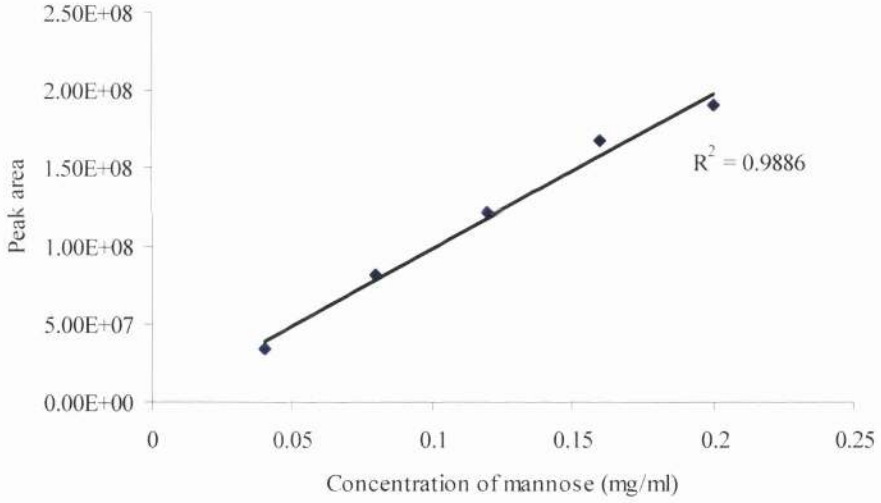
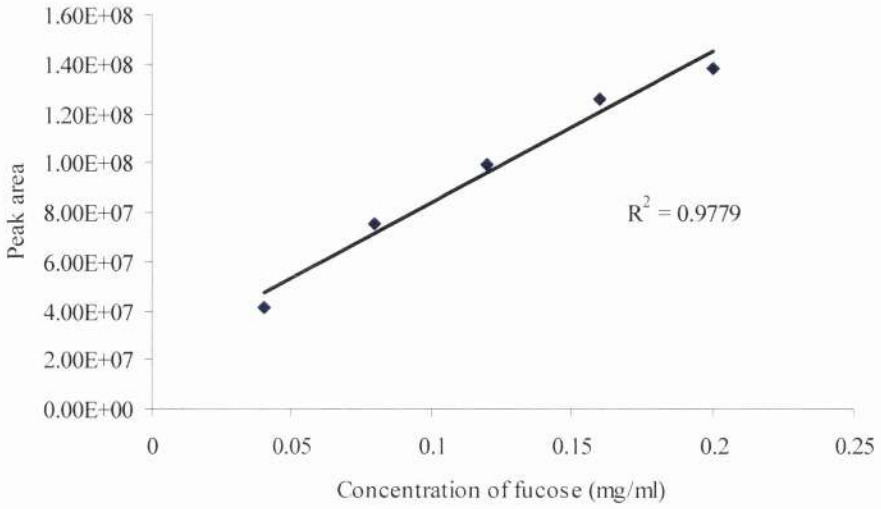


Figure 3.3 1 Standard curves for galactose and mannose respectively which were used to calculate the concentration of monosacchardie residues in each individual sample.



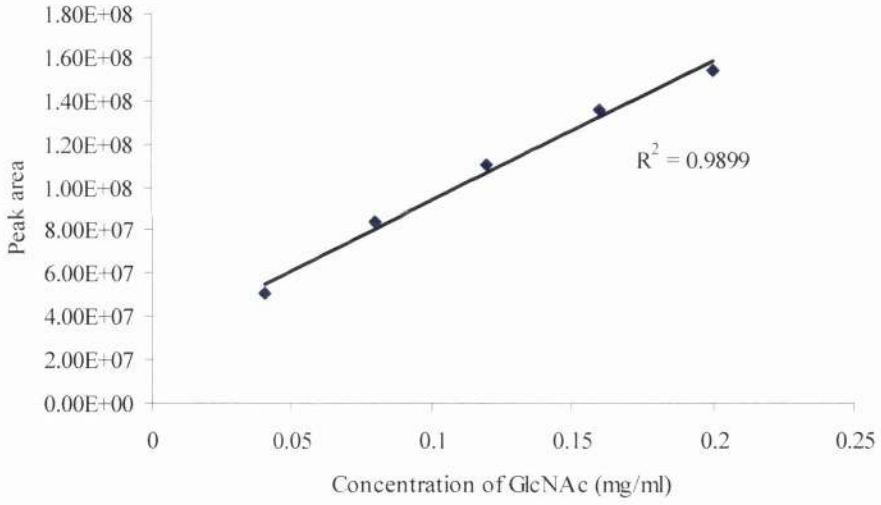


Figure 3.3 2 Standard curves for fucose and N-acetyl glucosamine respectively that were used to calculate the concentration of the monosaccharides in the samples.

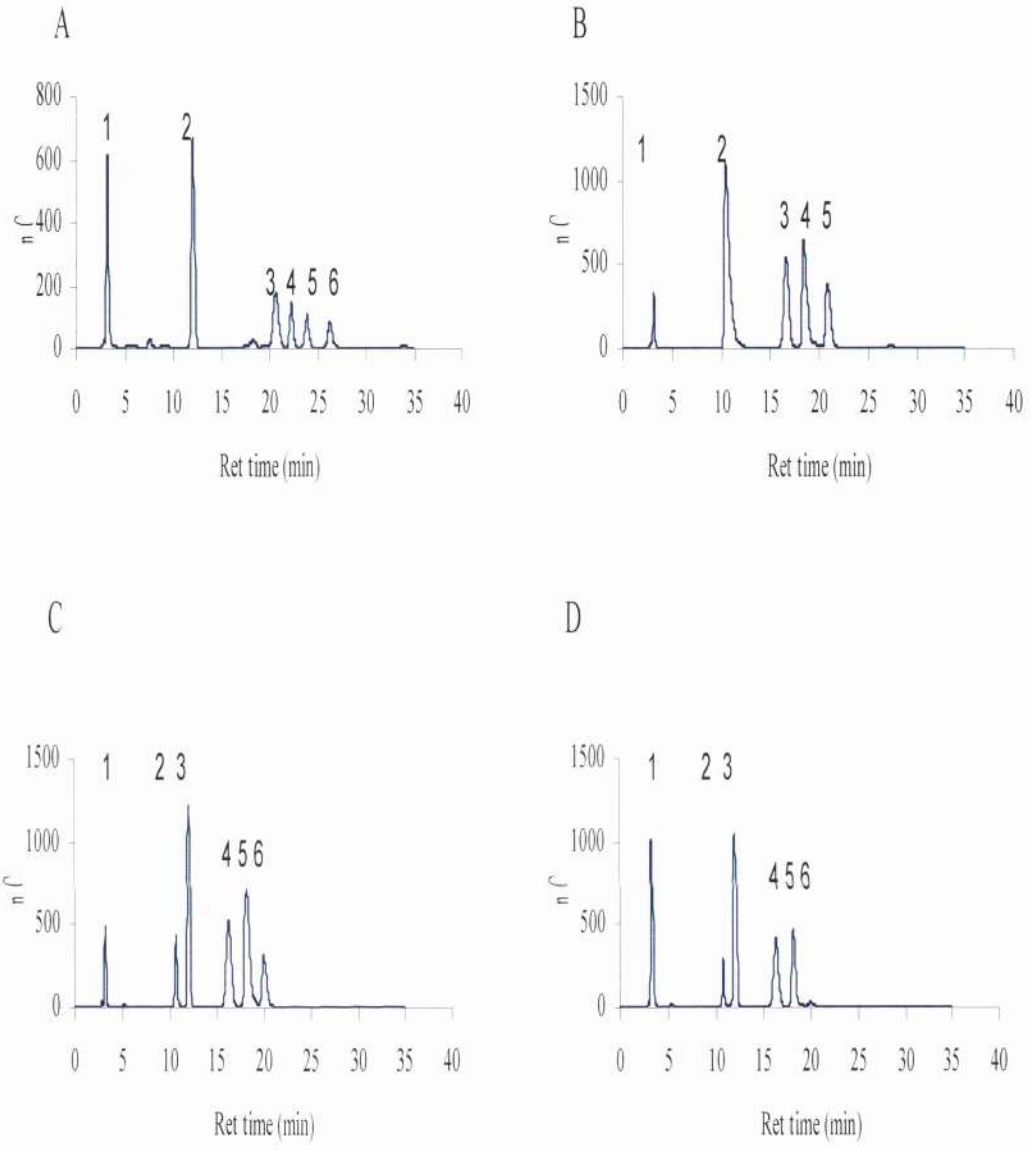


Figure 3.4 HPAEC-PAD chromatograms for A) NP2 B) cat 7 C) cat 5 D) cat 9

Cat No	FIP or Non FIP	AGP Conc	Mol/mol AGP			
		mg/ml	Fucose	GlcNAc	Gal	Man
5	FIP	NA	0.35	22	27	11
6	FIP	NA	0	15	13	3
9	FIP	2	0.16	16	12	1
7	FIP	2.2	0	23	22	13
8	FIP	1.7	0	22	16	10
2	FIP	0.6	0	17	17	14
10	FIP	2.86	0	19	20	14
17	FIP	NA	0	14	16	14
13	FIP	1.1	0	17	16	11
16	FIP	NA	0	23	19	14
18	Non	0.25	0	5	6	6
NP1	Non	0.1±0	0	11	8	5
NP2	Non	0.16±0.02	0	5	3	3

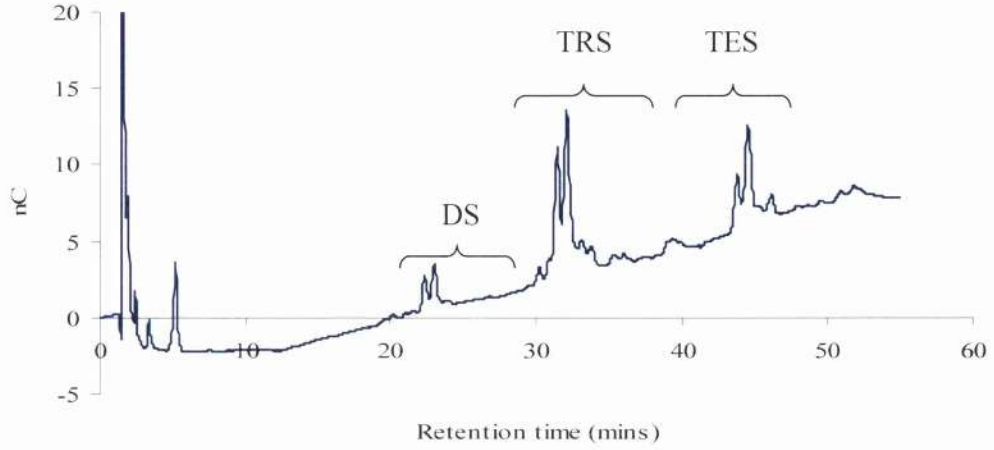
Table 3.3 The calculated concentrations of each monosaccharide in individual samples

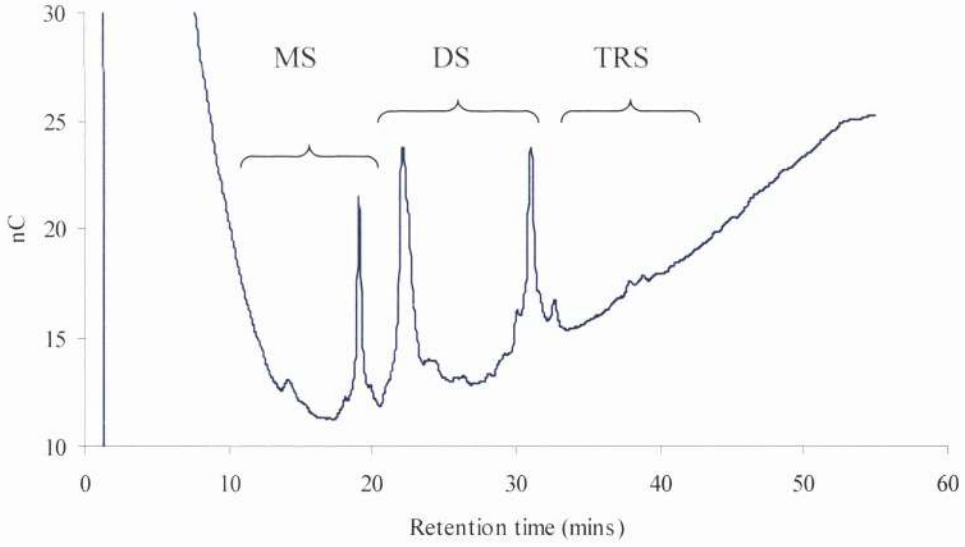
	mol/mol AGP			
	Fucose	GlcNAc	Gal	Man
FIP	0.051±0.04	18.8±1	17.8±1.4	10.5±1.5
Non-FIP n=3	0±0	7±2	5.8±1.4	4.7±0.89

Table 3.4 The mean ± standard error of each monosaccharide for disease groups.

3.3.2 Oligosaccharide analysis

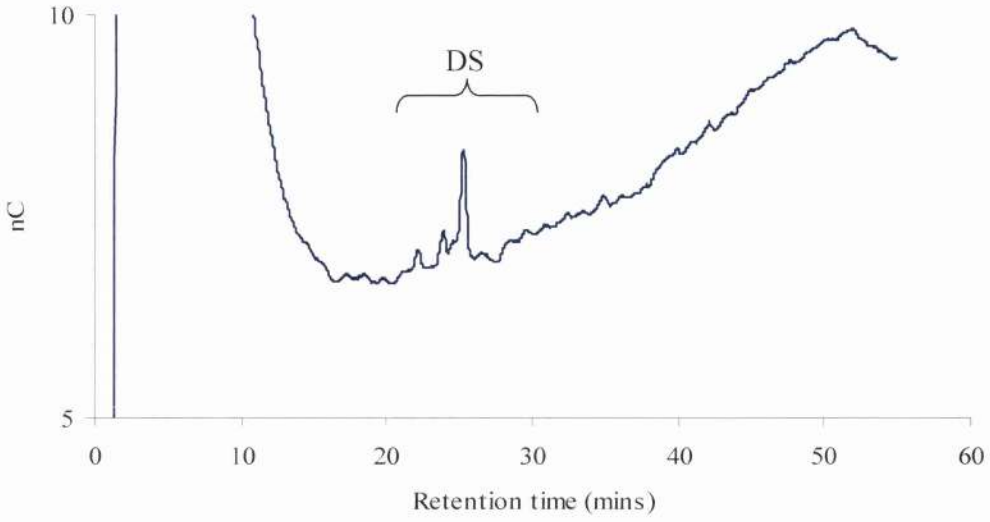
The HPAEC-PAD profile of oligosaccharides from bovine fetuin is shown in Figure 3.5.1 to show the elution times of the standard sialylated glycoforms. Following PNGaseF action on the fAGP it was found that AGP from pools 3 + 4 carried solely disialylated glycan chains (Figure 3.5.2). The oligosaccharide profiles for each individual cat within the FIP group (cats 6, 7, 10,11, 12) are shown in Figures 3.5.3 - 3.5.5 all have two major peaks, one in the disialylated (retention time 20-30 mins) range and one in the trisialylated (retention time 30-40mins) range which are eluted 8-9 mins apart for all samples. Cat 18 which was also used in the non-FIP group however had a different oligosaccharide profile to both the pools and the FIP group in that three major peaks where detected, one in monosialylated range, one in disialylated and one in the trisialylated range.

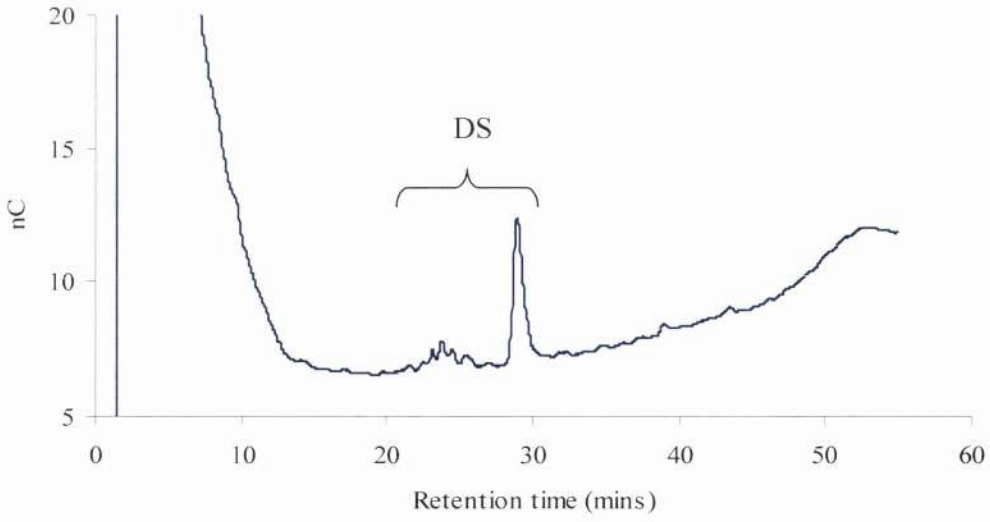




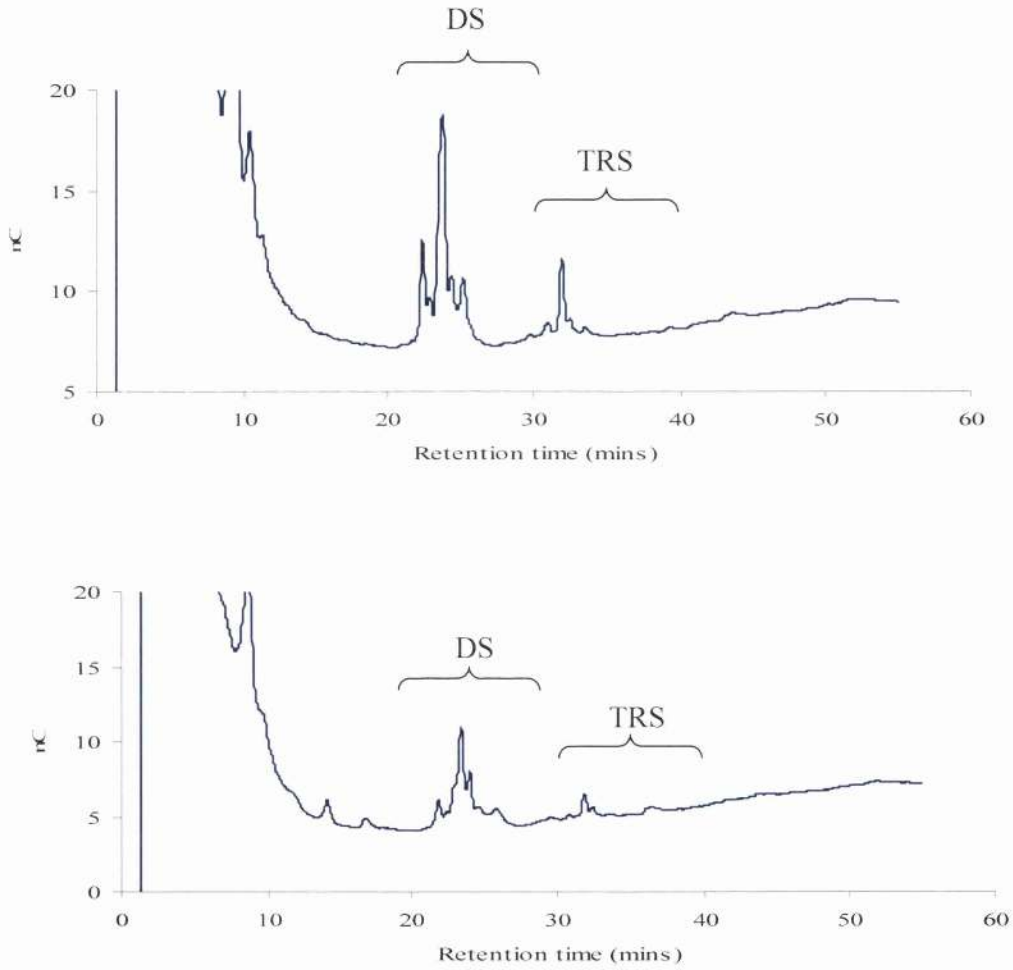
3.5 1 HPAEC-PAD chromatogram of the library obtained for bovine feutin and cat 18 respectively.

MS= monosialylated DS=disialylated TRS=trisialylated TES=tetrasialylated.



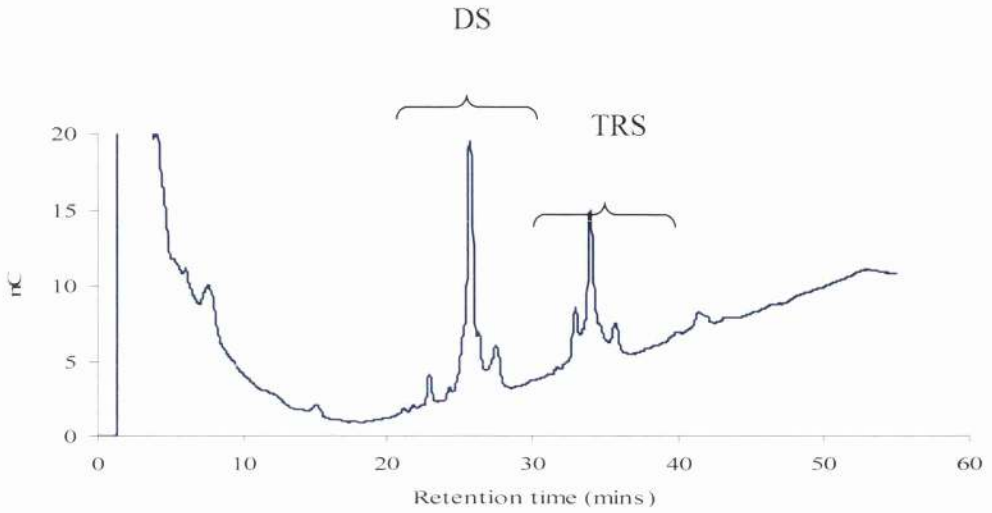


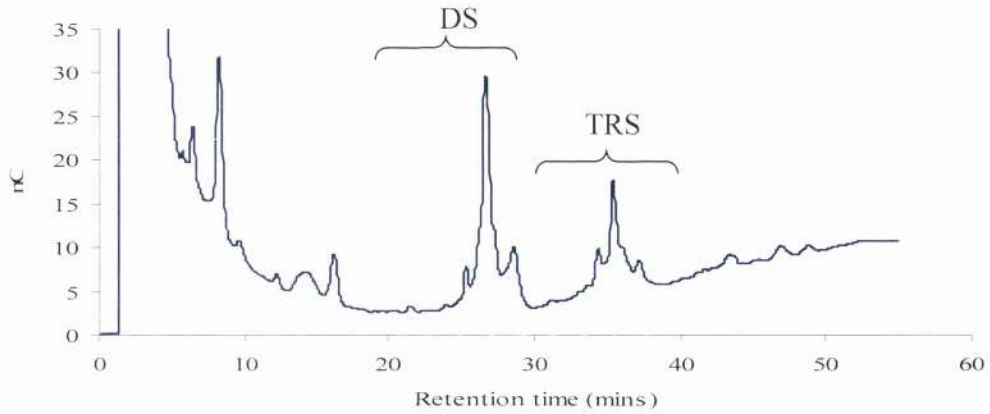
3.5 2 HPEAC-PAD chromatogram of oligosaccharide profile for non-FIP pools 3 and 4. MS= monosialylated DS=disialylated TRS=trisialylated TES=tetrasialylated



3.5 3 HPAEC-PAD chromatogram for cats 10 and 11 respectively.

