

**A Study of the Effect of Physical Sports Injury on
the Glycosylation Patterns of Alpha-1-Acid
Glycoprotein.**

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Declaration

It is hereby declared that this thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has lead to the award of the degree.

Signed: William Surradge

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WS

Abstract

Alpha-1-acid glycoprotein (AGP) is a 43kDa glycoprotein, so called due to the post-translational addition of carbohydrate units to the main protein. AGP is the second most abundant protein found within the serum of humans at normal physiological conditions, and is a positive acute phase protein produced by hepatocytes within the liver. As AGP is a positive acute phase protein, the concentration of AGP has been found to increase when a person is experiencing an acute phase response (APR); this is the body's first line of defence against stressful stimuli, such as bacterial and viral infections, strenuous exercise, and physical injury.

As a result of the APR, it has been found that the structure of the carbohydrate units attached to the protein backbone can be altered. During a normal physiological state, 12-20 glycoforms of AGP can exist, however, this number can increase during the APR. A single molecule possesses five branching sites along the protein backbone, where the monosaccharide units bond to the AGP molecule in the form of branches; these branches can either be bi, tri, or tetra-sialylated arrangements. It is this attribute of AGP that is the focus of this research within this project. Previous studies have shown that the altered glycosylation of AGP has the potential to differentiate between different types of liver diseases and breast cancers. While it has been shown that the APR can be induced by physical injury, no studies have been carried out to determine whether or not a physical injury induced APR can alter the glycosylation patterns of AGP.

Physical injury samples for the project were collected via venepuncture from volunteers who were injured while taking part in the sport of downhill mountain biking; a sport chosen due to its inherent dangers and the potential for injury. A two fold analysis of these samples was then carried out, by analysing the monosaccharide composition of the physical injury samples, before analysing the oligosaccharide structure of these samples, both being achieved through high pH anion exchange chromatography. The collected injury samples were then compared against collected normal blood samples, healed samples from previously injured volunteers while one sample remained unknown for the duration of the project.

Of the collected samples, it was seen that physical injury does have an effect on the glycosylation patterns of AGP. Furthermore, it was seen throughout the study that different injury types can produce different effects on the glycosylation patterns of AGP. Finally, the diagnostic potential of AGP was explored by comparing the monosaccharide and oligosaccharide compositions of the unknown injury sample against the compositions of the known injury samples. Once comparisons had been

completed, it was found that the unknown shared a great deal of homology with known fracture injuries, before the unknown was confirmed as a fracture injury itself.

In conclusion, the aim of the project was to determine whether or not physical injury induced APR can affect the glycosylation patterns of AGP. This project has confirmed that physical injuries can affect the glycosylation patterns of AGP. Further research within this area can then be carried out, such as studying the changes in glycosylation patterns throughout the recovery process.

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Abbreviations

4X- Four Cross

AAT- Alpha-Anti-Trypsin

AGP- Alpha-1-Acid Glycoprotein

APP- Acute Phase Protein

APR- Acute Phase Response

Asn- Asparagine

BMX- Bicycle Motocross

Da- Daltons

DH- Downhill

Dol-P- Dolichylphosphate

EDTA- Ethylenediaminetetraacetic acid

ER- Endoplasmic Reticulum

GalNAc- N-acetyl-Galactosamine

GDP- Guanine Diphosphate

GlcNAc- N-acetyl-Glucosamine

HPAEC- High pH Anion Exchange Chromatography

HPLC- High Performance Liquid Chromatography

IL- Inter Leukins

MTB- Mountain Biking

MW- Molecular Weight

NAAP- Negative Acute Phase Protein

NaCl- Sodium Chloride

NaOH- Sodium Hydroxide

N-lib- N-Linked Library

PAAP- Positive Acute Phase Protein

PED- Pulsed Electrochemical Detection

PEG- Polyethylene Glycol

pI- Isoelectric Point

PNGase- peptide-N-glycosidase

PSC- Primary Sclerosing Cholangitis

RER- Rough Endoplasmic Reticulum

SBC- Secondary Biliary Cirrhosis

TFA- Trifluoroacetic Acid

TNF α - Tumour Necrosis Factor Alpha

UCI- Union Cycliste Internationale

UDP- Uridine Diphosphate

UV- Ultraviolet

XC- Cross Country

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1.1 Alpha-1-Acid Glycoprotein