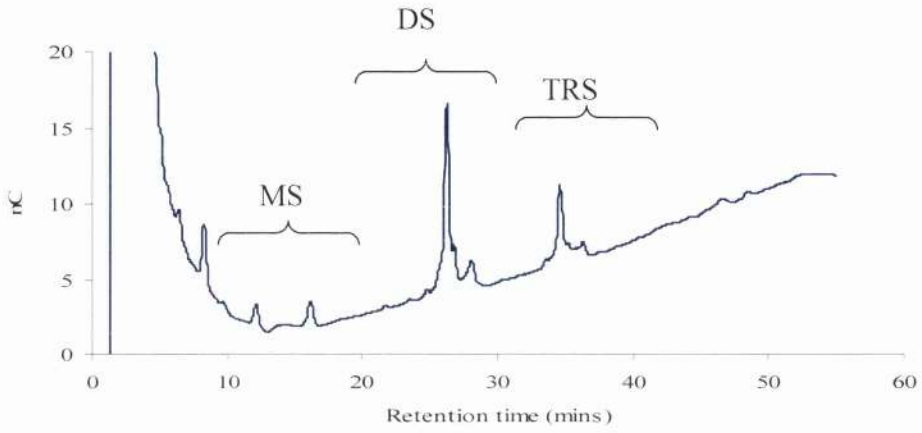
MS= monosialylated DS=disialylated TRS=trisialylated TES=tetrasialylated





3.5 4 Shows HPAEC-PAD chromatogram of oligosaccharide profile for cats 7 and 12 respectively

MS= monosialylated DS=disialylated TRS=trisialylated TES=tetrasialylated



3.5 5 HPEAC-PAD chromatogram of oligosaccharide profile for cat 6

MS= monosialylated DS=disialylated TRS=trisialylated TES=tetrasialylated

3.4 Discussion

The oligosaccharide chains covalently attached to glycoproteins are essential for a number of important functions including cell-cell interactions and a role in non-specific immunity. Therefore in order to fully elucidate the structure function relationship within a glycoprotein it is necessary to gain a complete understanding of their glycosylation pattern which should include analysis of the monosaccharide composition of the oligosaccharide chains as the presence or absence of terminal monosaccharides such as fucose and sialic acids can have marked effect on the physio-chemical properties of the glycoprotein and could changes its the function and antigenic properties. Compositional analysis of the monosaccharide composition of AGP using HPAEC is only achieved after prior acid hydrolysis of the intact glycoprotein. Incubation with 2M TFA and 4M HCl for 4 hrs at 100° attains optimal conditions for cleavage of all the intrachain linkages in oligosaccharides and with the exception of Neu 5 Ac which is destroyed (McGuire, Stewart, & Smith 1999). TFA releases all neutral monosaccharides including fucose, galactose and mannose but poorly cleaves the amino sugars due to their high acid resistance. In particular it fails to hydrolyse the bond between the asparagines residue and the innermost GlcNAc residue in N-linked glycoproteins (Fan *et al.* 1994). The solution to this problem is to use hydrochloric acid which easily cleaves the bonds with the amino sugars, in combination with TFA. It should be noted however using HCl can result in decomposition of neutral monosaccharides. TFA hydrolysis has also been shown to give a low yield in mannose using this procedure (Hardy & Townsend 1988;McGuire, Stewart, & Smith 1999).

Post hydrolysis, the hydrolysate is applied to an acidic cation exchange resin (Dowex 50) in order to prevent interference of amino acids, peptides or salt with the separation of the monosaccharides (Hardy & Townsend 1988).

HPAEC allows for highly specific resolution of monosaccharides including those that are anomeric or have positional isomers with a successful resolution of galactose, mannose and glucose despite the similarities in size and number of hydroxyl groups (Lee 1996). Hydroxyl groups act as weak acids in highly basic conditions therefore monosaccharides which are polyhydric compounds will also be weakly acidic with a pKa of between 12 and 14. This fact is exploited in HPAEC by eluting the monosaccharides with strong, isocratic alkaline gradient which causes the monosaccharides to become negatively charged through the formation of oxyanions.

The monosaccharides are detected as oxyanions and it has been found that the elution order of the compounds correlates well with the pKa values (Hardy & Townsend 1994a). The difference in pKa value for each monosaccharide is explained through differences in the positions of the hydroxyl groups. It is the subtly different pKa values of the oxyanions and their different interactions with the strong anion exchange groups that results in the monosaccharides having different elution times. While most of the acidity of monosaccharides is due to the anomeric hydroxyl group, the final pKa is dependant on a number of other factors including the acidity of other hydroxyl groups which is not uniform. The 2-OH is the most acidic with the other groups in the order 6-OH >3-OH > 4-OH (Lee 1990).

HPAEC-PAD has been successfully applied to the study of glycoproteins including hAGP (Anderson et al. 2002;Elliott et al. 1997). Therefore it has been employed in this investigation to determine the monosaccharide composition of fAGP using the previously described method of Smith *et al.*, (1994).

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Previous studies have revealed that fucose content on AGP displays the greatest variability in disease conditions (Biou et al. 1987;Listinsky, Siegal, & Listinsky 1998). It is generally accepted that fucose is an inflammatory marker and a possible measure of tumour growth (Thompson & Turner 1987;Turner et al. 1985)(Turner *et al*, 1985; Thomson *et al*, 1987). However fAGP from only 2 (cats 5 +8) of the cats with FIP contained fucose residues. Possible explanations for the AGP from these two cats expressing fucose residues may be either due to a more advanced stage of the disease or the presence of an underlying disease that was masked by the clinical signs of the FIP.

Theoretically each complex type diantennary chain N-linked chain would give 4molGlcNAc, 3molMan and 2molGal while all tetra-antennary chains would give 6 molGlcNAc, 3molMan and 4molGal. Therefore it is expected that if only complex type N –linked glycans chains are attached to fAGP then a 3:2 ratio of GlcNAc:Gal residue would be obtained when diantennary and tetraantennary chains are present. However analysis of monosaccharides from fAGP did not follow the theoretical expected pattern as four of the cats were found to have more detectable galactose residues resides per mol of AGP than GlcNAc while one sample had equal numbers of GlcNAc and galactose.

N acetyl glucosamine is found within the peripheral branches and the pentasaccharide core of glycan chains. Therefore the increase in N acetyl glucosamine such as was

found for all 10 of the FIP compared to the three non-FIP could be due to an increase in branching or the addition of an another chain on the polypeptide backbone.

Galactose is only found in the outer branches of AGP oligosaccharide chains and never in the core region of N-linked glycan chains. Thus in a manner similar to N acetyl glucosamine, if AGP galactose levels were increased, this would suggest there was a corresponding increase in the proportion of tri and tetra antennary oligosaccharides present on AGP. However the addition of another chain to the polypeptide would also result in increased Gal residues.

Unlike GlcNAc and Gal, mannose is not found in the peripheral branches of complex type N-linked chains, so any change found in the expression of mannose would be due to the addition or removal of chains from the polypeptide backbone.

Since AGP from FIP had significantly higher expression of GlcNAc and galactose residues accompanied by no significant increase in mannose expression, it would seem more likely that that the increase seen in the GlcNAc and Gal residues is due to increased branching and not the addition of glycan chains to the polypeptide backbone. However these results must be interpreted with caution because, as stated previously studies have shown that hydrolysis method used to remove the monosaccharide residues from the protein, may degrade some of the mannose residues (Hardy & Townsend 1988;Hardy 1989;McGuire, Stewart, & Smith 1999). Though not statistically different from normal pools the mannose was higher in 9 of 11 samples from FIP cats and analysis of a greater sample of the population might have yielded significant results.

HPAEC-PAD can also be successfully applied to the study of intact oligosaccharides as well as the investigation of constituent monosaccharides. HPAEC coupled with PAD can efficiently resolve positional isomers of oligosaccharides, including those differing in just a single linkage, in the pmolar range with no prior need for derivatization (Hardy & Townsend 1988). The principal of separation of the intact oligosaccharides is identical to that described for monosaccharide analysis. The charge on the oligosaccharide chains varies not only with the composition of the monosaccharide constituents but also the linkages they form. The involvement of certain hydroxyl groups in the formation of glycosidic bonds between the monosaccharides can alter the overall charge of the oligosaccharide such that they can be resolved by HPEAC-PAD. Thus oligosaccharides with identical monosaccharide content but with a difference in one linkage can be successfully separated.

The use of HPEAC-PAD in the analysis of glycan chains is not quantitative but rather allows a qualitative determination of the pattern of N-linked oligosaccharides in comparison to a library sample of bovine fetuin (Smith *et al*, 1997).

N-linked glycan chains were released from the polypeptide backbone of isolated fAGP using peptide-N4-(N-acetyl β-D-glucosaminyl) asparagines amidase (PNGaseF EC number 3.5.1.52), which is an amidase that catalyses the hydrolysis of the glycosidic bond between the asparagine and the innermost GlcNAc residue of N-linked glycan chains (Davies *et al.* 1993). The cleavage results in the formation of a glycosylamine as the amino group of the asparagine residue remains attached to the oligosaccharide (Hardy & Townsend 1994).

The initial separation of the oligosaccharide, and thus the major determinant of their elution times, is the formal negative charge on the oligosaccharide principally determined by the number of terminal sialic acid residues present on the chain. The greater the number of sialic acid residues the stronger the interaction with the resin. An acetate gradient is required to displace the sialylated oligosaccharide chain. The greater the number of sialic acid residues present on the oligosaccharide the greater the interaction with the resin and the higher the concentration of acetate required to displace the oligosaccharide chain. For example trisialylated glycans are retained on the column longer than disialylated glycans. Within each charge band with the same charge further separation is on the basis of size, intermonomeric linkages and fucosylation (McGuire, Douglas, & Smith 1996).

Non-FIP samples were analysed in order to determine any changes which occurrence of FIP induces in the oligosaccharide pattern as determined by HPAEC-PAD. It was shown that AGP isolated from 2 pools of non-FIP serum had only disialylated oligosaccharides chains attached in contrast although each individual cat in the FIP group had a unique oligosaccharide profile all had 2 major peaks one in the disialylated range and one in the trisialylated which were eluted 8-9 mins apart. Overall the extent of structural heterogeneity within the glycan chains of fAGP from FIP patients is greater than that observed within the glycan chains of fAGP from non-FIP patients.

In summary, it was found that the presence of FIP had a profound effect on the carbohydrate moiety of fAGP, with increased expression of Gal, GlcNAc and trisialylated glycan chains. If the increased expression of Gal and GlcNAc residues is in fact due to increased branching of the glycan chains this may have a significant effect

on the functions of fAGP. Although very little is actually known on the function of fAGP if it has similar functions to hAGP then increased branching of the glycan chains of fAGP may result in an increased ability to inhibit proliferation of lymphocytes as, it has been reported that highly branched glycoforms of hAGP have a greater inhibitory effect on this function of AGP (Pos et al. 1990).

CHAPTER 4

4. Alpha-1-acid glycoprotein and bovine mastitis

4.1 Introduction

Bovine mastitis arises through intramammary infection with many different pathogens and remains the principal economical problem for dairy farmers worldwide. Many studies have been carried out in an effort to identify a diagnostic marker for mastitis that can be used to positively identify mastitis quickly and reliably (Conner et al. 1986;Conner et al. 1988;Eckersall et al. 2001;Eckersall et al. 2006;Gronlund et al. 2003;Martin et al. 2002;Mottram et al. 2007;Paape et al. 2002). At present the most effective measurement is the somatic cell count (SCC) which is the measure of somatic cells per ml of milk. Recently studies have targeted the acute phase proteins as possible diagnostic markers and in these studies it has been found that serum alpha -1 acid glycoprotein behaves as in humans as a moderate acute phase protein with serum levels rising 2-5 fold in mastitis (Conner et al. 1988). As has been described in previous chapters AGP undergoes remarkable structural changes with in the glycan moiety in the presence of specific diseases in humans and cats. These findings have enormous implications on the functioning of AGP and offer the potential of diagnostic and prognostic markers for diseases such as rheumatoid arthritis, cancer and feline

infectious peritonitis (Anderson et al. 2002;Ceciliani et al. 2004;Hashimoto et al. 2004;Pawlowski, Mackiewicz, & Mackiewicz 1989). It is possible that similar phenomenon occurs with serum AGP in cows with mastitis.

4.1.1 Mastitis

Mastitis simply means inflammation of the udder. Most farmers associate mastitis with an inflamed udder quarter together with a change in the appearance of the milk. Symptoms usually include redness, tenderness, heat and clumping of the milk. These changes are due to the effect of the cow's inflammatory response to infection. However mastitis can also occur in a subclinical form. This means that although infection is present in the udder there are no visible external changes to indicate its presence and this can persist for days leading to a large loss of production. Over 200 different organisms have been reported to cause bovine mastitis and there are substantial differences in epidemiological and predisposing factors with different types of infection (Dodd 1970). 'Contagious mastitis' and environmental mastitis' are labels used to classify the epidemiology of pathogens that cause intramammary infections in dairy cows (Bramley & Dodd 1984;Smith & Hogan 1993). Contagious mastitis is transmitted from cow to cow during milking, whereas exposure to environmental pathogens can occur at any time during a cow's life (Smith & Hogan 1993).

It is unlikely that mastitis will ever be eradicated. There are too many different bacteria involved many of which are continually present while antibiotic treatment has varying degrees of effectiveness. The approach to management of mastitis must therefore be to

control the disease and with increased milk production rates producing ever higher susceptibility, control will become increasing important in the future with detection and diagnosis being of the utmost importance.

4.1.2 Clinical mastitis

Clinical mastitis is characterised by abnormal milk of varying degrees depending on the severity of mammary gland inflammation. Signs of inflammation include heat, pain, redness, swelling and loss of function of the mammary gland (O'Rourke 1992). Clinical signs alone are considered to be insufficient to differentiate between infections caused by Gram positive and Gram negative bacteria with certainty; although recent work indicates that some clinical signs are more likely to be associated with certain types of pathogens (Milne *et al.* 2002).

4.1.3 Subclinical mastitis

Subclinical mastitis occurs when the mammary gland is infected and the number of leukocytes in the milk (SCC) is increased, but where there is no visible abnormality of the milk and there are no clinically detectable signs of inflammation in the mammary gland (Cullor 1996;Sears *et al.* 1993). Subclinical infections are, therefore, difficult to detect and repeated or persistent bouts may lead to fibrosis of the mammary tissue, resulting in further reduction in milk production (Cullor *et al* 1996).

4.1.4 Contagious pathogens

Contagious mastitis is caused by organisms that colonise the mammary gland and can be spread during the milking process (Cullor *et al* 1996). Colonies become established at the teats and slowly grow through the canal over a few days. For the most part, successful control of contagious mastitis is by dry cow therapy and post milking dipping. The most commonly found contagious pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae, Streptococcus dysgalactiae* (Bramley 1984).

Staphylococcus aureus is a haemolytic gram positive coccus often visualised as white colonies on blood agar. *Staphylococcus aureus* is a coagulase positive bacteria and as so is often referred to as the coagulase positive staphylococci (Blowey & Edmondson 2000b). Staphylococci are notoriously difficult to treat for two reasons. Once infection has been established in the udder they become surrounded by a fibrous tissue which makes penetration with antibiotics more difficult. *Staphylococcus aureus* also has the ability to infect macrophages and epithelial cells which makes them inaccessible to antibiotics. Infection with *S. aureus* usually gives rise to a chronic subclinical infection however cases of mild and moderate clinical infection can occur (Cullor & Smith 1996). Under certain circumstances *S. aureus* can also cause an acute gangrenous mastitis. This occurs following the production of a large quantity of toxin. In most cases cows suffering for gangrenous mastitis would be culled.

In contrast to *S. aureus, Strep.agalactiae* is a non-haemolytic coccus gram positive bacteria and is a highly contagious cause of mastitis. *Streptococcus agalactiae* is visualized as small colonies which are blue in appearance on Edwards medium. The

primary reservoir of infection is in the udder although it may colonise the teat canal and even the teat skin. Unlike *S aureus, Strep agalactiae* responds well to antibiotic treatment and almost all antibiotics are effective. The level of *Strep. agalactiae* infection in an individual cow is closely associated with cell counts. Although *Strep. agalactiae* is mainly colonised in the udder it has been reported that it can survive in the environment.

Streptococcus dysgalactiae is the third major cause of contagious mastitis, like *Staphylococcus aureus, streptococcus dysgalactiae* is a haemolytic gram positive coccus and can be seen as small colonies green in appearance on Edwards medium. *Streptococcus dysgalactiae* can survive well in the environment and for this reason some consider it be an environmental pathogen as well as contagious pathogen. *Streptococcus dysgalactiae* is most commonly found on the teat skin particularly when the integrity of the skin is compromised. This bacteria is also commonly found on the tonsils and so licking may be a route of infection.

4.1.5 Environmental Pathogens

Environmental mastitis is caused by pathogens that do not necessarily colonise the mammary gland and exposure is not limited to the milking process. Dry cow therapy is of no value in the control of environmental mastitis as they do not persist subclinically and are not carried from one lactation to the next. Gram negative coliforms and *Streptococcus uberis* are the predominant environmental pathogens (Smith, Todhunter, & Schoenberger 1985).

Escherichia coli is the prevalent environmental organism causing mastitis. *Escherichia coli* are gram negative bacilli of which there are haemolytic and non-haemolytic strains. *Escherichia coli* can be visualised as creamy white mucoid colonies on blood agar. It is present in large numbers in faeces and hence infection occurs primarily in housed animals. The symptoms of typical *E.coli* mastitis are a hard, hot swollen quarter with a watery discharge. The toxic effect of *E.coli* mastitis is due to the release of an endotoxin, which is a lipopolysaccharide (LPS) derived from the bacterial wall. Most cases of *E.coli* mastitis are restricted to the teat and gland cistern. If *E.coli* reaches the smaller ducts and lactiferous sinuses of the main gland, then a massive multiplication of bacteria occurs, and this leads to a severe response in the cows.

Streptococcus uberis is a widely occurring environmental organism causing mastitis and is particularly associated with straw yards. *Streptococcus uberis* is a non-haemolytic gram positive coccus which appears as brown colonies on Edwards medium due to the splitting of aesculin. *Streptococcus uberis* is responsible for the majority of clinical and subclinical cases of mastitis in the UK (Hillerton *et al.* 1993). Unlike *E.coli* high numbers of *Strep. uberis* are not found in the faeces however it is found naturally on mucous membranes and skin (Blowey 1990). Mastitis caused by *Strep. uberis* is often sudden in onset, giving a hard, swollen quarter, clots in milk and a high or very high, body temperature. In most instances of this infection response to antibiotic treatment is good.

4.1.6 Detection of mastitis

Detection of clinical mastitis by visual inspection and palpation is relatively easy, but diagnostic problems arise when dealing with sub-clinical mastitis, where an increased SCC is the only finding. The SCC is the number of somatic cells present per ml of milk. It is used as an indicator of udder infection. Somatic cell counts are made up of a combination of white blood cells and epithelial cells (Nickerson 1985). White blood cells enter milk in response to inflammation which may occur due to disease or occasionally injury. Epithelial cells are shed from the lining of the udder tissue. White blood cells make up nearly all of the somatic cells.

Despite more than 30 years of research into mastitic diagnostics, there are few alternatives to the SCC in practical use for identification of subclinical mastitis apart from the California mastitis test which is an on farm test that only give qualitative results.

Dairy cooperatives give farmers a premium quality payment to encourage low SCC. Furthermore EC directive 853/2004 states that the SCC of the bulk tank milk must be less than 400,000 cells/ml. Somatic cell count measurement is the most common tool used in mastitis diagnostics as somatic cells in the milk give a good indication as to the degree of infection in the udder (Sears, Gonzalez, Wilson, & Han 1993), however the SCC can remain elevated for weeks after the infection is eliminated and the gland is recovering (Petersen, Nielsen, & Heegaard 2004).

Mastitis is by far the most important factor that causes increased SCC. When mastitic causing agents enter the udder, the defence mechanisms send vast numbers of white blood cells to the area to try and kill the bacteria. If the infection is eliminated the cell counts will return to normal levels. If the white blood cells fail to eliminate the infection then a subclinical infection is established. White blood cells are continually transferred in to the milk leading to the increased cell count. For some organisms there is a relationship between cell counts and the level of infection in the udder.

There are a number of ways in which SCC can be measured. The most informative but perhaps less practical way is at the quarter level when individual milk samples from each udder quarter of a cow is analysed i.e. individual quarter SCC (IQSCC). This method has the ability to identify which individual cow is infected and in which quarter. Somatic cell counts can also be measured at the cow level where a composite sample made up of equal volumes of milk from each quarter is analysed i.e. individual cow SCC (ICSCC). This method of SCC is also very informative in that it can identify which cows are infected and so they can be withdrawn form the herd. However as it is known that each quarter is immunologically completely separate and there is no flow between each quarter then ICSCC can mask individual quarter counts and so may mask infection. More often than not SCC is analysed from the bulk tank i.e. bulk milk SCC (BMSCC). This is useful in identifying the prevalence of infection in the herd but the BMSCC is limited as it is unable to identify individually infected cows or quarters.

4.1.6.2 California mastitis test

This is a simple test that is useful in detecting subclinical mastitis by crudely estimating the cell count of milk. The CMT test does not give a numerical result rather an indication whether the count is high or low. The California Mastitis test is based on bromcresol-violet reacting with nuclear content of the somatic cells to give a viscous gel (Bramley, 1992). The benefits of CMT are that is cheap to use, it can be carried out by the milker during milking and results are available immediately and it gives an indication of the level of infection in each quarter.

4.1.6.3 The total bacterial count (TBC) or Bactoscan

The bacterial count of milk is a measure of the bacteria grown from an aliquot of milk over a fixed period of time (Blowey & Edmondson 2000). The bactoscan measures the number of bacteria in the milk using an electronic method. High TBCs effect the farmer in two ways directly in the form of financial penalties and indirectly through the production of a poor quality milk, and are measured regularly by the dairy companies. However the benefits to the farmer are limited as they are unable to identify the bacteria present or the source. There are four main sources of bacteria in the milk; mastitis causing organisms, environmental contaminants, dirty milking equipment and failure to refrigerate. Mastitis should be suspected if the TBC fluctuate dramatically. When quarters become infected with clinical or subclinical mastitis the numbers of bacteria can increase substantially. *Streptococcus agalactiae* and *Strep. uberis* in particular are shed in extremely high numbers.

4.1.6.4 APPS in detecting Mastitis

Acute phase proteins such as Hp, SAA, and AGP have been identified as markers of inflammation in cattle. Many studies have reported increased serum levels of the mentioned APPs in mastitis caused by various pathogens (Eckersall et al. 2001;Gronlund et al. 2003;Hirvonen, Pyorala, & Jousimies-Somer 1996;Horadagoda et al. 1999). Haptoglobin and SAA are major APPs in cattle with serum concentration increasing 100 fold for Haptoglobin and 5-20 fold for SAA: AGP is a moderate acute phase protein in cattle, which has a low relative rise and indicates more chronic inflammation (Eckersall et al. 2001;Eckersall & Conner 1988). However Hp, SAA and AGP are also raised in the serum in the presence of other infections and trauma making them non-specific and reduce their value in the diagnosis of mastitis.