1.1.1 Discovery of AGP

Alpha-1-Acid Glycoprotein (AGP, also known as Orosomucoid) was first discovered in 1950 by Schmid and colleagues (Schmid, 1950). AGP is a 43kDa glycoprotein so called due to the 45% of post-translationally added carbohydrate units to the molecule (Schmid *et al.*, 1977). At the time of its discovery, AGP was thought to possess the largest amount of post-translationally added carbohydrate in the form of oligosaccharide chains (glycan chains) within the human body; this would later be refuted with the discovery of galactoglycoprotein of which possessed the post-translational addition of carbohydrate equating to 76% of its total molecular weight (Schmid *et al.*, 1980). AGP is still the second most abundant protein within the serum of humans, with only albumin present more within the body at normal physiological conditions (Gallacher, 2009). The majority of AGP within the body is produced by the hepatocytes within the liver, however, centralised production of AGP within other organs has been noted (although not to the same concentration as within the liver), such as within the prostate and granulocytes (Poland *et al.*, 2002).

1.1.2 The Acute Phase Response

The Acute Phase Response (APR) is one of the body's first line of defence against trauma, pathogens and other harmful stimuli. Specifically, the APR can be activated through bacterial and viral infections, such as leprosy (Gupta *et al.*, 2010), disease, diabetes (Poland *et al.*, 2001), childbirth (Smith *et al.*, 2002), the first few days of life (Kushner, 1982), strenuous exercise, and, importantly within this project, physical injury (Kushner and Rzewnicki, 1994). The APR begins minutes after the introduction of the stressful stimuli, and will continue for 1-2 days after the event (Kushner, 1982). AGP is a Positive Acute Phase Protein (PAPP), meaning that the serum level of AGP increases after the initiation of the APR (Clementson, 1997). AGP's typical concentration within the blood out with this event is that of 0.5-1mg/ml (Kremer *et al.*, 1988), however, during the APR the concentration of AGP can increase between two and ten times (Petersen, *et al.*, 2004). The production of AGP during this event is

controlled by interleukins 1 and 6 (IL-1 and IL-6) and glucocorticoids (Baumann *et al.*, 1989). Increases in AGP have been reported in diseases such as cancer and rheumatoid arthritis (van Dijk, 1995), during periods of stress, pregnancy and heart attacks (Duche *et al.*, 2000), although the exact function of the glycoprotein remains unknown.

The APR begins with the introduction of the harmful stimuli (for example, a pin to the thumb); immediately, pain is felt within the individual, and stimulation of the endothelial cells, fibroblasts and macrophages within the thumb begins. This in turn leads to the stimulation of IL-1, IL-6 and tumour necrosis factor Alpha (TNF α); the response of this then feeds back to the stimulation of the endothelial cells, fibroblasts and macrophages at the site of injury (Goldsby *et al.*, fifth edition). The production of IL-1, IL-6 and TNF α then leads to the increase in secondary systemic effects within the individual; this includes fever (to inhibit the growth of a pathogen), loss of appetite, an increase in tiredness, an increase in white blood cell production (leukocytosis), and an increase in glucose production for use as energy (gluconeogenesis) to aid in the healing process (Goldsby *et al.*, fifth edition). The increase in IL-1, IL-6 and TNF α also leads to the alteration in the acute phase proteins (APPs) within the serum of the individual; this includes the increased presence of AGP within the blood (due to its status as a PAPP), while also leading to a decrease in the serum concentration of negative acute phase proteins (NAPP), such as albumin. Following this, the body then increases the level of fluid, called exudate, leading to swelling in the affected area, called edema, which begins to isolate the stimuli and prevent it from spreading to other parts of the body (Goldsby *et al.*, fifth edition); typically, this process is categorised physiologically as a redness, pain and swelling due to increased vascular permeability (possibly due to the effects of AGP) around the area of stimulation (Haraldson and Rippe, 1987). The healing process itself can therefore begin. The APR associated increase in AGP remains at an increased level for 1-2 days after the initial introduction of the stimulus (Kushner, 1982). In addition to the increased production of serum AGP, however, there is also an alteration in the glycan chain arrangements along the polypeptide backbone of the AGP molecule (Ceciliani and Pocacqua, 2007); i.e. there will be an appearance of new types of chains that would not be expected in the AGP molecule, like an

increased appearance in tetra-antennary chains, or a decrease in bi-antennary chains when compared to resting levels.