Acute phase proteins Hp, SAA and AGP have been found in the bovine milk during mastitis implying that it is possible that measuring APP's in milk will be of more use than measuring APPs in the serum for diagnosis of mastitis (Ceciliani et al. 2005;Gronlund et al. 2003;Hirvonen, Pyorala, & Jousimies-Somer 1996). This will be discussed in more detail in Chapter 5

The main objective of this study was identifying any changes within the carbohydrate moiety of bovine serum AGP that are due to the presence of mastitis. In order to achieve this objective the serum obtained from control cows and cows with mastitis had to be prepared in such a way as to enrich the AGP concentration present in the serum. This was achieved by fractionation precipitation with polyethylene glycol followed by

anion exchange chromatography. Once the samples where successfully prepared they where probed with lectins specific for defined antigens within the glycan chains attached to bovine AGP.

4.2 Materials and methods

4.2.1 Materials

4.2.1.1 Samples

All samples were kindly provided by Dr F Young, Department of Clinical Studies, Faculty of Veterinary Medicine, University of Glasgow and detailed in Eckersall *et al* 2001)

All reagents were obtained from Sigma (Poole, UK) unless otherwise stated.

4.2.2 Sample preparation

Polycthylene glycol (PEG) Mwt and 8000, Trizma base, sodium chloride (NaCl). A 5ml Hitrap Mono Q chromatography column was used on the AKTA FPLC system and was supplied by Amersham Pharmacia Biotech UK. Centricon, centrifugal filter units were supplied by Millipore Corporation (Massachusetts, USA).

Dialysis tubing used for buffer exchange was purchased from Medicell (London, UK) . The Akta Fast Protein liquid chromatography system was supplied from Amersham Pharmacia Biotech (Buckinghamshire, UK) and controlled by a Compaq deskpro EN, which runs Unicorn version 4 software.

4.2.1.2 Lectin Blots

Sodium dodecyl sulphate (SDS), ammonium persulphate (APS), Trizma base, bromophenol blue, mercaptoethanol, TEMED, Coomassie blue, NaCl, Tween-20, magnesium chloride, manganese chloride, calcium chloride, and bovine serum albumin were all supplied by Sigma (Poole, UK). Hydrochloric acid (HCl), methanol and acetic acid were supplied by BDH. Glycerol was purchased from Fisher Scientific (Loughborough, UK). Fluorescein labelled Sambucus Nigra, Concanavalin A, Maackia Amurensis and biotin labelled Aleuria aurantia lectin where purchased from Vector laboratories (California, USA).

30% Acrylamide mix, Standard marker and nitrocellulose membrane were purchased from BioRad (Hemel-Hempstead, UK).

4.2.1.3 Measurement of AGP concentrations.

AGP concentration in bovine serum samples were measured using a commercially available radial immunodiffusion kit supplied by the Institute for Metabolic Ecosystems (Seratoni, Japan). SAA was measured by ELISA and Hp was measured by colorimetric assay on a MIRA biochemical analyser. Kits for Hp and SAA were obtained from Tridelta Development (Eire).

4.2.2 Methods

4.2.2.1 Samples

Serum samples from 8 healthy control cows, 7 cows diagnosed as suffering from mild mastitis and 7 cows diagnosed with moderate mastitis were used in this study. Mild and moderate mastitis was graded on the basis that the presence of clots accompanied by visible signs of inflammation of the udder were in the moderate group whereas cows which only had clots in the milk and no visible signs of inflammation were grouped in the mild mastitis category.

Somatic cell counts, SAA, Hp and AGP levels had previously been measured by Dr Fiona Young using methods detailed elsewhere (Eckersall *et al*, 2001) with brief outline given below.

4.2.2.2 Somatic cell counts

Milk samples were taken from infected quarters and submitted to the Veterinary Science Division, Scottish Agricultural College, at Auchincruive for automated counting.

4.2.2.3 Haptoglobin concentrations

Serum haptoglobin levels were determined using the 'Phase' haptoglobin kit on a MIRA biochemical analyser according to the manufacturers instructions

4.2.2.4 SAA Concentrations

SAA concentrations were determined using a commercially available ELISA kit (McDonald, Weber, & Smith 1991) and run according to the manufacturer instructions.

4.2.2.5 AGP concentrations

A radial immunodiffusion technique (Mancini, Carbonara, & Heremans 1965) was used to determine the quantity of AGP in each sample. Standards of AGP at concentrations of 1, 0.5, 0.25 and 0.125 mg/ml were applied to the precast antibody containing gel. The gel was then incubated overnight at 37°C for 24-48hrs and the diameter of the precipitate rings measured. A calibration curve from the results of the standards (diameter (mm) vs concentration (mg/ml)) was drawn to determine AGP concentrations in the samples.

4.2.2.6 Sample preparation

In order to undertake a comparison of the glycan formation on bovine AGP the protein had to be partially purified from each sample. Depending on the concentration of AGP in the serum 2-5 ml of bovine serum was precipitated with PEG 8000 using 400mg of PEG per 1ml of sample (40%w/v) then stored overnight at 4°C before being centrifuged at 3000g for 30 mins. The supernatant was aspirated for further purification.

The supernatant from the PEG precipitation was dialysed against 50mMTris HCl pH 7.4 overnight at 4°C before being applied to a 5ml Mono Q column that was equilibrated with 2 CV of 50mM Tris HCl pH 7.4. Once sample was injected it was washed through the column with 2 CV of 50mM Tris HCl pH 7.4 before a continuous gradient of buffer 1M NaCl 50mMTrisHCl pH 7.4 was applied. The gradient used was 0-50% over 10 CV and during this period 3 peaks eluted and were retained. The column was regenerated using 100%. 50mM Tris HCl 1M NaCl pH 7.4. Gel electrophoresis (SDS-PAGE) was carried out to determine which peak contained AGP. The middle peak after the initiation of the gradient was found to contain AGP so this fraction was retained and desalted using centricon centrifugal devices. The concentration of AGP present in each sample was then measured by RID.

4.2.2.7 Lectin Blots

Following desalting the concentration of AGP was diluted appropriately to give 1 g/L solution. A 10µl of the 1mg/ml solution was reduced in 10µl of sample buffer and 10µl was applied to each well of a SDS-PAGE gel and run according to the method described in Chapter 2 and transferred to nitrocellulose membrane (see 2.2.1.5).

After electroblotting on to nitrocellulose, the nitrocellulose was then incubated in 0.5% (w/v) BSA diluted in TBS (0.15M NaCl 50mM Tris HCL pH7.5) for 30 mins at room temperature, the membrane was then washed twice with TBS containing 1% (v/v) Tween-20 (TTBS) and once with TBS containing 1mM $MnCl_2$, 1mM $CaCl_2$, 1mM $MgCl_2$. The membrane was then incubated with either 5μg/ml of fluorescein labelled Sambucus nigra lectin (SNA), 10μg/ml fluorescein labelled Maackia amurensis (MAA), or 25 μg/ml fluorescein labelled Concanavalin A lectins for 1hr in the dark. The membrane was again washed with TTBS and subsequently scanned at 580nm using a Storm 840 scanner (GE Health Care). To assess binding of Aleuria aurantia which was labelled with biotin the membrane was incubated for 1 hr with 10μg/ml of the Aleuria aurantia lectin and washed repeatedly with TTBS before being incubated with a 1:1000 dilution of fluorescein labelled Streptavidin and subsequently washed and scanned as above. The bands were quantified using the imaging programme called Scion Imaging, (http://www.scioncorp.com/) giving a mean pixel density for each band. The mean pixel density was obtained by highlighting a defined area and measuring the intensity of the band.

4.3 Results

4.3.1 Acute phase proteins in serum samples

The acute phase protein concentrations and SCC were measured in order to identify animals with mastitis so the animals could be grouped appropriately for glycan analysis. The acute phase protein concentrations and SCC data for the individual samples used in this study are given in Table 4.1 (courtesy of Dr F Young Department of Clinical Studies, Faculty of Veterinary Medicine, University of Glasgow). The samples were from 3 groups of cows control (no clinical signs of mastitis), mild mastitis (clots in the milk) and moderate mastitis (clots in milk accompanied with visible signs of inflammation in the udder). The mean values ± the standard error of each group is recorded in Table 4.2 and histograms of this data is shown in Figure 4.1. Control cows had a mean somatic cell count of 1183 ± 260 $x10^3$ cells/ml compared to 8251±2628 $x10^3$ cells/ml and 10758±1477 $x10^3$ cells/ml for the mild and moderate mastitis groups respectively. Both mastitic groups had higher somatic cells when compared to the control group however only the moderate mastitis group had statistical significance with $P<0.05$ there was no significance difference between the mild and moderate mastitic subgroups ($P= 0.61$).

The mean AGP concentration levels of 337±57.5, 503±90 and 619±41 µg/ml were measured for the control, mild and moderate mastitic groups respectively. Only the moderate group was statistically different from the control group with a $P<0.05$.

Cow No	Health status	SCC (10^3cells/ml)	AGP (μg/ml)	HP (μg/ml	SAA (μg/ml)
8c	control	300	300	0	3.8
1 (9c)	control	1820	300	0	6.1
2 (10c)	control	457	780	0	4
3 (12C)	control	1593	340	0	5.1
4 (13C)	control	593	440	0	11
5 (15C)	control	1398	150	0	5.1
6 (16C)	control	2585	230	13.6	5.7
18c	control	160	420	0	6.6
7 21C)	control	2022	210	15	4.9
8 (24C)	control	898	200	0	7.3
9 (11A)	mild	16053	490	67.5	15.3
10 (13A)	mild	400	350	0	8.6
11(14A)	mild	9900	1000	27.7	25.5
12 (18A)	mild	13900	280	33.2	13.4
13(19A)	mild	975	570	122	21.7
14 (20A)	mild	1940	410	0	9.2
15 (28A)	mild	14590	420	173	14.4
16 (6B)	moderate	7150	640	833	5
17(9B)	moderate	8622	540	320	15.5
18(12B)	moderate	14428	540	638	18.9
19 (14B)	moderate	10692	840	730	20.4
20 (15B)	moderate	6400	590	973	12.6
21(18B)	moderate	17210	540	665	12.1
22(24B)	moderate	10805	640	758	45

Table 4.1 The SCC and acute phase protein concentration for the individual animals used in the study of glycan analysis

	SCC ($x10^3$ cells/ml)	Serum AGP (μg/ml)	Serum Hp (μg/ml)	Serum SAA (μg/ml)
Control (n=8)	1183±260	337±57.5	2.86±1.9	5.96±0.66
Mild mastitis (n= 7)	8251±2628	503±90	138±49*	15.4±2.3*
Moderate mastitis(n=7)	10758±1477*	619±41*	702±77* +	18.5±4.8*

Table 4.2 The mean ± Standard error (SE) of the groups. * indicates statistical significance p<0.05 compared to controls.

Mean SAA levels of 5.96±0.66 μg/ml, 15.4±2.3 μg/ml and 18.5±4.8μg/ml were recorded for control, mild and mastitic groups respectively. Both mild and moderate mastitic groups had statistically significant higher SAA levels with P<0.05 respectively there was no difference between the mastitic groups.

Mean Hp levels were 2.86±1.9 μg/ml, 138±49 μg/ml and 702±77μg/ml for control, mild mastitis and moderate mastitis groups respectively. As with SAA the Hp levels of mild and moderate mastitis groups where significantly higher than those of the control group with P< 0.05. However unlike SAA, Hp concentrations in serum from the moderate mastitis group was statistically higher than the mild mastitis group with P< 0.05.

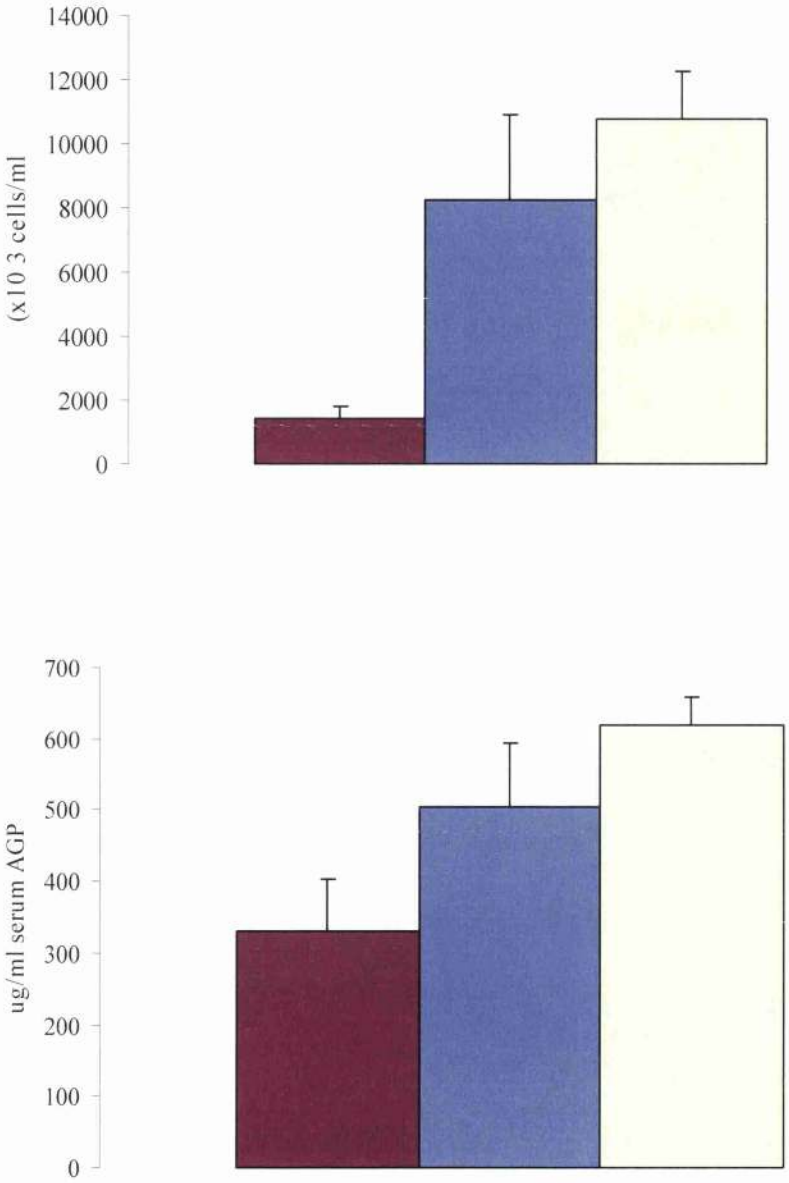


Figure 4.1 A) histogram of means ± SE of the SCC for control (Red), mild (blue) and moderate (Yellow) mastitic groups. B) histogram of mean ± SE of the AGP levels for control (Red), mild (blue) and moderate ( ) mastitic groups

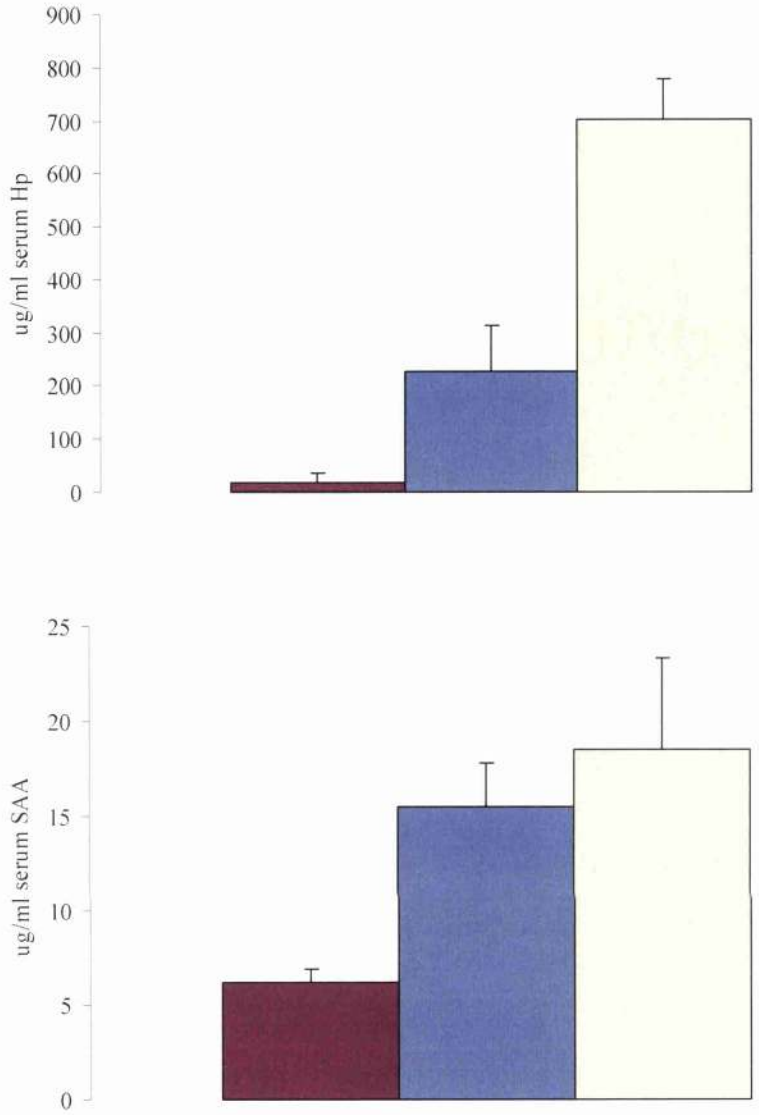


Figure 4.2 A) histogram of mean ± SE of the haptoglobin levels for control (Red), mild (blue) and moderate ( ) mastitic groups. B) histogram of mean ± SE of the SAA levels control (Red), mild (blue) and moderate ( ) mastitic groups.

4.3.2 Lectin blot analysis

A blot of bAGP against SNA for the detection of α2-6 sialic acid in control, mild and moderate mastitic groups can be seen in Figure 4.4A. The intensity of the bands from the animals in the mild and especially the moderate mastitic groups are visually more intense than those from the control group. Bovine AGP purchased from sigma was run alongside the samples to ensure the correct band was analysed. A similar pattern was seen when blotted with Con A (Figure 4.4B) however the reaction of animals was less pronounced than that seen with SNA.

The individual mean pixels measured for each sample when blotted against Con A and SNA are shown in Table 4.3 and Table 4.4 shows the mean ± standard error of the mean pixel density of each disease group. The mean pixel densities for Con A were calculated as 151±8.6, 195±5.2 and 199±4.4 for control, mild and moderate mastitis groups respectively. The density of the AGP bands for mild and moderate mastitic patients when blotted with Con A being significantly higher than the control groups with $P<0.05$. However although blotted bands from samples in the moderate mastitic group had slightly higher pixel density values when compared the animals in the mild mastitic group this was not significant with $P>0.05$.

When bovine AGP was probed with SNA the mean pixel density where calculated as 171±8.9, 189±4.4 and 196±8.15 for control mild and moderate mastitic groups respectively. Although mild mastitic samples had mean pixel densities high than the controls no statistical significance was found $P>0.05$ however samples in the moderate

group showed significantly greater reactivity ($p<0.05$) with SNA than the controls. No significant difference was detected between the mastitic groups ($p>0.05$).

Bovine AGP was also probed with AAL and MAA lectin for the detection of fucose and α2-3 linked sialic acid. No reaction was found (Figure 4.4 C and D respectively). The blots contained positive controls which were run along side the bovine samples and showed positive reaction confirming that the procedure had worked. Human AGP was used as the positive control when blotting with AAL and fAGP was used as the positive control when blotting with MAA lectin and gave positive binding reaction with the respective fluorescent lectins.

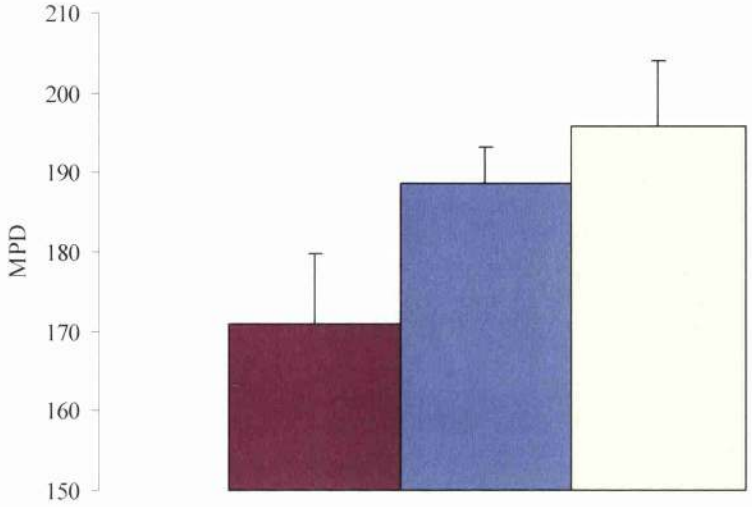
Cow No	Health status	AGP levels	Con A (mean pixels)	SNA (mean pixels)
1 (8c)	control	300	136.03	136.46
2 (9c)	control	300	106.36	222.03
3(10c)	control	780	123.92	158.76
4(12C)	control	340	169.6	150.22
5(13C)	control	440	120.24	187.27
6(15C)	control	150	176.53	148.76
7(16C)	control	230	175.25	na
8 18C	control	420	171.64	189.77
921C)	control	210	153.6	162.2
10(24C)	control	200	179.43	182.4
11(11A)	mild	490	185.63	203.94
12(13A)	mild	350	210.09	199.11
13(14A)	mild	1000	198.88	182.4
14(18A)	mild	280	199.69	187.5
15(19a)	mild	570	198.3	170.57
16(20A)	mild	410	202.15	195.26
17(28A)	mild	420	168.34	180
18(6B)	moderate	640	183.58	195.95
19(9B)	moderate	540	191.22	165.37
20(12B)	moderate	540	205.41	199.48
21(14B)	moderate	840	191.13	177.04
22(15B)	moderate	590	195.93	190.25
23(18B)	moderate	540	213.28	212.19
24(24B)	moderate	640	213.22	230.39

Table 4.3 The mean pixel density of each animal when probed with Con A and SNA lectins

Disease group	AGP µg/ml Mean±SE	Con A (MPD) Mean±SE	SNA (MPD) Mean±SE
Control	337±57.5	151±8.6	171±8.9
Mild	503±90	195±5.2*	188±4.4
moderate	619±41*	199±4.4*	196±8.1*

Table 4.4 The mean ± SE of the mean pixel density of bovine AGP bands when probed with ConA and SNA for each disease group. * indicates statistical significance $p<0.05$ compared to controls

A



B

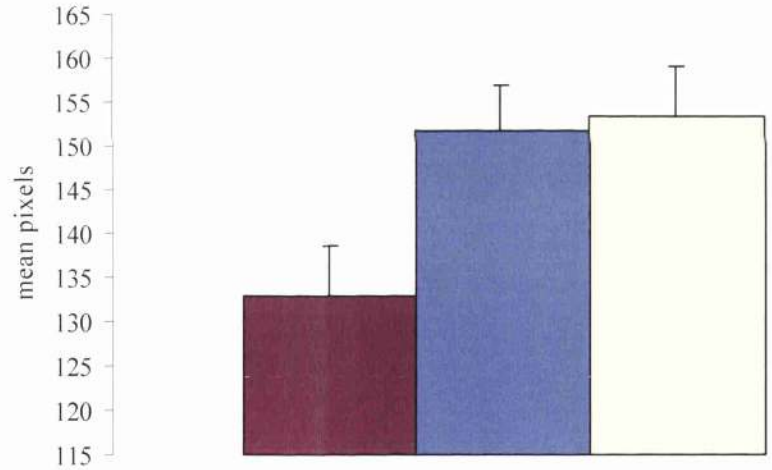


Figure 4.3 Histogram showing mean ± SE values for density of A) SNA B) ConA

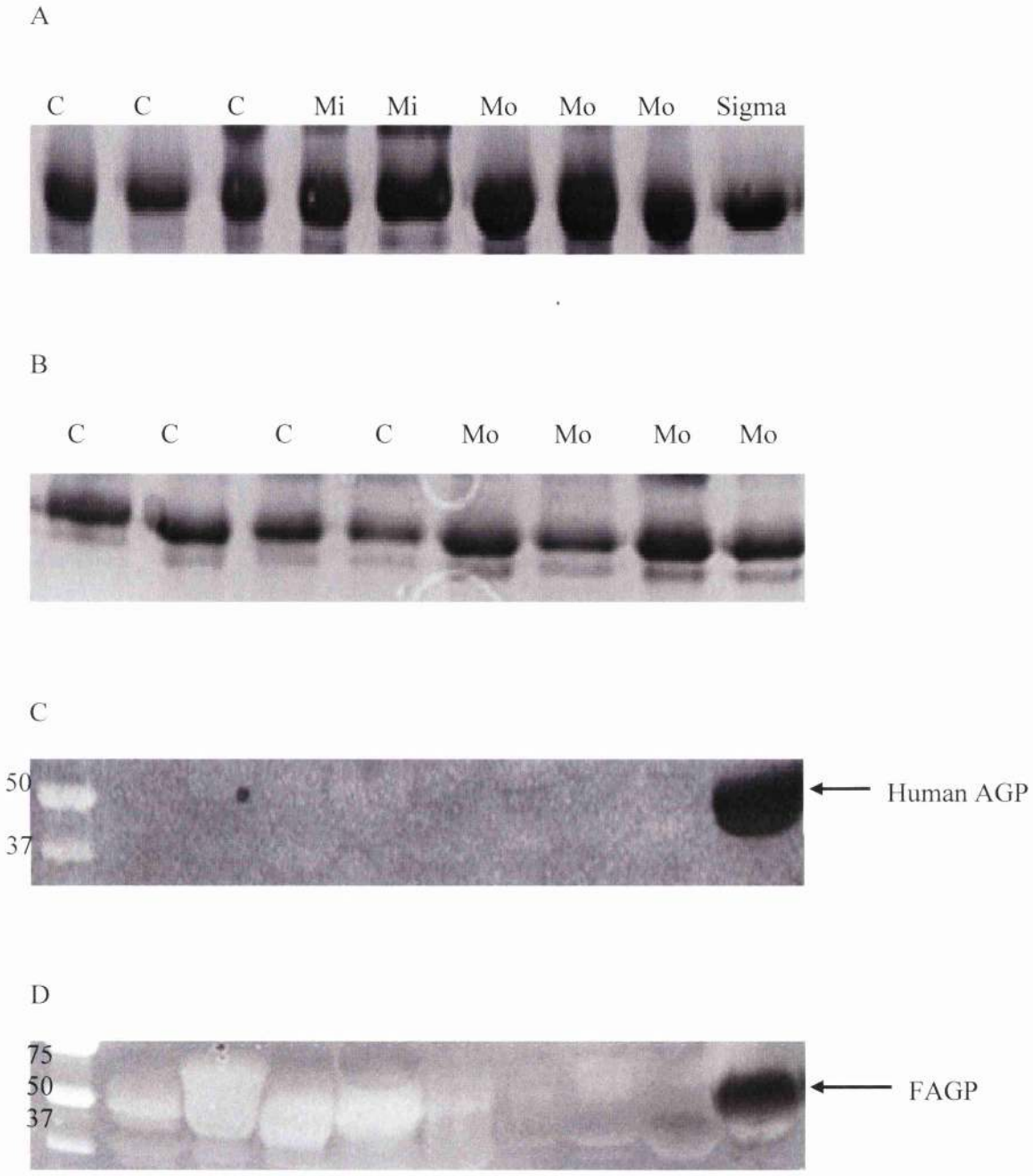


Figure 4.4 A) A blot of bovine AGP from the three groupings against SNA B) a blot of bovine AGP from the three groupings against Con A C) blot of bovine AGP samples blotted against AAL, with human AGP used as a positive control D) blot of bovine AGP samples blotted against MAA, with feline AGP used as a positive control. C = control, Mi =mild mastitis, Mo= Moderate mastitis, Sigma = Bovine AGP purchased form Sigma.

4.4 Discussion

Mastitis remains the greatest health related economical problem for dairy farmers and affects the farmer economically in two ways, both directly and none directly. Direct costs of mastitis include discarded milk, veterinary care and medication and indirect costs include penalties for decreased milk quality and decreased milk yield. Early diagnosis of mastitis is important in both economical and welfare terms as there would be reduced spread of disease, lower reduction in milk yield and treatment would be effective more quickly .

Somatic cell counts are used routinely in the diagnosis of subclinical mastitis. However they are less efficient in the diagnosis of clinical mastitis as the visible changes to milk such as clots make it difficult to measure SCC accurately via an automated system (Blowey & Edmondson 2000;O'Rourke 1992). The importance of SCC to the dairy companies can be seen from the large financial penalties that are imposed for having an increase in BMSCC. Therefore it is important that the farmer can take action to reduce the SCC. The relationship between bulk milk somatic cell counts (BMSCC) and the prevalence of mastitis has been the focus of numerous studies. Holdaway *et al* (1996) reported a correlation of 0.84 ($p<0.001$) between BMSCC and the percentage of infected quarters. Consequently BMSCC have become universally adopted as a screening test for mastitis in herds (Holdaway, Holmes, & Steffert 1996). A EC directive requires bulk milk to have a total bacteria count <100,000 bacteria /ml and SCC <400,000 cells/ml. However BMSCC of <250,000 cells/ml is considered to be a realistic upper limit for UK herds with good mastitis control (Blowey & Collis 1992). Individual cows SCC are obviously more reliable than BMSCC at identifying increased

SCC levels however it is not practical to measure ICSCC regularly. Most ICSCC levels are obtained from experimental investigation and the level that is most often used as the upper limit is 100,000 cells/ml. The mean SCC count of the normal cows used in this study group was higher than expected and may be explained by the fact that foremilking was not performed during the collection of samples (Eckersall et al. 2001). Foremilking can be used to remove the non-mastitic bacteria from the teat canal before sampling. Bacteria was not isolated from the majority of the samples in the control group.

Haptoglobin is a major APP in cattle in which it has a negligible circulating level in blood of normal animals (Conner *et al*, 1988), but increases up to 300 fold during the APR from a concentration of less than 0.01g/L to reach 2-3 g/L within 48hrs of infection (Eckersall 1999). Heegaard et al (2000) even reported Hp levels of up to 8-9mg/ml. In cattle and other ruminants, Hp has been the acute phase protein most commonly monitored as a marker of inflammation (Skinner, Brown, & Roberts 1991;Wittum, *et al* 1996). Serum amyloid A is also a major APP in cattle with concentrations in serum ranging from 3.6-11μg/ml in normal cows and raising rapidly to levels of 115μg/ml (Eckersall et al. 2006). Alpha-1-acid glycoprotein is not a major APP in cattle but it falls into the moderate APP category will levels only raising 2-5 fold.

The detection of SAA and Hp as well as high levels of AGP in serum from the mild and moderate mastitic groups indicate that an acute phase response was present and highlights the potential use of these parameters as diagnostic markers (Hirvonen, Pyorala, & Jousimies-Somer 1996). However although the moderate mastitis group tended to have higher serum concentrations of the acute phase proteins the finding that

these where not significantly different rule out their use in the discrimination between the two groups. On the other hand although there were raised levels of AGP in the mild mastitic group statistical significance was not seen between the mild and control groups but was apparent when comparing the moderate and control groups suggesting that maybe AGP to differentiate the two categories of mastitis. However this can not be concluded from the present study due to small sample populations. The data is not in total agreement with Eckersall *et al* 2001 as they reported that AGP levels where significantly greater in mild and moderate mastitis when compared to control animals (Eckersall et al. 2001). The difference in the findings may be due to the fact that the sample population used by Eckersall *et al* 2001 was greater than that used in this study as only a random selection of samples were analysed here.

Most studies which have examined the structure of the oligosaccharide chains of AGP and tracking disease associated changes within the chains have been carried using human AGP. Many studies in human disease have examined the extent of sialylation, fucosylation and branching of the glycans on AGP. The most common finding in human disease is the detection of alterations in the extent of branching. In acute inflammatory conditions such as bacterial infection, surgery and trauma, there is an increase in AGP glycoforms containing bi-antennary chains (De Graaf et al. 1993;Mackiewicz et al. 1987;Pawlowski, Mackiewicz, & Mackiewicz 1989;Pos et al. 1990). In contrast chronic inflammatory conditions such as alcoholic liver cirrhosis and rheumatoid arthritis, an increased appearance of tri and tetra-antennary structures is observed (Elliott et al. 1997;Jezequel et al. 1988;Serbource-Goguel, Durand, Corbic, 1986;Smith et al. 1994).

Recently a few studies have examined the oligosaccharide chains of fAGP, and in particular the branching fucosylation and sialylation of the glycan chains. It was found that fAGP from cats suffering from FIP had decreased levels of sialic acid whilst cats infected with feline leukaemia virus had increased levels of sialic acid when compared to non diseased cats. Few studies have analysed the structure of bovine AGP, though it has been reported that bovine AGP consists of multiple glycoforms as a result of having 3 N-linked glycosylation sites (Hunter & Games 1995)and Choe *et al* 2000 reported that bovine AGP had a fourfold higher percentage of diantennary glycans chains compared to human AGP (Choe *et al.* 2000).

Nakano *et al* (2004) has also shown that bovine AGP has di-anntenary chains as its major oligosaccharide chains (Nakano *et al.* 2004). However hyperasialylated di-anntenary chains were detected, some having four sialic acid residues and even more surprisingly glycan chains were found with four N glycoyl neuraminic acid (NGNA) residues. Triantennary glycan chains were a lot less abundant than the di-anntenary chains however these too were hypersialylated with the occurrence of triantennary chains carrying five NGNA residues.

The present study investigated the structure of the glycan chains attached to bovine AGP in clinically healthy dairy cows as well as those suffering from mild and moderate mastitis.

The lectins Con A, SNA, MAA and AAL were chosen to look for changes within the oligosaccharide chains of bovine AGP during an acute phase response caused by the presence of mastitis. Con A was used to look at branching, SNA and MAA were used

to analyse sialic acid content and AAL was used to probe for the presence of fucose residues. Sambucus nigra (SNA) preferentially binds α 2-6 bound sialic acid whilst MAA preferentially binds α 2-3 sialic acids. Nakano *et al* (2004) showed using mass spectrometry that bovine AGP contains novel sialic acid containing bi antennary chains composed of only n-glycoyl neuraminic acid which had not been detected in AGP from other species (Nakano, *et al* 2004). It was found here that bovine AGP reacted very strong with SNA indicating that the glycan chains of bovine AGP have abundant quantities of α 2-6 sialic acid, however bovine AGP did not bind MAA suggesting that α2-3 sialic acid was not present in significant quantities on the oligosaccharide chains. The study carried out by Nakano *et al.*, (2004) was not able to distinguish between positional isomers of sialic acid (Nakano, *et al* 2004). Examination of AGP from animals classified as having moderate mastitis bound significantly more of the fluorescently labelled SNA than control AGP indicating that more α2-6 sialic acid residues are present on AGP during the acute phase reaction. However animals with mild mastitis, although they had higher reactivity with SNA there was no significant difference when compared to the control animals or the moderate group. This may be due to the small sample groups or that the increase in α2-6 sialic acids is a gradual process as the severity of disease progresses.

In human disease a change in branching results in a change in sialic acid content because in human N linked glycan chains are almost always terminated with one sialic acid. Therefore an increase in branching would automatically be followed with an increase in sialic acid and on the other hand if a decrease in branching was observed then a decrease in sialic acid would ensue. However the results here showed an increased reaction with Con A when mastitis was present suggests lower triantennary

and higher diantennary chains are present, and so we would expect a decrease in sialylation. However in this study an increase in α2-6 sialylation was observed during mastitis, but it has been observed that the branches of the oligosaccharide chains attached to bovine AGP may have more than one sialic acid residue attached (Nakano, Kakehi, Tsai, & Lee 2004). It is possible that a decrease in branches and an increase in sialic acid could occur if the branches of bovine AGP during an acute phase reaction contained increasing number of sialic acid linked by α2-6 bonds.

Although the fucose content of normal human AGP is relatively low it is commonly found to increase in diseases such as rheumatoid arthritis, acute inflammation and cancer. However fucose was not detectable on bovine AGP in control or mastitic samples. Human AGP was run along side bovine when probing with AAL as a positive control and as this was detectable in the blotted gels this would confirm that fucose was not present within the glycan chains of bovine AGP. Monosaccharide and oligosaccharide analysis with HPAEC-PAD would be a possible next step in this study.

In conclusion it was shown that the carbohydrate of bovine AGP has increased numbers of diantennary chains and increased numbers of α 2-6 sialic acid residues expressed when mastitis is present.

CHAPTER 5

5. The structural heterogeneity of bovine Haptoglobin in mastitis

5.1 Introduction

As outlined in Chapter 4 bovine mastitis is a major problem for farmers worldwide. Although bovine mastitis been investigated for many years numerous questions remain unanswered, one of which is, what are the changes in the components of milk and more specifically what changes occur in the whey proteins. The presence of blood proteins in the whey fraction of milk during mastitis has been widely accepted to be due to leakage from the blood system into the mammary gland. However in the last ten years, studies investigating the origin of proteins such as the acute phase proteins SAA and Hp in milk have shown that the mammary gland is itself capable of synthesising these proteins. In 2001 McDonald reported that SAA present in milk was in fact a mammary associated isoform of SAA which is now referred to as M-SAA3 (McDonald et al. 2001). Therefore the aim of this investigation described in this chapter was to compare serum Hp with milk Hp in an effort to identify any mammary associated isoforms particularly related to glycan modification

5.1.2 Milk Components

5.1.2.1 Introduction

Mastitis leads to a reduction in the useful components of milk and increases the levels of undesirable elements. Overall mastitis results in a less acceptable product for the consumer and so the value of this mastitic milk is reduced (Hortet *et al.* 1999;Hortet & Seegers 1998;Yalcin *et al.* 2000). Bovine milk from a healthy gland contains 3.9% fat, 3.3% protein, 5% lactose, and 0.7% minerals (Wong, Camirand, & Pavlath 1996). (Kehrli, Jr. & Shuster 1994;Lindmark-Mansson *et al.* 2000). Changes in milk composition during mastitis have been widely studied (Kitchen 1981;Watanabe *et al.* 2000). The most documented change in milk observed during infection of the mammary gland is the increased number of somatic cells. This is accompanied by variation within the protein constituents of milk, including an influx of serum proteins, such as bovine serum albumin (BSA) and acute phase proteins due to the increased capillary permeability or intra mammary synthesis of the infected gland. A substantial reduction in lactose and butterfat also occurs.

The changes in butterfat and lactose quantities are of great economic significance to the farmer as they make up the basis of the milk price. During mastitis increased levels of the enzymes lipase and plasmin are produced and these breakdown milk fat and casein which therefore has a detrimental effect on manufacturing yield and keeping quality of the milk. A list of changes seen in mastitis are outlined in Table 5.1

Components	Desirable/undesirable	Effect of mastitis
Total proteins	desirable	Remains unchanged
Casein	desirable	decreases 6-18%
Lactose	desirable	decreases 5-20%
Butterfat	desirable	decreases 4-12%
Calcium	desirable	decreased
Phosphorus	desirable	decreased
Potassium	desirable	decreased
plasmin	undesirable	increased
lipase	undesirable	increased
immunoglobulins	undesirable	increased
Sodium	undesirable	increased

Table 5.1 Desirable and undesirable changes seen in the components of milk when the mammary gland is infected.

5.1.2.2 Lactose

The milk sugar lactose is a disaccharide of α or β glucose and β galactose. Glucose is produced by the liver and after it is transferred to the udder, a proportion of the glucose is converted into galactose. Then one molecule of glucose combines with one molecule of galactose to produce lactose by the action of lactose synthatase. The lactose content of milk varies between 3.5-5.5% (w/v). In milk lactose is free or bound into oligosaccharides. Milk lactose is capable of reacting with free amino groups of proteins. Lactose is the main osmotic determinant of milk therefore in order to maintain

milk at the same osmolarity as blood, lactose concentration increases and decreases as the concentration of the other milk components vary (Blowey & Edmondson 2000).

5.1.2.3 Fat

Milk fat is formed in the udder secretory cells when fatty acids are esterified with glycerol and converted into the neutral fat called triacyl glycerol or triglycerides. Milk fat is a mixture of various fatty acid esters, the main group of which are the triglycerides (97-98%) (Blowey & Edmondson 2000).

5.1.2.4 Protein

Milk proteins include α_{s1}, α_{s2} and κ-caseins, β-lactoglobulin (β-LG), α-lactalbumin (α-LA), BSA, lactoferrin and immunoglobulins (Wong, Camirand, & Pavlath 1996).

The majority of protein in the milk is in the form of casein which is synthesised in the udder cells before transfer into the ducts, with approximately 80% of milk protein being casein and the remaining 20% being the whey proteins. Casein is a complex of at least four components; α casein, β casein, γ-casein and κ casein. Casein refers to that part of proteins that can be precipitated at pH4.6 and then separated from the whey. Casein is found only in milk to which it gives its white colour due to the casein complex reflecting light of all wavelengths. Casein occurs mainly as calcium caseinate in fresh milk. After precipitation of casein the supernatant is referred to as 'whey'. β-lactoglobulin and α-LA are the major whey proteins found in bovine milk accounting for between 7-12% and 2-5% respectively of total protein content.

Although β LG represents the major whey protein no definitive biological function has been described. β-Lactoglobulin is a member of the lipocalin family and shares similarity with retinol-binding protein which has led to suggestion of it having a role in transport of hydrophobic molecules (Sawyer *et al.* 1998). β Lactoglobulin also takes part in the phosphate metabolism of the mammary gland. α-lactalbumin is the second most abundant whey protein and is a small (Mwt 14 200), acidic (pI 4-5), Ca^{2+} binding protein, which is involved in lactose biosynthesis. It is one of two components of lactose synthase, which catalyses the final step in lactose biosynthesis in the lactating mammary gland (Permyakov & Berliner 2000).

5.1.3 Changes in the milk components due to mastitis

5.1.3.1 Lactose

During periods of mastitis lactose concentrations are reduced which results in increases in the concentration of sodium and chloride to maintain osmolarity and hence results in the bitter and slightly salty tasting milk often associated with mastitis (1995).

5.1.3.2 Fat

Mastitic milk has increased levels of the enzyme lipase (Lin & Randolph 1977). This leads to degradation of the milk fat releasing fatty acids and thus results in the rancid flavour associated with mastitic milk. Increased levels of fatty acids can inhibit starter culture in cheese and yogurt manufacture so these industries need to use milk from cows which do not have mastitis.

5.1.3.3 Enzymes

Many indigenous enzymes increase in milk during bovine mastitis. Enzymes that are involved in the synthesis of milk decrease and enzymes related to inflammation are increased. Enzymes such as N-acetyl-β-D-glucosamindase (NAGase) which originate from phagocytes (i.e. the somatic cells) are increased exponentially while enzymes that originate in the blood such as plasminogen are also increased. The use of NAGase activity in the detection of mastitis has been investigated and found in many studies to reliably detect intramammary infection (Kitchen 1981;Mattila & Sandholm 1986) and to reflect the degree of inflammation (Mattila, Pyorala, & Sandholm 1986). The blood enzyme plasminogen is also increased in mastitic milk most likely due to leakage from the blood. It is then locally activated to plasmin, a proteolytic enzyme capable of degrading fibrin and caseins (Kaartinen & Sandholm 1987).

5.1.3.2 Protein

Casein concentrations in bovine milk can drop by up to 18% during periods of mastitis and the concentration of whey proteins are also decreased in mastitic milk. β-Lactoglobulin and α-LA can drop by more than 70% of their normal level during mastitis. The decomposition of the casein fraction is due to increased proteolytic activity rather than decreased synthesis. Unlike caseins however the major whey proteins are relatively resistant to proteolytic attack. Their reduction could be due in part to impaired cellular synthetic and secretory function, but is more likely due to leakage of these proteins out of the milk and into the extracellular fluid (Auldist & Hubble 1998). This theory is supported in a study which reported elevated concentrations of α-LA in the blood of cows with increased SCC (Mcfadden, Akers, & Capuco 1988).

However as the concentration of milk proteins such as the caseins and whey proteins are decreased during mastitis increased levels of serum proteins are observed in the milk at the same time therefore total protein content may remain unchanged. However the milk is of lower quality especially for cheese and yogurt manufacturers as the coagulation of casein is important as the starting process in the manufacture of these dairy products (Blowey & Edmondson 2000;Lindmark-Mansson *et al.* 2000;Verdier-Metz, Coulon, & Pradel 2001).

5.1.4 Acute phase proteins in milk

It is known that intramammary infection results in increased permeability of the blood mammary barrier. Many serum proteins have been measured in milk including albumin, alpha-1antitrypsin (Sandholm & Mattila 1985), haptoglobin (Eckersall et al. 2001h), CRP (Hamann *et al.* 1997), and M-SAA3 (Eckersall et al. 2001). However recent work has focused on the major acute phase proteins Hp and M-SAA3 because they are not usually present in the serum or the milk from healthy cows.

The use of measuring the concentration of APPs such as Hp and M-SAA3 in milk as biomarkers for the earlier diagnosis of mastitis was suggested by Eckersall *et al.*, (2001) and Pedersen *et al.*, (2003) (Eckersall et al. 2001;Pedersen et al. 2003) .

Following the initial reports of APP in mastitic milk the potential for APPs in the diagnosis of mastitis was quickly recognised and numerous studies have examined the APP response in naturally and experimentally induced clinical and subclinical mastitis. Eckersall *et al* 2001 reported that SAA and Hp were present in the milk from naturally occurring cases of clinical mastitis. In this study determination of the diagnostic values of APPs in differentiating between healthy and mastitic cows gave sensitivities and specificities for milk Hp 86% and 100% respectively and 93% and 100% respectively for milk SAA. The SAA levels correlated with severity of disease as they were able to differentiate between mild (clots in milk) and moderate (clots accompanied by visible signs of inflammation in the udder) mastitis.

Pedersen *et al* 2003 reported elevated levels of Hp and M-SAA3 levels in milk when cows where experimentally infected with *S. uberis*. Increased levels of SAA were detectable in both milk and serum 6hrs and 11 hrs post inoculation respectively whereas increased levels of Hp where detected after10h of infection in serum only. These observations support the potential role of milk APPs as good early indicators of mastitis. The finding that M-SAA3 and Hp levels in milk were elevated without elevation of serum concentrations suggested that not all APPs in milk are a result of leakage from the blood system and may in fact be secreted more locally. A similar result was also reported by Hiss *et al* 2004 who showed that increased Hp levels in the milk were detectable 3 hrs post inoculation with LPS whereas changes in the concentration of Hp in the blood was not evident until 9 hrs post-inoculation (Hiss et al. 2004). Eckersall *et al* 2006 also reported that increased levels of Hp and SAA where detectable before increased levels in milk (Eckersall et al. 2006). These results have lead to the hypothesis that the Hp present is milk is not simply due to leakage from the blood but that cells within the mammary gland are synthesising and secreting Hp.

Most studies examining the levels of APPs in bovine mastitis have focused on individual cows milk. More recently Akerstedt *et al* 2006 reported that Hp and SAA are detectable at quarter, cow and bulk tank levels (Akerstedt *et al.* 2006). Significant relationships were found between HP, SAA and SCC at quarter and cow levels however only between SAA and SCC at the bulk tank milk level.

5.1.4 Extra hepatic expression of Hp

The principal site of haptoglobin synthesis is the liver (Urieli-Shoval *et al.* 1998) however extra hepatic expression has been reported for many species. In addition to the liver, Hp has been found to be synthesised in the lung (Yang *et al.* 1995), adipocytes (Friedrichs *et al.* 1995) endometriotic tissue (Sharpe-Timms et al. 1998;Sharpe-Timms et al. 2000a) the uterus (Hoffman et al. 1996) as well as the mammary gland of cows (Eckersall et al. 2001).

The finding that Hp levels in milk were increased before increases were evident in serum prompted Hiss *et al* 2004 to examine the expression of Hp mRNA from different regions of the mammary gland as well as liver using RT-PCR. Haptoglobin mRNA transcripts where detected by RT-PCR in RNA extracts from the teat, the cisternal region, the glandular parenchyma as well as the liver and Hp mRNA was upregulated following inoculation with LPS. Further support for mammary gland expression of APP's was provided by Eckersall *et al* 2006 who reported increased expression of mRNA for SAA and Hp in both liver and mammary tissue 48h after infusion with *S. aureus*.

Yang *et al* (1995) investigated the presence of Hp mRNA in lung epithelium. This group reported results from an *in vivo* experiment in which an APR was stimulated in mice by treatment with lipopolysaccaride (LPS). Thirty hours after induction of inflammation with LPS, a four-fold increase in Hp mRNA was observed in the lung and was equivalent to the level observed in the normal unstimulated liver. *In situ* hybridization was used to investigate the cellular location of Hp mRNA expression. A

strong signal for Hp mRNA was found on epithelial cells, while no significant signal was found in endothelium, smooth muscle cells, alveolar epithelium or macrophages. It was therefore concluded from this experiment, that Hp is synthesized by the respiratory epithelium.

Another similar *in vivo* experiment examined the response of mouse adipose tissue subjected to LPS in order to induce inflammation, and demonstrated a significant increase in Hp mRNA. Thirty hours after LPS injection, a six-fold increase in Hp mRNA was seen in the adipose tissue. *In situ* hybridization confirmed that the Hp expression within adipose tissue was confined to adipocytes (Friedrichs, Navarijoashbaugh, Bowman, & Yang 1995). These findings suggest that adipose tissue may contribute to the presence of Hp in extravascular body fluids as well as in the serum.

Piva and Sharpe-Timms, (1999) showed that Endo-1 a unique glycoprotein that had previously been shown by the group to be synthesised and secreted by rat and human endometrial explants shared 99.4% homology with human Hp (Piva & Sharpe-Timms 1999). Subsequently Sharpe-Timms *et al* (2000) looked at the expression and protein localization of endometrial haptoglobin using in-situ hybridisation and immunohistochemistry in endometriotic lesions (Sharpe-Timms et al. 2000;Sharpe-Timms et al. 2002). It was reported that Hp mRNA and protein were observed in the functionalis zone of endometrial stroma and the stroma of endometriotic lesions.

Ping *et al* 2005 reported that skin cells can synthesis and secrete haptoglobin. Using in situ hybridization it was shown that Hp mRNA was expressed in the epidermal

keratinocytes, the epithelial cells of hair follicles and sebaceous glands of normal skin and various skin complaints. However using immunohistochemistry with antisera to Hp protein only stained positively in epidermal keratinocytes of patients with inflammatory skin disorders such as psoriasis and erythroderma.

5.1.5 Extra Hepatic glycoforms of Hp

Following the research outlined above it is clear that Hp is not only synthesised by the liver but can be synthesised by many different cell types. It is apparent that extrahepatically expressed Hp differs from hepatically expressed Hp with most documented differences being due to differences within the carbohydrate moiety of Hp. A study looking at human endometrial form of Hp with comparison to the hepatic form reported that Hp secreted by the endometrial tissue had an analogous nucleotide sequence to hepatic haptoglobin but was 3kDa smaller than hepatic Hp (Piva *et al* 2002). Enzyme digestion carried out with recombinant flavobacterium meningosepticum N-glycase, which cleaves intact N-glycan chains from glycoproteins, resulted in both Hp isoforms appearing at 27kDa when analysed on a SDS-PAGE gel. Therefore it was concluded that the 3kDA weight difference between hepatic Hp and endometrial Hp was due to differences within the carbohydrate moiety with the suggestion that endometrial Hp carries one less glycan chain than hepatic Hp.

A study in rabbits looking at local production of haptoglobin following arterial balloon dilation reported that locally produced haptoglobin consisted of a unique set of glycoforms compared to haptoglobin synthesised by the liver. It was shown that aterial

Hp had a lower pI values than the hepatic Hp with the former having a pI at 4.5 and hepatic Hp being around pI at pH 5.5. It was also shown that arterial Hp had terminal mannose and fucose residues present whereas hepatic Hp did not have these residues present (Smeets *et al* 2003).

Mastitis is a major economical problem for farmers worldwide and research into this disease has been extensive. It has been known for many years that acute phase proteins are present in the milk, though it was originally thought that serum proteins such as the acute phase proteins where present in the milk due to leakage from the blood system. However more recently it was shown that the mammary gland itself has the ability to synthesis acute phase proteins such as Hp and SAA. The objective of this study was to determine if the glycosylation of mammary gland Hp is the same as hepatic Hp.

5.2 Materials and methods

5.2.1 Materials

All reagents were purchased form BioRad (Hemel-Hempstead, UK) unless otherwise stated.

5.2.1.1 Samples

Bovine serum haptoglobin was purchased from Life Diagnostics (USA). Milk haptoglobin was isolated from a pool of mastitic milk supplied by the Acute Phase Protein lab run by Professor Eckersall which had APP's concentrations previously measured and with Hp > 10μg/ml

5.2.1.2 Isolation of HP from milk

Bovine hemoglobin agarose, guanadinium hydrochloride, phosphate, were purchased from Sigma Aldrich.

A Beckman model J2-21 centrifuge was used to separate the whey fraction. The Akta Fast Protein liquid chromatography system used to run the bovine haemoglobin affinity

column and was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK) and controlled by a Compaq deskpro EN, which runs Unicorn version 4 software.

5.2.1.3 SDS-PAGE/Western blots

Criterion XT 4-12% precast gels, XT Mes running buffer, immunoblot PVDF membrane, extra thick criterion size blotting paper, 4-CN substrate. Sheep anti bovine haptoglobin was supplied by Life Diagnostics (USA) methanol was supplied by BDH. SDS-PAGE and western blots were carried in a criterion system supplied by BioRad (Hemel-Hempstead, UK)

5.2.2 Methods

5.2.2.1 SDS-PAGE/Western blot

SDS-PAGE was carried out using a 4-12% bis tris precast Criterion gel in the Criterion electrophoresis buffer tank. The gel was run using XT (2-N-Mopholino ethanesulfonic acid) MES buffer. Protein (5-10μg) in 5-10μl aliquots was added to equal volumes of sample buffer containing mercaptoethanol and heated at 95°C before loading 10μl of the mixture to each well of gel. A protein plus prelabelled molecular weight marker was run along side samples in order to estimate the molecular weight. Separation of the protein in the gels was achieved at 200V for 45-60 mins. Following separation the proteins were transferred to PVDF membrane which had previously been submerged in

methanol. The transfer was carried out at 100V for 1 h in transfer buffer. The membrane was then blocked with 10% dried milk (marvel) in Tris buffered saline buffer pH7.4 with 1% tween (TTBS) for 30 mins. Haptoglobin was then detected by incubation in a 1:1000 dilution of Life diagnostic anti-bovine Hp followed by incubation with a horse radish peroxidase labelled antibody to sheep IgG (1:1000 in TBS) and was then detected using a 4 CN substrate for peroxidase visibility. The membrane was washed 3X in TTBS for 5 mins between each step to remove excess reagents.

5.2.2.2 Isolation of HP from milk

Pooled mastitic milk was centrifuged at 50,000 g at 4°C for 30 mins to remove cells and lipid. The infranatant was carefully aspirated and then applied to a 1ml haemoglobin affinity column equilibrated with PBS and the unbound proteins were washed through with approximately 10 CV of PBS or until the absorbance returned to baseline levels. Haptoglobin was eluted using 100% 4M GnHCl then the buffer was exchanged with phosphate buffer saline pH 7.4 using centricon centrifugal devices. Isolation of Hp was confirmed by SDS-PAGE and western blotting.

5.2.2.3 Lectin blots

Lectin blots were carried out according to the method outline in section 4.2.2.7, with the modification that electroblotting was carried out in a Criterion system using PVDF membrane, which was soaked in methanol before the transfer.

5.2.2.4 PNGase F treated

Bovine Hp (100μg) from serum and milk was digested with 100 units PNGase F (EC number 3.5.1.52) according to the method outline from the supplier in order to cleave the N-linked chains attached to the polypeptide backbone as described in section 3.2.2.3.

5.2.2.5 MALDI-TOF Analysis

The protein band of interest was excised from the gel after staining by Coomassie blue staining and transferred to the proteomics unit of the University of Glasgow, (Dr R Burchmore) in the Sir Henry Wellcome Functional Genomic Facility, where the protein was digested with trypsin and analysed by matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF). The peptide mass fingerprint, obtained by MS was subjected to search by pasting the peptide masses into a protein database (MASCOT, Matrix Science), which uses a probability-based scoring system to define a significant match.

5.3 Results

5.3.1 Immunoblotting of Hp

Comparison of the blot of mastitic milk and serum from the same cow (Figure 5.1), with antisera to bHp showed 3 bands at 40kDa, 37kDa and 18kDa in mastitic milk whilst only the 37kDa and 18kDa bands were detectable in the serum.

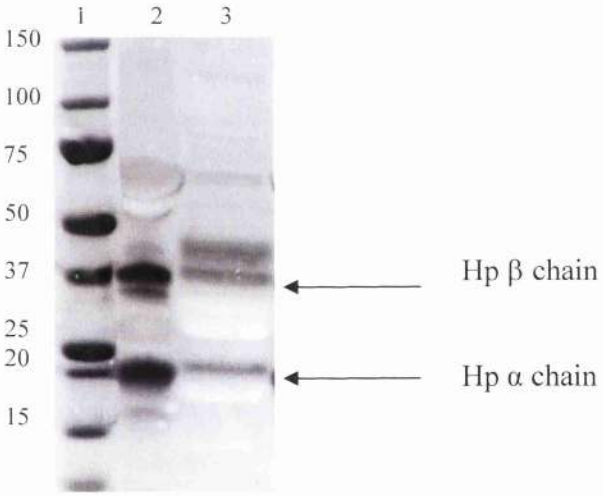


Figure 5.1 Western blot of serum and milk from the same cow probing for the presence of bovine haptoglobin. Lane 1 shows the precision plus molecular weight marker lane 2 serum and lane 3 milk from the same cow.

5.3.2 Isolation of Hp

In order to examine the carbohydrate moiety of milk haptoglobin it was first isolated using an affinity column of immobilised haemoglobin. Haptoglobin was initially retained on the column while fraction 3 shown in the FPLC trace in Figure 5.2 was found to be the haptoglobin containing fraction. Identification of the Hp containing fraction was carried out by SDS-PAGE and also confirmed by proteomic analysis by tryptic digest, MS and fragment fingerprinting (Figure 5.3 (37kDa band) + Figure 5.4 (40kDa band)).

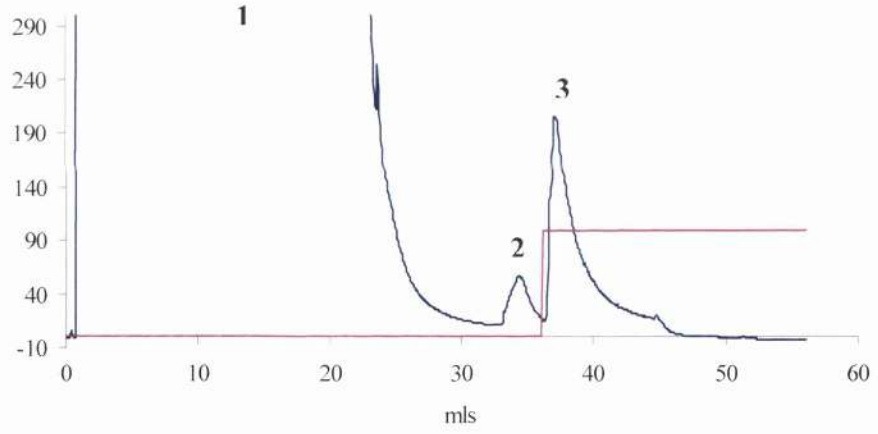


Figure 5.2 A representative chromatogram for the purification of haptoglobin from milk. The (pink) line represents the elution buffer.

Q9MYV8_BOVIN Mass: 11289 Score: 197 Queries matched: 7

Haptoglobin (Fragment).- Bos taurus (Bovine).

☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
	28	408.3110	814.6075	814.4549	0.1526	0	16	43	4	K.DITPTLR.L
	46	416.2917	830.5689	831.4854	-0.9165	0	11	1.6e+02	4	R.WLLTTAK.N
☑	155	460.8312	919.6478	919.4552	0.1926	0	13	64	1	K.GSFPWQAK.M
☑	184	479.8551	957.6956	957.5131	0.1825	0	23	5.9	1	K.NQLVEVEK.V
☑	219	507.8942	1013.7738	1013.5869	0.1869	1	31	0.95	1	K.AKDITPTLR.L
☑	507	942.1715	1882.3284	1881.9730	0.3554	0	103	2.9e-08	1	K.MVSQHNLISGATLINER.W
☑	508	950.1722	1898.3298	1897.9679	0.3619	0	(101)	5e-08	1	K.MVSQHNLISGATLINER.W + Oxidation (M)

Figure 5.3 The mascot test carried out on the 37kDa band of the doublet from milk haptoglobin following MALDI-TOF analysis

Q9MYV8_BOVIN Mass: 11289 Score: 167 Queries matched: 4

Haptoglobin (Fragment).- Bos taurus (Bovine).

☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	25	408.3020	814.5895	814.4549	0.1347	0	24	7.2	1	K.DITPTLR.L
	145	480.3227	958.6308	957.5131	1.1178	0	20	16	2	K.NQLVEVEK.V
☑	534	942.1715	1882.3284	1881.9730	0.3554	0	(92)	3.6e-07	1	K.MVSQHNLISGATLINER.W
☑	536	950.1722	1898.3298	1897.9679	0.3619	0	124	2.4e-10	1	K.MVSQHNLISGATLINER.W + Oxidation (M)

Figure 5.4 The mascot test results for the 40kDa band of the doublet from milk haptoglobin following MALDI-TOF analysis.

5.3.3 Effect of PNGase F digestion

In order to identify if the difference between the βHp chain of the milk and its serum counterpart is due to the amino acid or the carbohydrate moiety, oligosaccharide chains where cleaved from the polypeptide backbone using PNGase F. The polypeptide backbone was then run out on an SDS-PAGE gel in order to see the effect on the milk Hp. As seen in Figure 5.5A that milk haptoglobin (lane 3) has major bands at 37kDa and 18kDa while in serum multiple bands were seen when Coomassie blue stained. However following enzyme digestion serum and milk haptoglobin has a β chain molecular weight of approximately 30kDa. The bands seen at approximately 35kDa represent the PNGase F and the band seen at 37kDa in the serum is probably due to incomplete cleavage. The band at 18kDa in both is the α Hp chain.

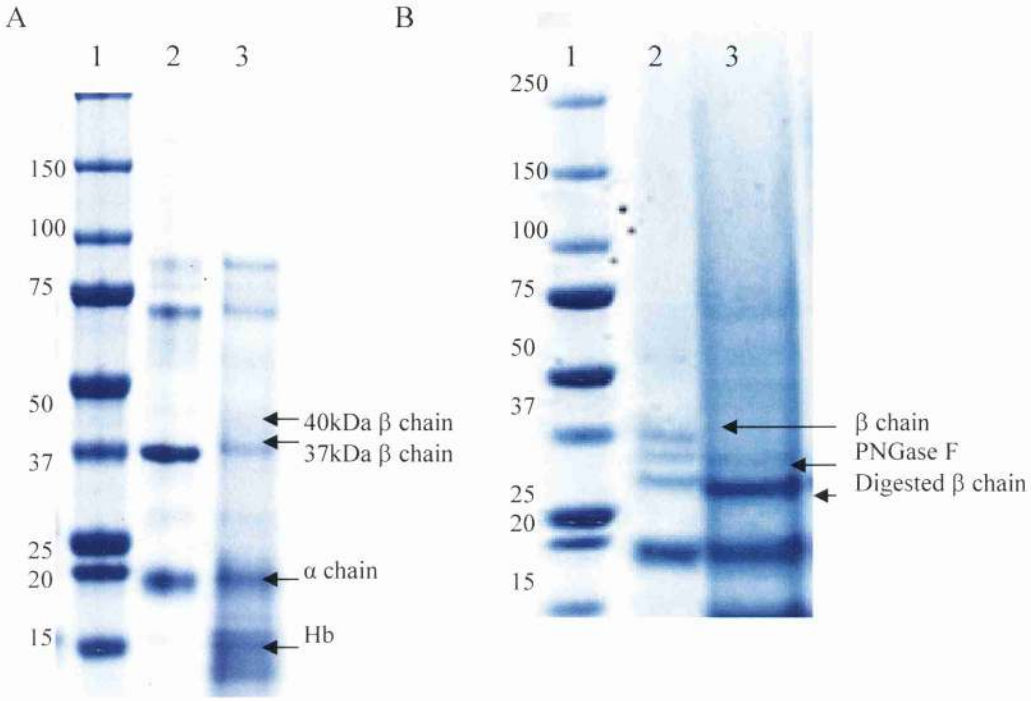


Figure 5.5 A) SDS-PAGE of isolated bovine haptoglobin from serum in lane 2 and bovine haptoglobin present in milk in lane 3. B) shows the SDS-PAGE of bovine serum and milk haptoglobin following enzyme digestion with PNGase F.

5.3.4 Lectin binding of bovine Hp

The carbohydrate moiety of the βHp bands found in milk and serum were examined using lectins following western blotting (Figure 5.6). Figure 5.6A shows bovine haptoglobin from serum and milk which was probed with Con A for oligosaccharide branching (diantennary) examination and both milk and serum reacted strongly with Con A but only the lower (Mwt 37kDa) band of milk βHp gave a reaction and the upper band of the doublet did not react with Con A. When bovine Hp from serum and milk was probed with SNA (Figure 5.6B) for the presence of α2-6 linked SA it was shown that both milk and serum bovine Hp reacted with SNA however it was also shown that that the 40kDa and 37kDa bands of milk reacted with SNA. When bovine Hp was probed for the presence of fucose using AAL it was found that only the 40kDa band of milk Hp reacted with AAL see Figure 5.6C lane 2. Bovine Hp from milk and serum was also examined for the presence of α2-3 linked SA by MAA lectin however no reaction was seen.

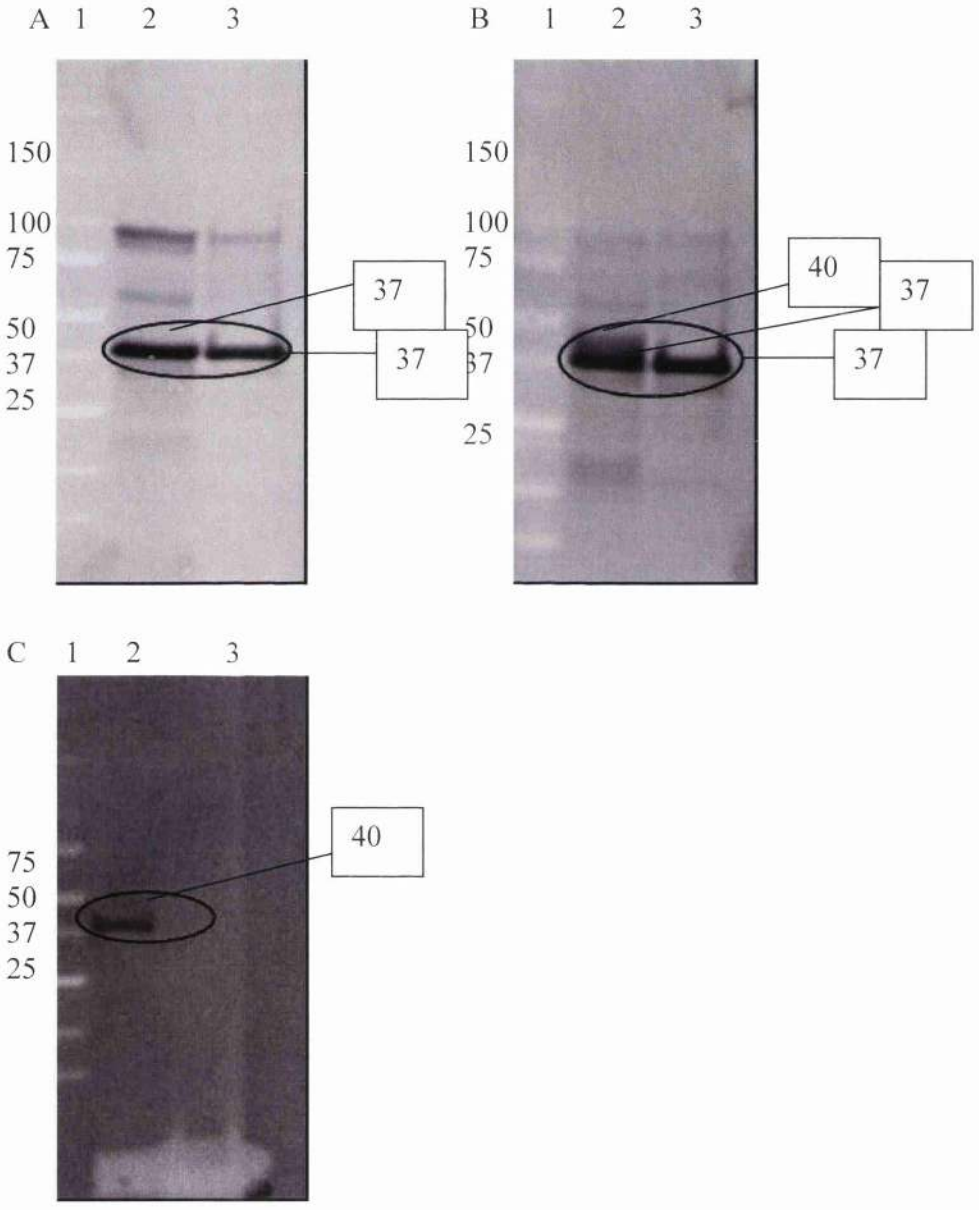


Figure 5.6 Shows the fluorescent lectin blots of milk Hp versus serum haptoglobin from cows A) against Con A B) against SNA C) against AAL. Track 1 Mwt, Track 2 milk Hp, Track 3 Serum Hp.

5.4 Discussion

It has been extensively reported that Hp is a major acute phase protein in cattle (Eckersall 1995;Gruys, Obwolo, & Toussaint 1994;Schroedl *et al.* 2001). Until recently most studies looking at the concentration of bovine Hp in various disease conditions such as mastitis have focused on serum levels of the protein (Conner et al. 1986;Conner et al. 1989;Hirvonen, Pyorala, & Jousimies-Somer 1996;Horadagoda et al. 1999;Makimura & Suzuki 1982) however the discovery of Hp in milk (Eckersall, *et al* 2001) means that a number of studies have recently focused on the levels present in milk. It was initially thought that the presence of serum proteins in the milk wholly originated from the blood and had simply leaked into the milk through a disrupted blood mammary barrier. However McDonald *et al* (2001) reported a mammary associated form of SAA that was produced by mammary epithelial and was the SAA3 isoform. It has been reported that mammary associated SAA3 differs from hepatic SAA sharing only 80%similarity (McDonald et al. 2001).

Although Hp is present in milk during mastitis it remains unclear whether or not the Hp isoform in the milk is a mammary associated isoform of Hp that differs from Hp synthesized by the liver. For this reason milk and serum from a dairy cow with mastitis was probed for the presence of haptoglobin by western blotting with a polyclonal antibody to bovine haptoglobin. It was found that milk had three haptoglobin bands compared to two in serum. The α subunit showed no difference in Mwt between serum and milk. The extra band in milk creates a doublet at 37kDa and 40kDa whereas in serum only the band at 37kDa was present. The lower band of the doublet corresponds

with β subunit band seen in serum. The novel band of the doublet seen in the milk could be due to de novo synthesis of Hp in the mammary gland. As the new band is a higher Mwt than the beta subunit of serum, it is unlikely to be due to secretion modification as this would mostly likely lead to a lower Mwt due to deglycosylation. The presence of the second band of βHp could be explained as being the product of intra mammary synthesis (Eckersall, Young, McComb, Hogarth, Safi, Weber, McDonald, Nolan, & Fitzpatrick 2001a;Hiss et al. 2004). A remaining question is whether the mammary produces both the 37kDa and 40kDa isoforms detected in the milk or whether the 40kDa isoform is the sole produce of the milk and the 37kDa form is a serum protein leaking from the circulation. However before this can be addressed it is important to verify that the 40kDa band is indeed Hp and identify the cause of the increase in size. To undertake this task, Hp had to be purified from mastitic milk and serum to allow analysis to proceed.

Haptoglobin has been isolated from the sera of many species using many different approaches however many procedures have involved multiple steps. To date no one has published data outlining a method for the isolation ofHp from bovine milk.

Morimatsu *et al.*, (1991) isolated bovine haptoglobin from sera by initially fractionating using ammonium sulphate followed by a complexed 3 step chromatography procedure making it time consuming and impractical (Morimatsu *et al.* 1991). Yang and Mao, 1999 isolated porcine haptoglobin using a novel HPLC chromatography procedure using superose 12 gel permeation chromatography. An ammonium sulphate fractionation step was required before chromatography was carried out (Yang & Mao 1999). Haptoglobin was isolated from human plasma by Liau *et al* 2003, isolated haemoglobin from human blood was coupled to CNBr-activated sepharose-4b. Human

plasma was then passed through the column with PBS. The haptoglobin remained bound to the hemoglobin column and was then eluted with 5M urea (Liau *et al.* 2003).

In the same year Hiss *et al* 2003 reported isolation of porcine haptoglobin from sera using a haemoglobin affinity column followed by gel filtration (Hiss *et al.* 2003) which was then repeated for the isolation of Hp from bovine sera (Hiss et al. 2004a).

In this study haptoglobin was isolated from bovine milk using a method similar to that used by Liau *et al* 2003 with the difference that in this method Guanadinium HCl was used to elute the haptoglobin rather than urea (Liau, Chang, Pan, Chen, & Mao 2003). Haptoglobin is however only present in cattle when an acute phase response is present therefore haptoglobin had to be isolated from milk from cows with mastitis rather than from healthy cows but such milk often has large clots making it incompatible with chromatography. Hence the centrifugal step before application to the FPLC system. The method of isolation used in this study was very effective and relatively simple having only two steps. The method enhanced the purity of milk HP sufficiently from characterisation of the glycan component. However as the haemoglobin agarose started to near its shelf life, haemoglobin was also found in the haptoglobin containing fraction. Although the novel 40kDa isoform of Hp, seen in mastitic milk was identified by reaction with antisera to bovine Hp, there could be a possibility that the commercial antisera used was cross reacting with other proteins in milk. The identity of the upper band (40kDa) was verified by MALDI-TOF which showed that both bands of the doublet were indeed Hp.

Enzymatic digestions and lectin binding blots were performed to determine if the difference in the Mwt of the novel β subunit is due to carbohydrate content or the polypeptide backbone and secondly to identify any differences between the carbohydrate moiety of bovine serum Hp and bovine milk Hp, which may provide insight to milk Hp functions.

The β chains of human hepatic Hp each have four Asn-X-Ser/Thr sequons (Asn 23, Asn 46, Asn50, and Asn 80) all of which may be occupied by N-linked glycan chains (Kurosky *et al* 1980: Black *et al* 1970). The carbohydrate content of hepatic Hp is approximately 20% of β chain mass (Black & Dixon 1970) and is found external to the protein, exclusively as N-linked, complex oligosaccharides chains (Kurosky et al. 1980d;Turner 1992) Digestion of bovine serum Hp and milk Hp using PNGase F to cleave the N-linked glycans from the polypeptide backbone revealed that the protein core of both the 37 and 40 kDa forms of βHp have a similar size of 30kDa and that approximately 20% of the 37kDa form of βHps and 25% of the 40kDa form of βHp was accountable to N-linked glycan chains. Therefore the size difference seen between the two β chains of milk Hp is due to differences within the carbohydrate chain. This is not unexpected as studies of hepatically and extra hepatically expressed Hp have identified differences that are due to the carbohydrate moiety rather than the polypeptide backbone (Piva, Moreno, & Sharpe-Timms 2002).

Possible differences between the glycoforms of milk and serum Hp were examined by probing with the lectins SNA, Con A and AAL which are used for the detection of α2-6 SA, diantennary branching and Fuc residues respectively. From the study it was shown that both bands of the milk β chain and the serum β chain had α 2-6 linked sialic acid

present on their glycan chains as shown by binding with SNA. Only the 37kDa βHp in milk and serum reacted with Con A while the 40kDa βHp in milk had no reaction with this lectin suggesting the glycan chains attached to this β chain isoform are more highly branched than diantennary chains with perhaps three or four branches. This would account for the molecular weight difference between the two forms of β chain seen in milk and is a novel glycosylation as Hp of various species studied to date have been shown to have diantennary N-linked glycan chains. For instance HPLC analysis of glycans derived from immunopurified human Hp revealed the presence of diantennary complex glycans in a neutral, monosialylated and bisialylated forms and lower amounts of triantennary complex glycans that were present in disialylated and trisialylated forms. About 75% of charged Hp glycans were of diantennary complex structure some with one terminal sialic acid missing. Triantennary structures made up 25% and highly branched glycan pools did not exceed 1%. (Ferens-Sieczkowska & Olczak 2001).

The novel milk Hp isoform had fucose residues present within the glycan chains whereas serum Hp appeared to have no Fuc residues present as the latter had no reaction with AAL. Increased fucosylation is a common finding in disease states. This can be used to differentiate different diseases for example fucosylated Hp is useful for discriminating between active and inactive RA as 94% of patients with active RA have high fucose content on Hp versus 5% of inactive RA (Thompson et al. 1993;Thompson, Stappenbeck, & Turner 1989). Fucosylated Hp has also shown potential as a prognostic marker as it can be used to monitor disease activity in cancer. Levels of abnormally fucosylated forms Hp have been measured in blood specimens from women with carcinoma of the ovary or breast that are undergoing chemotherapy. The levels of fucosylated Hp increased if the women had progressive disease and decreased if they showed complete response to therapy (Thompson, Dargan, & Turner 1992a).

In conclusion Hp in milk comprises two distinct forms a serum derived form and a mammary derived form. The mammary derived isoform of Hp has more highly branched glycoforms than serum Hp that carries α2-6 linked SA and Fuc residues.

CHAPTER 6

6. Comparative investigation of the glycosylation of AGP

6.1 Introduction

Alpha-1-acid glycoprotein has been identified in the serum of all mammalian species studied to date including pig, cow, sheep, horse, dog, cat, rat, mouse and man. It has been shown that AGP from all of these species is heavily glycosylated with 3-6 N-linked glycans attached (Alava et al. 1997;Duthie et al. 1997f;Hunter & Games 1995;Matsunaga, Sadakane, & Haginaka 2004;Pocacqua, Provasi, Paltrinieri, Gelain, Comunian, & Ceciliani 2005;Taira et al. 1992;Yoshima et al. 1981) Alpha-1-acid glycoprotein has been recognized as an important drug binding protein in chickens, dogs and cows as well as humans (Matsumoto *et al.* 2002;Sadakane *et al.* 2002). Alpha-1-acid glycosylation is often grouped as an immunocalin due to its immunomodulatory activities which have been discussed at length in chapter 1 with regards to human AGP. Very little work has been carried in the veterinary science field to identify the functional role of AGP in other species.

The drug binding capabilities of AGP and the binding site of human AGP have been well characterised. It is now widely accepted that AGP is an important drug binding protein in the serum as it binds both endogenous and exogenous ligands. Some research has also been carried out on this topic in the other species. Matsumoto *et al.,* 2002 carried out an experiment in order to examine the differences between the drug binding capabilities of bovine and canine AGP compared to human AGP. They reported that human AGP was capable of binding basic and acidic ligands as well as steroid hormones whereas both bovine and canine AGP were only able to bind basic ligands and steroid hormones (Matsumoto, Sukimoto, Nishi, Maruyama, Suenaga, & Otagiri 2002). Son *et al.,*(1998) also reported species specific differences in the binding of cow, dog, sheep and human AGP to lincomycin and clindamycin (Son *et al.* 1998).

Though many studies have reported immunomodulatory activities attributed to human AGP, recently bovine AGP has been studied by Ceciliani *et al.,* (2007) who reported that bovine AGP was capable of inhibiting monocyte apoptosis. It was also shown that the inhibitor effect of bovine AGP on monocyte apoptosis was not only dependant on plasma concentration but also on the presence of sialic acid on the glycan chains attached to bovine AGP (Ceciliani *et al.* 2007).

A few studies have examined the glycan chains attached to AGP from various species. Kuster *et al.,* (1998) used an in gel digestion followed by MALDI MS to characterise the glycan chains attached to AGP from man, cow, sheep and dog. Major species specific variations in glycan moiety were reported. Cow, sheep, and dog were reported to have predominantly diantennary glycans within the carbohydrate moiety whereas human AGP had triantennary chains as a major glycan type. Alpha-1-acid glycoprotein

from human and bovine was shown to express fucose residue and bovine and ovine AGP was reported to express NGNA and NANA.

Another study carried out by Nakano *et al.*,(2004) investigated the glycan chains of AGP from human, bovine, ovine and rat AGP using MALDI-TOF. It was reported that all species showed distinctive variation with the glycan chains from AGP (Nakano, Kakehi, Tsai, & Lee 2004). Human AGP was reported to carry di-tri and tetraantennary carbohydrate some of which had a fucose residue substituted which was part of the SLeX epitope. It was reported that the most abundant glycan types attached to sheep AGP were mono and disialylated diantennary chains, however the presence of tri and tetra antennary chains were detectable but were minor. Unlike human AGP sheep AGP had both NGNA and NANA within its glycan chains. Nakano reported that bovine AGP carried predominantly diantennary chains though some of the diantennary chains of bovine AGP were carrying three and four sialic acid residues and NGNA was the predominant sialic acid within the glycan chains.

It has been shown that biological functions of glycoproteins are dependant on the glycan chains attached to the protein. Therefore in order to truly understanding the biological function of a glycoprotein the structure of the glycan chains must be considered. Therefore it was the aim of this study to analyse the glycan chains attached to species such as cows, cats, and sheep and compare them to the more extensively studied human AGP.

6.2 Materials and methods

6.2.1 Materials

6.2.1.1 Samples

Feline AGP was isolated from ascitic fluid from cat 10 (section 2.3.1) kindly provided by Dr D Addie. Human, bovine and ovine AGP were purchased from Sigma (Poole, UK).

6.2.1.2 Monosaccharide and oligosaccharide analysis

All reagents and suppliers are listed in section 3.2.1.2 for monosaccharide analysis and section 3.2.1.3 for oligosaccharide analysis.

6.2.1.3 Sialic acid analysis

N acetyl neuraminic acid and N glycoyl neuraminic acid were supplied by Sigma (Poole, UK)

Sodium hydroxide (NaOH) 50% (w/v) was purchased from BDH (Poole, UK) and the HPLC grade water was obtained from Rathburn (Walkerburn, UK).

Neuraminidase (EC number 3.2.1.18) and buffers were supplied by New England Biolabs (Herts, UK). Ethanol was supplied by university stores.

Sialic acid analysis was carried out on a Dionex (California, USA) DX-500 High pH anion exchange chromatography system using pulsed amperometric detection (HPAEC-PAD), which utilizes a GP40 gradient pump and an ED 40 electrochemical detector controlled by a desktop computer running Peaknet software. Separation was carried out on a Carbopac-100 ™ column and guard column (250x4mm and 50x4mm respectively) purchased from Dionex (California, USA)

6.2.1.4 Isoelectric focusing

Clean IEF gel, Pharmalyte 2.5-5, Broad pI protein marker, Low pI protein marker, Ampholine PAg plate pH 3.5-9.5 Coomassie blue Tablets and electrode strips were purchased from GE healthcare (Buckinghamshire, UK). Orthophosphoric acid was supplied by Sigma (Poole, UK). Sodium hydroxide, acetic acid, urea and glycerol were purchased from BDH (Poole, UK).

Isoelectric focusing was performed using a LKB Ultrophor electrofocusing unit GE healthcare, (Buckinghamshire, UK) powered by a Consort E815 electrophoresis power supply from Sigma (Poole, UK).

6.2.1.5 SDS-PAGE

All reagents and suppliers are listed in section 2.2.1.3.

6.2.2 Methods

6.2.2.1 Monosaccharide and Oligosaccharide analysis

See section 3.2.2 for a detailed outline of the methods used to analyse the monosaccharides and oligosaccharides. Human, bovine and ovine AGP (100μg) and fAGP (66μg) was hydrolyzed by TFA and HCl prior to monosaccharide analysis, while 100μg of human, bovine and ovine AGP and 50μg of fAGP were digested with PNGase F prior to oligosaccharide analysis.

6.2.2.2 Sialic acid analysis

Human, bovine and ovine AGP (100μg) and 50μg of fAGP were digested with 100 units of neuraminidase in buffer supplied by the manufacturer in a total volume of 20μl overnight at 36°C before being precipitated with 500μl ice cold ethanol. Ethanol precipitation was carried out as was detailed in section 3.2.2. The supernatant from the ethanol precipitation was removed and dried under vacuum using a centrifugal evaporator (Jouan) and reconstituted in 25μl of HPLC grade water and then injected on

to the HPAEC-PAD system. The precipitant from the ethanol precipitation was retained for further analysis

6.2.2.3 SDS-PAGE

See chapter 2 for detailed description of method (section 2.2.1.5)

6.2.2.4 Lectin blots

Lectin blots were carried as described in section 4.2.2.7 with 10μg of AGP from each species being analysed.

6.2.2.5 Isoelectric focusing

Isoelectric focusing was carried out using preformed polyacrylamide gels (clean IEF gel) on plastic backing rehydrated overnight with 7.5% v/v pharmalyte pH 2.5-5 or a preprepared ampholine PAg plate pH 3.5-9.5. Electrode strips were soaked with 1M phosphoric acid and 1M NaOH and placed overlying the gel at the anode and cathode side of the gel respectively. Prefocusing was carried out at 650V, 30mA, 14 W for 20 mins before samples were applied. Samples were applied 1 cm from the cathode end of the gel using sample application strips and focusing was carried out at 1200V, 25mA, 10W for 2hrs 30mins to 3 hrs 30 mins dependant on sample composition. The gel was then fixed using a fixing solution made up of 2.9% (w/v) trichloroacetic acid and 0.85 % (w/v) sulphosalicylic acid for 30-45 mins. The gel was the rinsed with destain

solution made up of 25% v/v ethanol, 8% v/v acetic acid in $dH_2O$for five minutes before staining with Coomassie blue protein stain (1 R Tablet in 1L of destain solution) which was heated to 60°C prior to application and stained for 15 minutes. After staining the gel was incubated in destain solution overnight or until most of the background stain had been removed. The gel was then preserved in a 10% (v/v) glycerol solution for 1 hr before being scanned and stored at 4°C. Isoelectric focusing in flatbed polyacrylamide gels separates proteins by establishment of a pH gradient which allows proteins to migrate to their isoelectric point (pI), which is the point at which the protein has no net charge. The isoelectric point of sample bands was determined using a mixture of markers of known low pI, of which 10µl was applied to each gel. The migration from the cathodal end was plotted against the pI of the markers to construct a calibration curve. Through measurement of the migration distance from the cathode for the sample bands the isoelectric point was calculated from the calibration curve.

6.3 Results

6.3.1 Monosaccharide analysis

HPAEC-PAD analysis of the monosaccharides released from feline and bovine AGP showed very similar patterns on the chromatograms which can be seen in Figure 6.1B+C and 6.1D+E respectively. The chromatograms seen in Figure 6.1 B+C for fAGP where duplicate runs of cat 10 section 3.3.1. These runs were carried separately from the feline AGP study described in Chapter 3. The chromatograms from both these species had four peaks representing the internal standard, N-acetyl glucosamine, galactose and mannose respectively. The chromatograms of the monosaccharides released from ovine (Figure 6.1 F+G) and human AGP (Figure 6.1 H+I) differed in that they both had five monosaccharide peaks. In sheep the fifth peak which was eluted after the internal standard but before the N-acetyl glucosamine (denoted by * in Figure 6.1 F+G) and was identified as N acetyl galactosamine as N-acetyl galactosamine was run as a standard and eluted at the same time as the peak seen in sheep AGP. The fifth monosaccharide that was found in human AGP had the same elution time as peak one in the monomix and was therefore identified as fucose residues.

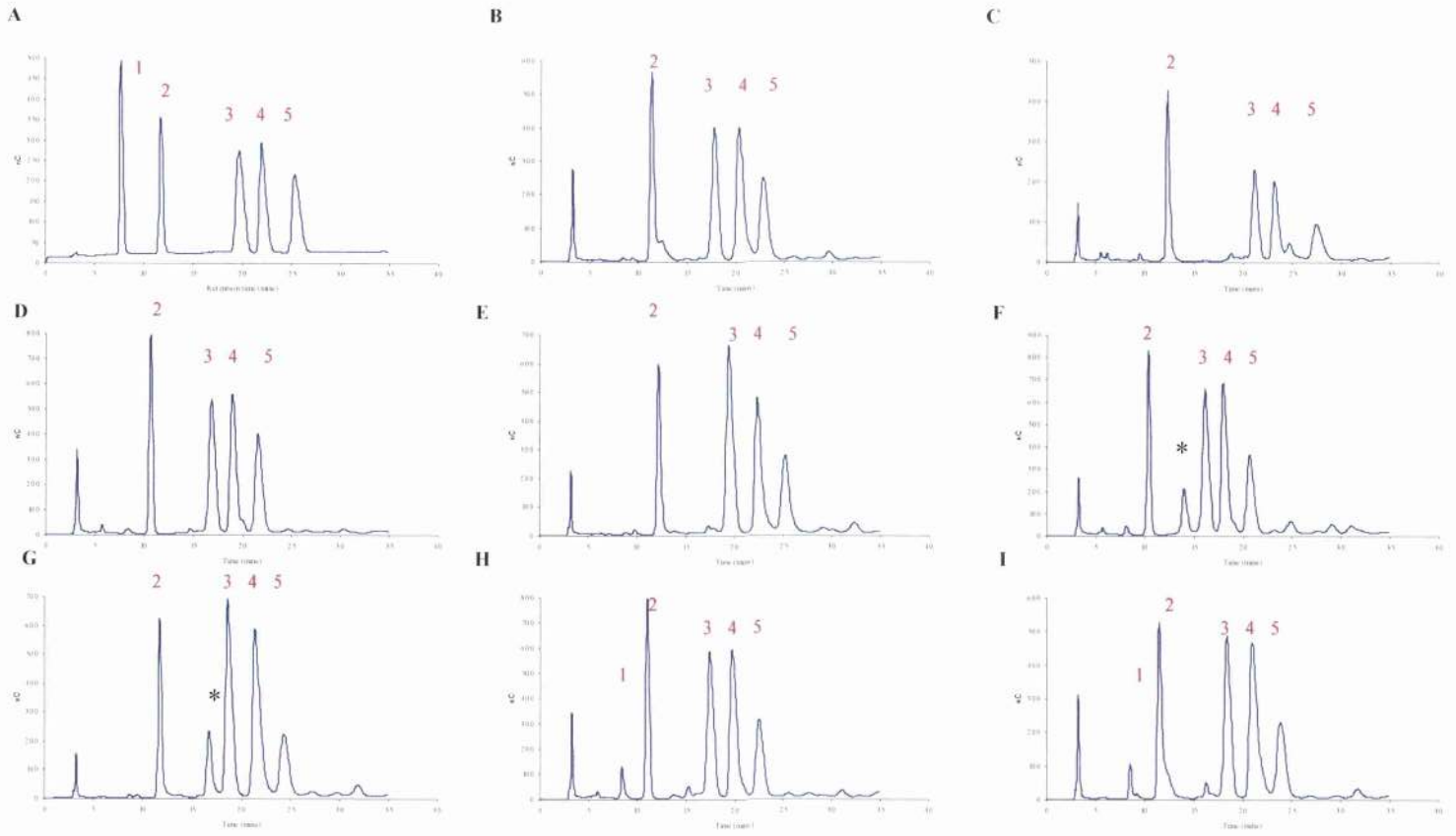


Figure 6.1 HPAEC-PAD chromatogram of monosaccharides A) Monomix B+C) Feline AGP D+E) Bovine AGP F+G) Ovine AGP H+I) Human AGP

1=Fucose, 2= Internal standard, 3= N-acetylglucosamine, 4=Galactose, 5=Mannose and *= N-acetylgalactosamine

6.3.2 Oligosaccharide analysis

HPAEC-PAD is also capable of determining differences in oligosaccharide profiles between individual samples. Oligosaccharide chains were released from AGP enzymatically using PNGase F. The detached oligosaccharides were separated from the protein in the sample by ethanol precipitation before being applied to the HPAEC-PAD. Oligosaccharide chains were primarily separated according to the amount of sialic acid residues present in each glycan chain. Thereafter the resolution of peaks within each charge band was on the basis of many subtle differences such as fucosylation, branching, and type of linkage between sialic acid and the galactose residue.

A standard oligosaccharide sample of bovine fetuin (Figure 6.2 A) was used to identify oligosaccharides carrying different numbers of sialic acid, with disialylated chains eluting between 20-30mins, trisialylated chains eluting between 30-40 mins and tetrasialylated at > 40 mins. Figure 6.2B+C shows the oligosaccharide chain profile chain of fAGP, which had oligosaccharide chains eluting between 20-40 mins, with three peaks between 20-30mins (disialylated) and three peaks between 30-40 mins (trisialylated). The oligosaccharide chains cleaved from human AGP (fig 6.2 H+I) showed a similar profile with all peaks being eluted between 20-40 mins. In contrast the oligosaccharide profiles of the carbohydrate moiety of bovine (fig 6.2 D+E) and ovine AGP (fig 6.2 F+G) showed a much more complex profile with over twenty peaks being eluted between 10 and 50 minutes after the initiation of the run. The bovine AGP profile showed five peaks eluting between 10-20 mins (monosialylated), eight separate peaks between 20-30 mins (disialylated), 6 peaks between 30-40 mins (trisialylated) and at least 3 peaks between 40-50 mins (tetrasialylated). The oligosaccharide profile of

ovine AGP showed a similar profile however subtle differences could also be observed, 3 peaks were eluted between 10-20 mins (monosialylated), 6 peaks between 20-30 mins (disialylated), 6 peaks between 30-40mins (trisialylated) however the presence of peaks eluting between 40-50 mins remains unclear. Although the oligosaccharide profile of each species was unique, similarities could be seen as the oligosaccharides profiles from all species showed that disialylated oligosaccharide are predominant, followed by trisialylated oligosaccharides.

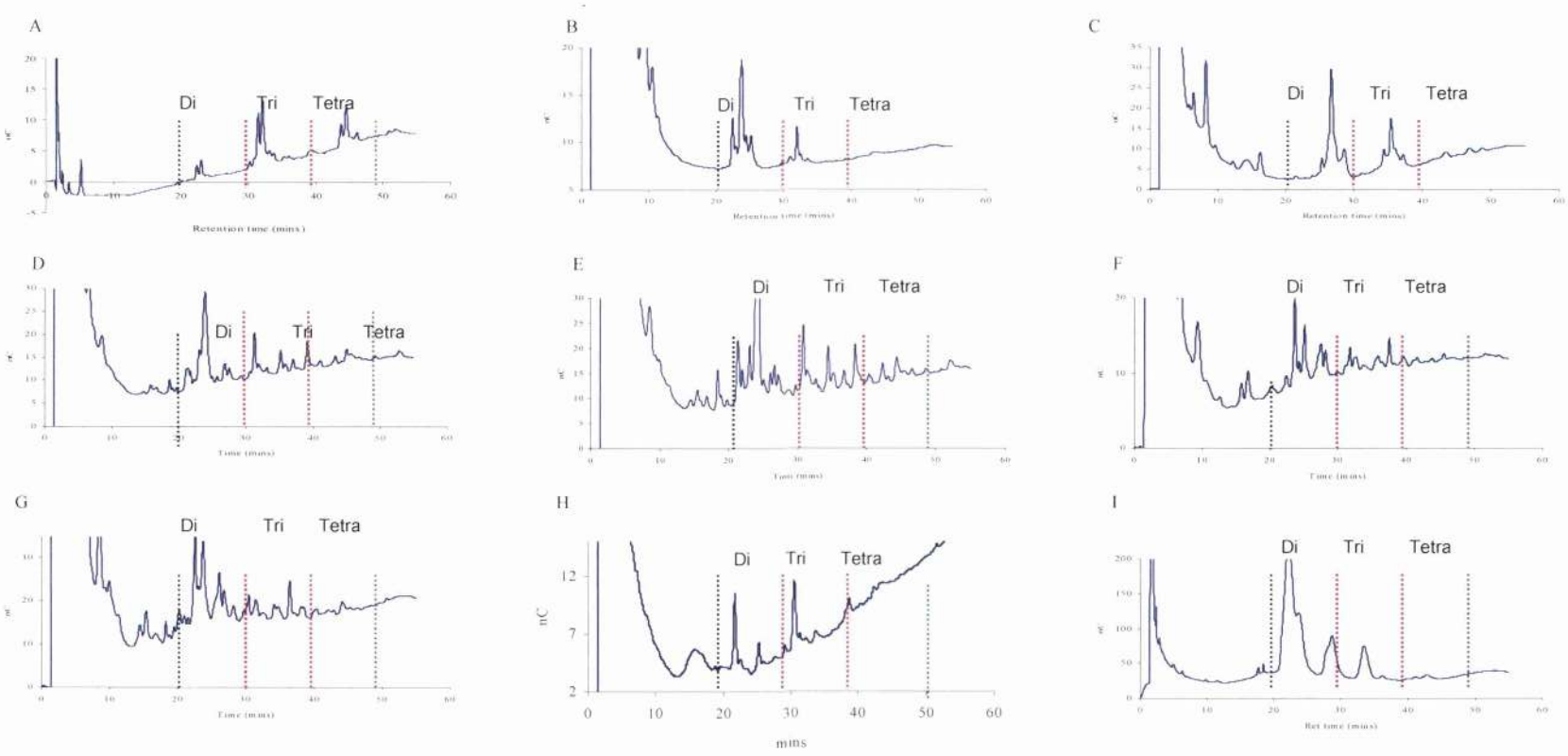


Figure 6.2 HPAEC-PAD chromatogram of monosaccharides A) oligosaccharide standard from bovine feutin B+C) Feline AGP D+E) Bovine AGP F+G) Sheep AGP H+I) Human AGP. Di=disialylated, Tri=trisialylated, Tetra = tetrasialylated.

6.3.3 Sialic acid analysis

Sialic acid was cleaved from the oligosaccharide chains attached to AGP from bovine, feline, ovine and human AGP using neuraminidase. Following enzyme digestion the sialic acids were subsequently characterised using HPAEC-PAD. Figure 6.3 A-I shows the chromatograms from HPAEC-PAD analysis of sialic acid. Figure 6.3A shows the chromatogram for standard N-glycoyl neuraminic acid in which a peak was observed at approximately 20 mins whereas the N-acetyl neuraminic acid standard eluted at approximately 5 mins (Figure 6.3 B). Comparison to these standards highlighted that bovine and ovine AGP both have large amounts of both N-acetyl neuraminic acid and N-glycolylneuraminic acid (in Figures 6.3 E-F and Figure 6.3 G-H respectively). FAGP was also shown to have both N-acetyl neuraminic acid and N-glycolyl neuraminic acid, however there were only trace amounts of the latter were found. Human AGP only had N-acetylneuraminic acid present in the glycan chains as a peak at 5 mins was the only one found (see Figure 6.3 I-J).

6.3.4 Lectin blot analysis of AGP.

From the lectin blot analysis of AGP it was shown that AGP from bovine, ovine, feline and human serum had high levels of α2-6 sialic acid present on the glycan chains (see Figure 6.4A) as SNA bound to all samples. It was also shown by reaction with Con A that all four species had diantennary chains within the carbohydrate moiety although human AGP reacted less with this lectin than AGP from the other species (Figure 6.4C lane 4). In contrast only human AGP reacted with AAL (Figure 6.4 B lane 5), suggesting that feline, bovine and ovine AGP had no fucose residues present within their carbohydrate moieties.

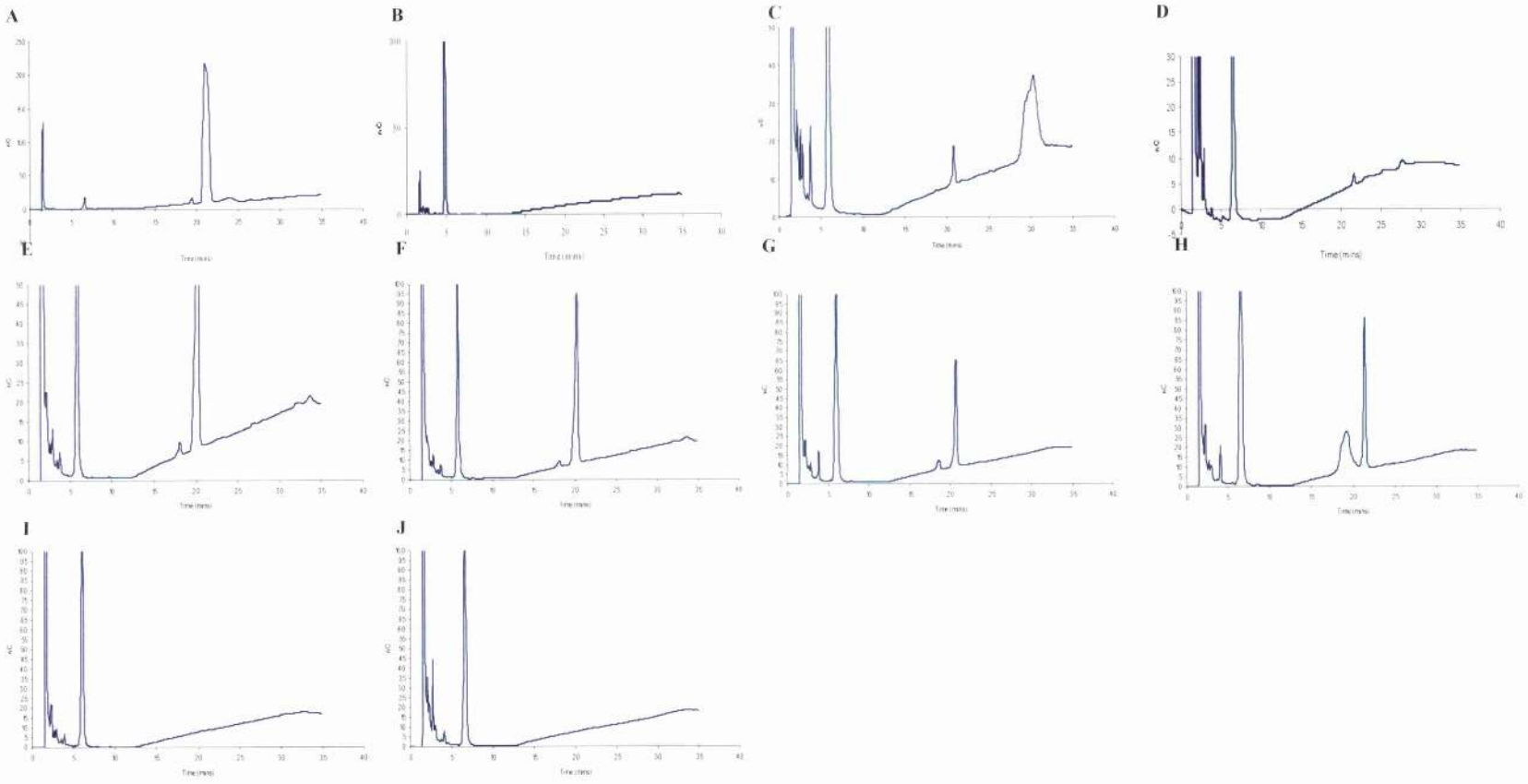


Figure 6.3 The HPAEC-PAD chromatograms of sialic acid A) N glycoyl standard B) N Acetyl Standard C+D) Feline AGP E+F) Bovine AGP G+H) Sheep AGP I+J) Human AGP

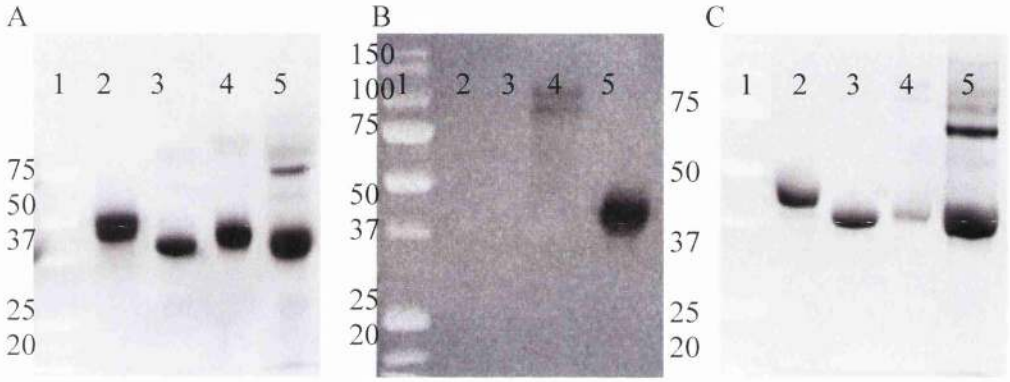


Figure 6.4 Lectin blots for A) SNA lane 1 Mwt marker, lane 2 feline AGP cat 14, lane 3 bovine lane 4 human lane 5 sheep B) AAL lane 1 Mwt marker, lane 2 feline AGP (Cat 14), lane 3 bovine AGP, lane 4 sheep AGP, lane 5 Human AGP C) Con A Lane 1 Mwt marker, lane 2 feline AGP cat (?), lane 3 bovine, lane 4 human AGP, lane 5 sheep AGP.

6.3.5 Isoelectric Focusing

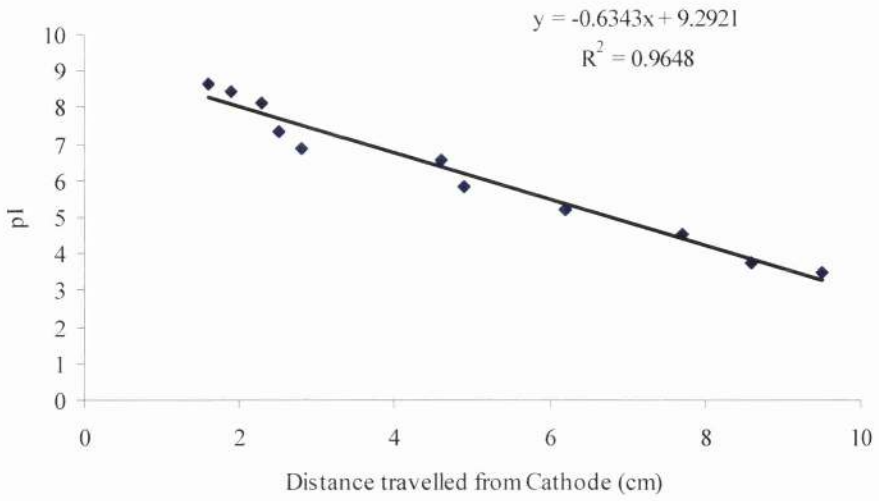
Calculation of pI was based on the calibration over the pH range 3.5-9.5 (Fig 6.5A) and in the narrow range gels pH 2.5-5.5 (Fig 6.5B)

The IEF pattern of a glycoprotein can be influenced by both the amino acid composition of the polypeptide backbone and the presence of sialic acid residues within the oligosaccharide chains covalently attached to the protein. The influence of sialic acid residues on the pI was examined by treating the AGP from the four species with neuraminidase and running them on a 3.5-9.5 IEF slab gel (Figure 6.6).

When run on this broad pI IEF gel it was revealed that removal of sialic residues resulted in a shift towards the cathode end of the gel. Feline AGP and human AGP without prior neuraminidase treatment (Figure 6.6 lanes 2 + 7) showed a similar high migration focusing at the anodal end of the gel and were seen as a single sharp band while untreated bovine and ovine AGP were seen as a broad band at the anodal end of the gel with predicted pI ranges of 3.42- 3.36 and 3.62-3.36 respectively (Figure 6.6 lanes 4 + 9). A low pI IEF gel was used to focus in and determine the pI values for the glycoforms of feline (Figure 6.7 lane 2), bovine (Figure 6.7 lane 5), ovine (Figure 6.7 lane 6) and human AGP (Figure 6.7 lane 3) prior to neuraminidase treatment. The pI were calculated as 1.6-2.8, 2.7-3.3, 3.2-2.8 and 2.3-3 for feline, bovine, ovine and human respectively. Following neuraminidase treated fAGP separated into four bands with pI's ranging from 3.84 to 4.36 while bovine AGP migrated to five pI values ranging between 3.65-4.64, neuraminidase treated human AGP had isoforms with pI

values ranging between 3.62-4 (Figure 6.6 lane 8) while sheep AGP had a broad smear ranging between 3.6-4.65 (Figure 6.6 lane 10).

The results of this comparative study of the glycosylation of feline, bovine, ovine and human AGP are summarised in Table 6.1.



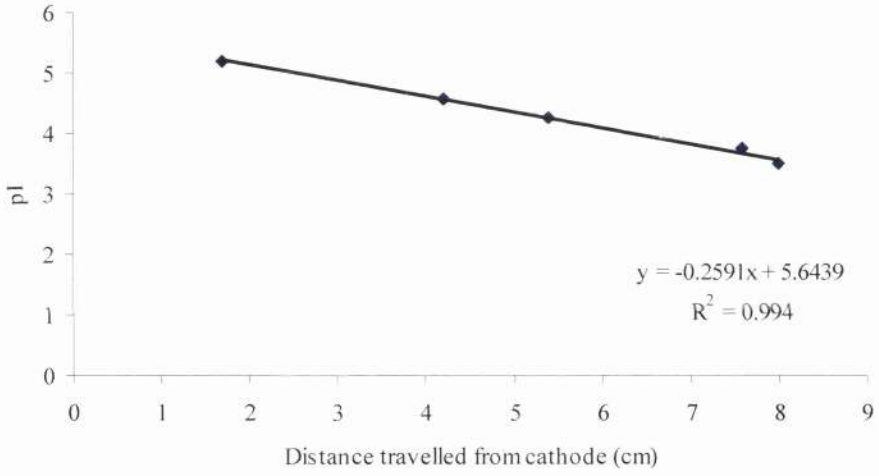


Figure 6.5 A) The calibration curve for pI markers used in the 3.5-9.5 IEF gels B) the calibration curve for pI markers used in the 2.5-5.5 IEF gels

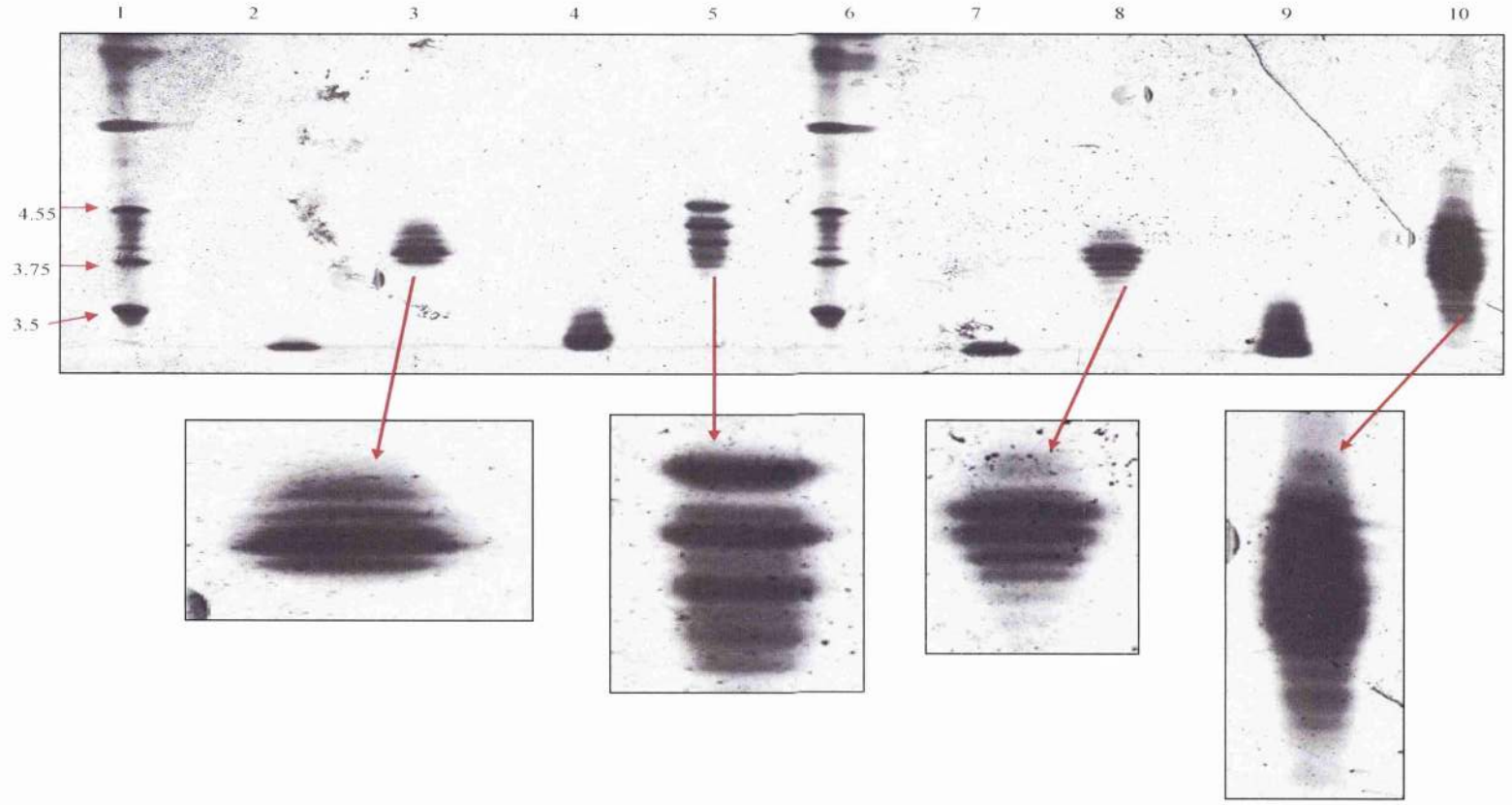


Figure 6.6 shows the broad range (pH3.5-9.5) IEF gel of feline, bovine, human and ovine AGP with and without neuraminidase treatment. 1+6=pI marker 2,4,7 and 9 show feline, bovine, human and ovine AGP prior to neuraminidase treatment respectively. Lane 3, 5,8,and 10 shows feline, bovine, human and ovine AGP following neuraminidase treatment respectively.

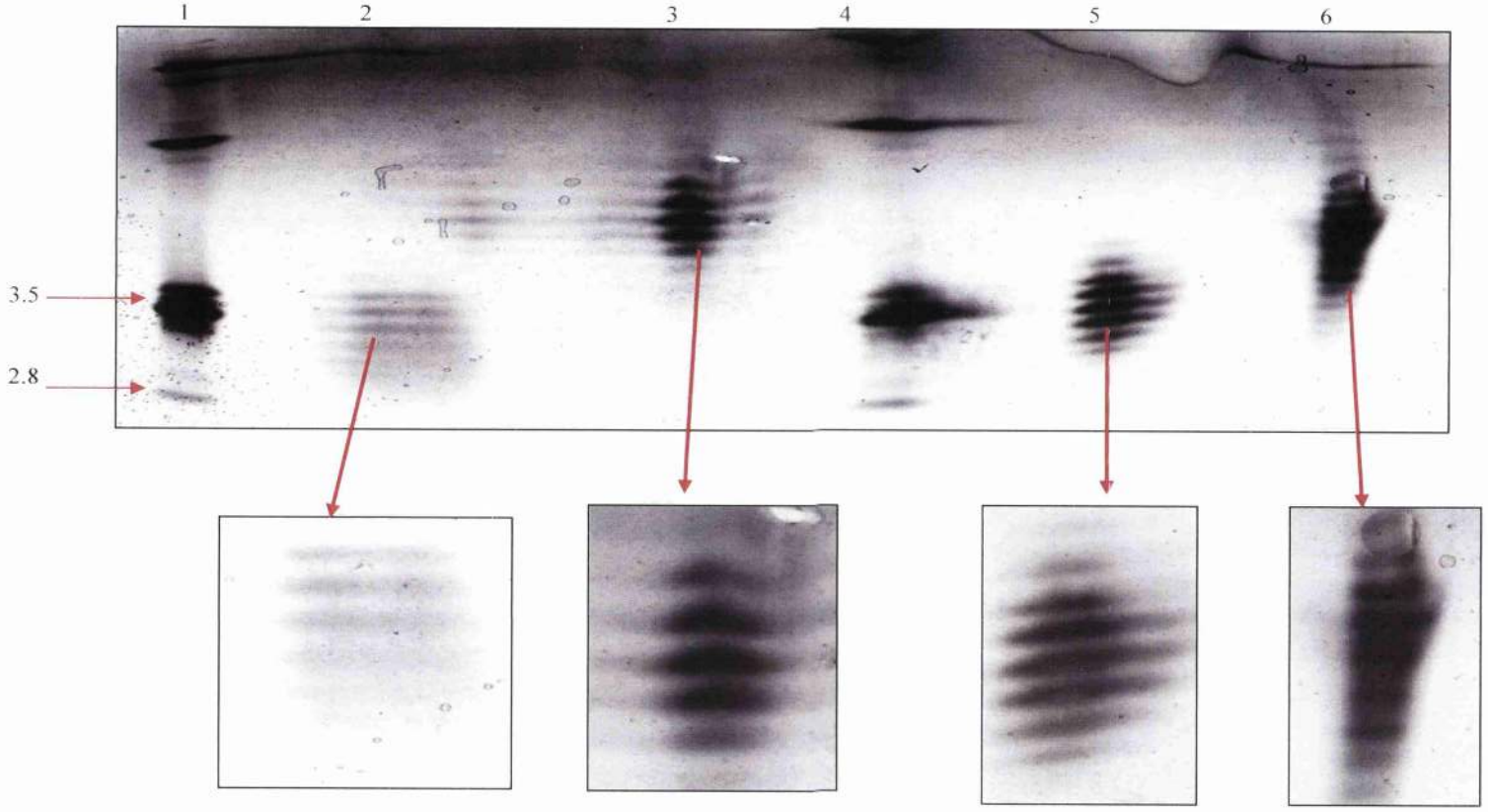


Figure 6.7 The low pI IEF for feline (lane 2), bovine (lane 3), human (lane 5) and ovine (lane 6) AGP. The low pI standard marker are shown in lanes1+4.

Glycan modification	Feline AGP	Bovine AGP	Ovine AGP	Human AGP
Fucose	N	N	N	Y
GalNAc	N	N	Y	N
Monosialylated	N	N	+	N
Disialylated	+++	+++	+++	++
Trisialylated	+	++	+	++
Tetrasialylated	N	N	+	N
NANA	+++	++	++	+++
NGNA	+	++	++	N
Con A	+++	+++	+++	+

Table 6.1 Differences within the glycan chains of AGP from human,bovine, ovine and feline AGP.

Y= present N= not present + = minor or weak reactivity ++ = moderate +++ = Major or strong reactivity

6.4 Discussion

The glycan chains covalently attached to glycoproteins play an essential role in a number of important functions including cell-cell interactions and modification of the non-specific immunity. Therefore to fully elucidate the structure-function relationship of a glycoprotein it is necessary to gain as full an understanding as possible of the glycosylation pattern of the glycoprotein. Thus it is important to determine the monosaccharide composition of the oligosaccharide chains as the presence or absence of terminal monosaccharides such as fucose and sialic acid can have a marked effect on the physico-chemical properties of the protein and in consequence on its function.

Alpha-1-acid glycoprotein is a glycoprotein and has been identified in most species including human, cow, sheep, dog, cat, chicken and rat but most studies on the structure and function of AGP have been carried out on the human protein. Nevertheless a few studies carried out in veterinary species have shown that AGP is also a functionally important protein in these species. Cecillani *et al.*,2007 showed that bovine AGP was capable of suppressing spontaneous apoptosis of bovine monocytes. Drug binding studies have shown that AGP is an important drug binding protein in dogs, cows and sheep (Matsumoto, Sukimoto, Nishi, Maruyama, Suenaga, & Otagiri 2002).

The use of HPAEC-PAD as a tool to analyse the monosaccharide composition and the method of monosaccharide identification was previously discussed in Chapter 3. In this comparative study of AGP monosaccharide analysis the glycan content of AGP from further species were compared to that of feline as described in Chapter 3. This revealed

that the monosaccharides within the glycan chains of bovine and fAGP showed similar patterns, both showing peaks representing Gal, GlcNAc and Man. Human AGP on the other hand also contained Fuc residues in addition to the three monosaccharides seen in bovine and fAGP. This finding is in agreement with Nakano *et al.*,(2004) who reported human AGP carrying fucose residues (Nakano, Kakehi, Tsai, & Lee 2004). Kuster *et al.*, (1998) reported fucose residues expressed on bovine AGP but this was not found here (Kuster B. *et al.* 1998). Fucose on human AGP is heavily influenced by the disease status of the individual. The bovine AGP use in this study was purchased from the same supplier as the study carried out by Kuster *et al.*,(1998) so in theory should be from animals with the same health status nevertheless it is possible that the health status of the animals donating was different (Kuster B., Hunter A P, Wheeler S F, Dwek R A, & Harvey D J 1998). Using HPAEC-PAD technique it was not possible to determine if the Fuc residue in human AGP was part of a SLe X epitope.

Many studies have reported the presence of fucose residues on the glycan chains of human AGP and it has been shown in some instances to be part of the SLeX antigen. The absence of fucose residues on the AGP from other species may suggest that these AGPs do not carry the SLe X antigen. However it has been reported throughout this thesis that the quantity of fucose residues and the expression of SLeX antigen is dependant on the pathophysiological condition of the patient and so the lack of fucose on the bovine, feline and ovine AGP may in fact be due to the disease status of the animals. It would be of interest to repeat this investigation with AGP from these species taken during an acute phase response.

Ovine AGP also has an extra monosaccharide present within the glycan chains which was identified as GalNAc. N-acetyl-galactosamine is not a commonly found monosaccharide in human AGP although it was reported that GalNAc was expressed on human AGP when a patient had hepatitis C (Anderson et al. 2002a).

HPAEC-PAD was also successfully utilised in the separation of oligosaccharides cleaved from the polypeptide backbone using PNGase F. The principal behind the separation of oligosaccharides using HPAEC-PAD has been discussed at length in Chapter 3 and the same technique was employed to analyse the glycan chains cleaved from the protein backbone of bovine, feline, human and ovine AGP. Using this method it was evident that, although there were similarities in the oligosaccharide profile between species, each pattern was unique. All four species had disialylated glycan chains as the prevalent form of oligosaccharide chains. Bovine AGP was the only protein that had a significant peak eluting 40 mins after the start of the run representing tetra-sialylated glycan chains. This is in agreement with Nakano *et al.*, 2004 who also reported that bovine AGP carried diantennary chains containing four sialic acid residues although it was not possible to identify how many antennae where present on the glycans within the tetra sialylated group (Nakano, Kakehi, Tsai, & Lee 2004).

As testament to the flexibility of HPAEC-PAD it was possible to utilise the same HPAEC-PAD column to separate sialic acids without base labile groups NANA and NGNA using an acetate gradient (Manzi, Diaz, & Varki 1990). Prior to HPAEC-PAD the sialic acids had to be cleaved from the glycan chains using neuraminidase. The basis of separation is identical to that described for the separation of intact oligosaccharides (Chapter 3).

The glycan chains of human AGP differed from all the other species in that the oligosaccharide chains only contain N-acetyl neuraminic acid whereas the other species also contained N-glycolyl neuraminic acid. Feline AGP only had trace quantities of N-glycolyl neuraminic acid whereas bovine and ovine AGP had approximately equal quantities of both sialic acid derivatives. Many groups have reported the lack of N-glycolyl neuraminic acid on human glycoproteins (Varki 2001) as well as the lack of sialyltransferase for NGNA. Different forms of sialic acids act as specific receptors for various pathogens and the lack of N-glycoyl neuraminic acid on human glycan chains suggests that some potential pathogens that infect a host via binding to NGNA such as certain influenza viruses, rotaviruses and *E.coli* may in fact be unable to infect human hosts due to the lack of the specific SA receptor.

In human disease during the acute phase response the three common modifications that are reported on AGP are changes in the quantity of SA, diantennary branching and the expression of Fuc residues. There are several ways in which these three modifications can be examined but one of the quickest and simplest methods is by way of lectin binding.

In nature there are many lectins that recognise specific glycans which can be used to probe glycoconjugates for the presence of specific epitopes. In this study SNA, Con A and AAL lectins where used to identify α2-6 linked SA, diantennary branching and Fuc respectively in human, bovine ovine and fAGP by way of electroblotting. This study confirmed the HPAEC-PAD results by showing that only human AGP expressed fucose residues. Blotting with Con A showed that human AGP had less diantennary chains

compared to the other species, however all species had considerable quantities of α2-6 linked sialic acid.

The net charge of proteins is ultimately decided by the relative proportions of amino acid residues with either positive or negative functional groups and, with regards to glycoproteins, the extent of sialic acid expression on the oligosaccharide chains.

The basis of protein separation by IEF is based on the existence of the isoelectric point (pI). All proteins have varying amounts of amino acid residues which have ionisable side chains which are dependant on the external pH. The isoelectric point is the exact pH at which the side chains have been ionised and the protein has no net charge, and at this point, due to the absence of charge, the protein has no electrophoretic mobility and hence remains static in the IEF gel (Righetti 1983). However the pI of a glycoprotein that carries SA on its glycan chains is not just determined by the amino acids but the presence of the negatively charged SA also is also a determining factor. In this study the effect of SA on the pI of AGP from human, bovine, ovine and feline was examined by running samples that had been treated with neuraminidase to remove SA alongside samples prior to neuraminidase treatment. It was found that removal of SA resulted in an increase in the pI's of all species examined, which was seen as a shift towards the cathodal end of the gel. Although SA was shown to be an influencing factor in the low pI of AGP from human, bovine, ovine and fAGP, it was also apparent from the multiple bands seen after neuraminidase treatment that other factors were involved in determination of AGPs' pI value. One possible explanation for the multiple bands seen in the enzyme treated samples is the difference within the amino acid sequence. To date, 2 forms of human AGP have been described that are due to the substitution of 22

amino acids. Isoelectric focusing also revealed that feline and human AGP were more negatively charged than bovine and ovine AGP which had some bands which migrated more towards the anodal end of the gel. However this pattern was even apparent following the removal of SA suggesting that the difference of pI values between the species is not completely due to differences in the quantity of sialic acid but is also due to the amino acid sequence.

In conclusion it was apparent that while the carbohydrate moiety of AGP from the four species shared similarity they were also unique, with differences evident in the SA, Fuc and branching quantities. Human AGP differed from bovine, ovine and fAGP as it contained fucose residues which were not detected within the oligosaccharide chains of AGP from the other species. Another difference between human AGP and the other species was the lack of NGNA. Human AGP also appeared to have more highly branched glycan chains than AGP from other species which was revealed by probing with Con A lectin.

CHAPTER 7

7. General discussion

The aim of this thesis was to examine the structural heterogeneity of AGP in veterinary species. This chapter is to review the important findings from preceding chapters and to highlight areas which are worthy of future research.

Glycosylation is an important modification to protein which occurs after translation. It has been extensively shown that the carbohydrate moieties of glycoconjugates play a crucial role in the functioning of the molecule so that even subtle changes such as the addition or the removal of one monosaccharide can have a profound effect on the ability of the glycoconjugates to function properly (Pos et al. 1990a;Pukhalsky, Shyian, Kalashnikova, Shmarina, Pukhalskaya, & Bovin 1998).

Acute phase proteins are a group of proteins that are produced mainly by hepatocytes in response to trauma infection or infections. Many of the APPs are glycoproteins and it is likely that the carbohydrate moiety of these proteins can influence their functions. Therefore in order to truly understand the importance of the APR and the APPs, the carbohydrate moiety of the APPs should be fully elucidated.

Prior to this investigation very little research had been carried out in veterinary medicine to elucidate the structure or the function of the carbohydrate moiety attached to the APPs of the domestic species. This thesis set out to examine whether changes in

structural heterogeneity of APPs occur and particularly whether they could be used as diagnostic markers in FIP in cats and mastitis in cows.

Feline infectious peritonitis is a well-known and widely distributed coronavirus induced systemic disease in cats, characterised by protein-rich effusions in body cavities and granulomatous inflammatory lesions in several organs (Kipar *et al.*,2005 Montali and Strandberg, 1972: Napoleone *et al.*,1997). Feline infectious peritonitis is now considered a very important disease world wide in cat populations. A few years ago it was reported that the AGP was significantly increased in the plasma of animals suffering from FIP even when compared to animals with other pathologies (Duthie *et al.*,1997).

The carbohydrate moiety of any glycoprotein is susceptible to degradation in harsh conditions such as extreme pH, therefore isolation of glycoproteins when planning to examine subtle changes within the oligosaccharide chains, as in these studies, requires careful manipulation. It was with this in mind that an improved method for the isolation of fAGP was developed which was described in detail in chapter 2. This involved initial precipitation with PEG followed by a three step chromatographic procedure. Although the yield gave a 26% recovery other techniques which have been described for the isolation of AGP from the serum of other species were ruled out due to the likelihood of degradation of the terminal SA residues during the prolonged use of buffers at acidic pHs. The conditions of the method developed were similar to a method described by Smith *et al.*, 1994 for the isolation of hAGP which was shown to keep the oligosaccharide chains intact. The only differences between the method described by Smith *et al.*, (1994) and the method used in this study is first that a higher molecular

weight PEG was used in this study and second the Sepharose red column used by Smith *et al.*, (1994) was substituted for the cation exchange column used in these studies. Therefore it was presumed that the method developed for this study would be suitably gentle enough to avoid degradation of the terminal SA residues and so was employed for the isolation of fAGP prior to analysis of the composition of the oligosaccharide chains attached to the protein backbone.

Chapter 3 described the monosaccharide and oligosaccharide analysis of fAGP using HPAEC-PAD. This technique has been increasingly used in the routine analysis of the glycosylation of glycoproteins. The versatility of the technique permits the analysis of glycoproteins in terms of both the oligosaccharide chains expressed and their monosaccharide content (Hardy and Townsend, 1988: Hardy *et al*, 1987: McGuire *et al*, 1999).

Previous studies (Anderson et al. 2002f;French et al. 2002)have shown that the HPAEC-PAD method used for monosaccharide analysis in this thesis was capable of separating out Fuc, Gal, GlcNAc, Glu, GalNAc and Man. However it is not suitable for the analysis of SA for two reasons first the harsh degradation conditions used to cleave the monosaccharides degrades SA and second the elution conditions would not be suitable for SA due to its negative charge. Therefore another HPAEC-PAD method had to be used to examine SA.

Monosaccharide analysis of AGP from cats with FIP and cats characterised as non FIP using HPAEC-PAD revealed that the group of cats with FIP had statistically significantly higher expression of GlcNAc and Gal residues when compared to the

group of cats with non-FIP diseases. This may be explained by increased branching or more N-linked chains. The ratio of GlcNAc to Gal residues which would be expected if fAGP, like human AGP carried only complex type N-linked glycans would be 4:2 for a diantennary chain, as 2 GlcNAc residues are within the common pentasaccharide core and the one residue per antennae whereas Gal is not present within the common core but is only found in ratio of 1:1 per antennae. Thus a possible explanation for the lack of continuity between the amount of GlcNAc and Gal expressed within the glycan chains of fAGP may be that fAGP carries a combination of complex, hybrid and high mannose type N-linked chains. The FIP group also had increased levels of mannose residues, however the increase was not statistically significant. The most interesting finding from the monosaccharide analysis of fAGP was that two cats (5 and 9) had fucose residues present on their oligosaccharide chains whereas no other cats including those in the non-FIP group, had any trace of fucose. Increased expression of fucose residues has been examined on human AGP in various diseases such as RA and cancer and shown to be of use in determining the prognosis of the disease. Unfortunately these cats where already deceased at the time of the investigation due to the high fatality associated with FIP and so it was not possible to take further samples to find a reason for the appearance of the fucose residue. Two possible explanations are first that the disease was at a more advanced stage when the sample was collected, and second that the cats had an underlying infection that was not noticed at the time of inspection. To clarify this, it would be interesting to assess the expression of fucose residues on glycoproteins in cats of different stages of FIP and with various pathologies.

Following analysis of the monosaccharide of fAGP it was important to determine how the monosaccharide formed the oligosaccharides chains attached to fAGP. Previous

studies have shown that HPAEC-PAD can be successfully utilised as a qualitative but not quantitative tool to examine the structure of the oligosaccharide chains attached to glycoproteins as well as monosaccharide analysis. Prior to oligosaccharide analysis using HPAEC-PAD the oligosaccharides have to be cleaved from the polypeptide backbone and in this study PNGase F was used for this purpose. Using HPAEC-PAD to examine oligosaccharides cleaved fAGP from cats with FIP and cats without FIP showed that in the latter case, all normal pool samples test two SA residues per chain whereas the cats with FIP had more complex HPAEC-PAD chromatograms with peaks representing oligosaccharides carrying one, two and three SA residues on the glycan chains. Each cat with FIP had two major peaks one representing a disialylated glycan oligosaccharides and one representing a trisialylated oligosaccharide which were eluted 8-9 minutes apart. This method of analysis would be a lot more informative if each peak could have been identified to highlight how many antennae each glycan had as well as the type of linkage between each of the monosaccharides. However it was not possible to run this type of analysis due to the lack of suitable standards available. This was a relatively small study but has shown that the oligosaccharide fingerprint of AGP from cats with FIP and cats without FIP is significantly different. It would be important to repeat this investigation in a larger study population with more non-FIP samples and also cats with clinically similar pathologies. A more informative technique that could have been used to characterise the structure of the glycan chains is mass spectrometry however this is a technically challenging and costly procedure and would have required a lot of optimization and for these reasons was not used. However this should be considered as an approach to be undertaken in future analysis of glycoproteins.

Since it was shown in Chapter 3 that the glycosylation pattern of fAGP is altered during disease and that is known that AGP from other species undergoes changes within its glycans during periods of inflammation it was thought possible that the same occurs in bovine mastitis. Therefore the probability of any changes within the glycan chains of serum AGP from healthy cows and cows with mild and moderate mastitis was examined using lectin blotting methods.

The chromatographic methods used to examine the structural heterogeneity of fAGP where not used to analyse the glycan chains of bAGP as time restraint meant that development and implementation of a method for the isolation of bAGP was not possible, as a pure preparation is needed for HPAEC-PAD analysis.

The study found that bAGP from cows with moderate and mild mastitis had statistically significantly higher expression of diantennary chains than control cows. While cows with moderate but not mild mastitis have statistically significant expression of α2-6 linked sialic acid when compared with control samples. It would be of interest to see if these structural alterations affect the function of bAGP.

It has been extensively reported that APPs such as SAA and Hp are expressed locally by the mammary gland in cows suffering from mastitis. Serum amyloid A produced in the mammary gland is a different isoform from that produced by hepatocytes however little work has been carried out to determine if the locally produced Hp is structurally identical to the hepatocytes produced counterpart. Therefore the Hp produced in the mammary gland was compared to the serum Hp produced by hepatocytes to examine whether or not they are identical.

Chapter 5 focused on looking at the structure of Hp from the serum and milk of cows with mastitis in a bid to see if the milk Hp is structurally similar to serum Hp. Western blotting revealed that the beta chain of milk Hp was a doublet whereas serum Hp was a single band. This suggested that milk Hp fraction may in fact have two isoforms. Therefore the difference between the isoforms was investigated employing an enzyme to remove the carbohydrate chains attached to Hp in order to determine if the difference was in the carbohydrate moiety or the amino acid sequence. The difference was found to lie within the carbohydrate moiety as following removal of the N-linked glycan chains with an endo-glycosidase (PNGase F) the doublet of milk Hp seen prior to enzyme cleavage was not seen and the βHp from both the milk and serum Hp were the same size when examined on a SDS-PAGE gel. These results suggests the difference in Mwt between the doublet bands seen in the SDS-PAGE of milk Hp is a result of an increase in the weight of the glycan chains attached to the polypeptide backbone. The glycan chains where then probed with fluorescent labelled lectins and it was found that the βHp from milk expressed fucose residues which were not on the serum equivalent. It was also apparent that only the 37kDa band of the doublet had diantennary glycan chains attached with the 40kDa band presumably having tri and tetra antennary chains. The results of this study suggest that the Hp present in milk is a mixture of 2 isoforms. Possibly these are liver derived Hp and a locally produced glycoform of Hp with altered glycosylation giving a higher Mwt. However this was a small pilot experiment and a more comprehensive study may be required in order to draw any definitive conclusions on the nature of the βHp isoform. However this could be a very interesting area to explore in the future, and in particular the effect of the different glycoforms on the function of Hp. It may also be worthwhile investigating the expression of the locally

produced Hp over a time course of the disease to determine if the presence of fucose on the Hp could be used as a diagnostic marker for subclinical mastitis.

With the development of powerful tools to analyse the glycosylation of serum proteins the opportunity was taken to examine the carbohydrate of bAGP, fAGP, oAGP and hAGP, to provide an initial insight into the comparative structure of the oligosaccharides present on these molecules in normal conditions and to identify any species related differences. From this study it was apparent that the carbohydrate moiety of AGP is substantially different between species.

The comparative monosaccharide composition of AGP from healthy cows, sheep human as well as cats was assessed. All four species were different but the most notable difference was that only human AGP expressed fucose residues which was confirmed by blotting with AAL. However a study detailed by Kuster *et al.*,(1998) who examined the glycan chains of hAGP, bAGP, oAGP and cAGP using mass spectrometry reported the presence of Fuc residues on bAGP as well as hAGP, but may be due to differences in source material. Further studies on AGP from animals of definitive provenance are warranted.

The SA content of human, ovine and bovine AGP was examined using HPAEC-PAD (chapter 6) and it was demonstrated that all species examined with the exception of hAGP had both NGNA and NANA but this is not surprising it has been reported that all mammals with the exemption of humans and apes express NGNA as well as NANA in their glycoconjugates (Varki 2001). This technique can be used to quantify the amount of NANA and NGNA present on the glycan chains of glycoproteins however this was

not carried out due to the lack of adequate amounts of normal standards. This could be further investigated as previous studies have shown that the sialic acid content of fAGP is dependant on the disease status of the animal (Cecilliani *et al.*,2004; Pocaque *et al.*,2005).

Another notable finding from this study is that oligosaccharide profile of AGP from the ruminants is more complex than that of human and cats with the former having a number of higher pI isoforms not present in the latter,

In conclusion the studies in this thesis have established that the structural heterogeneity of acute phase proteins in veterinary species, like humans, is dependant on the disease status of the individual but further work is required to determine its usefulness in diagnosis of diseases such as FIP and mastitis and the effect of the changes on the biological functions of the APPs.

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