1.1.3 Structure of AGP

As previously mentioned, most of the serum AGP within the body is produced within the liver. The production of the protein is coded by three genes on chromosome 9 of the human genome; AGP-A, AGP-B and AGP-B'. AGP-A codes for the ORM1 variant of the protein (Dente *et al.*, 1987), while AGP-B and AGP-B' (two identical genes) code for the ORM2 variant of the protein (Tomei *et al.*, 1989). AGP-A, however, produces the majority of the AGP within the liver, 100 times more so than the other two genes (Dente *et al.*, 1987). The ORM2 variant of AGP differs from the ORM1 variant of AGP in 22 bases.

The protein molecule of AGP is 183 bases long. Within this protein, there are five bonding sites, present on the amino acid asparagine (Asn), with which carbohydrate units can attach to the protein molecule within their glycan chains. It is within these N-linked bonding sites, that the variations within the molecule are introduced. Figure 1. shows a typical monosaccharide make up and arrangement of glycan chains on the protein backbone of the AGP molecule; this diagram also shows the three possible arrangements that each glycan chain can take, a bi-antennary chain (two branches), a tri-antennary chain (three branches) and a tetra-antennary chain (four branches). Each glycan chain is bound to one of these five sites, (positions -15, -38, -54, -75, -85). In a normal physiological state, only 10-20 glycoforms of an AGP exist due to the specific combinations of chains of which can appear on the Asn sites, however, during the APR, this number can increase further; theoretically, more than 10^5 glycoforms of AGP can exist (Cecliani and Pocaqua, 2007). Evidence has also been gathered to suggest that tissue specific AGP could also possess it's own modifications based on it's tissue of origin, i.e. AGP produced in the granulocytes have been found to possess a higher molecular weight than plasma AGP (50-60kDa as opposed to 43kDa), this is thought to be due to the strongly fucosylated and sialylated polylactosamine units that make up the carbohydrate additions to the molecule (Poland *et al.*, 2005).

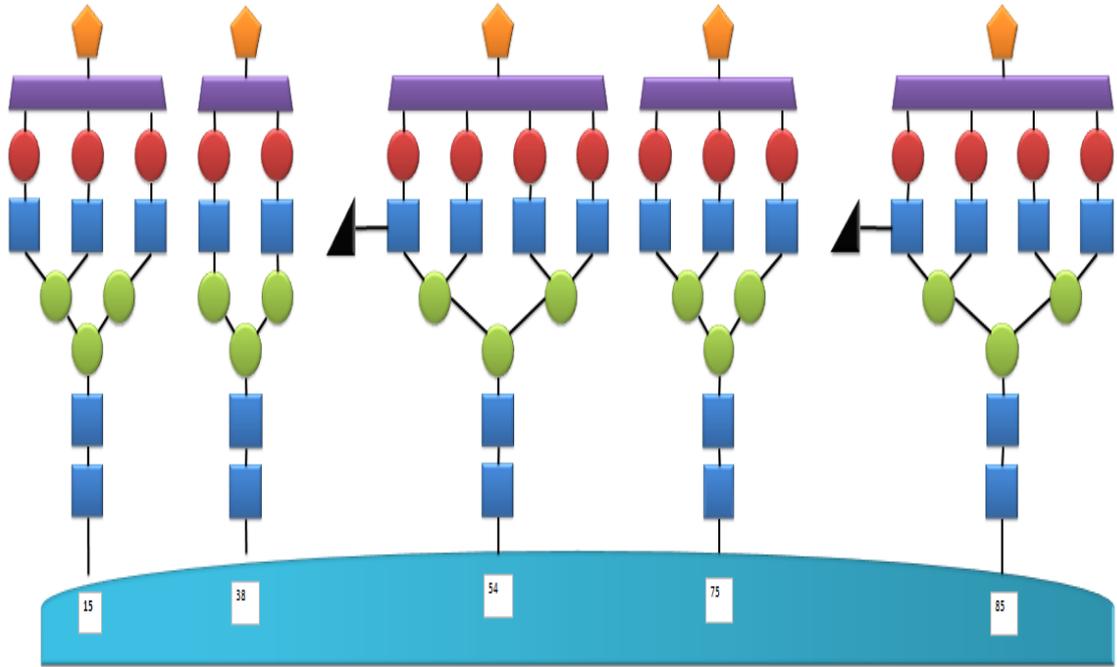


Figure 1. AGP Glycan Chain Diagram

Glucosamine

 Mannose

 Galactose

 Fucose

 Neuraminic Acid

In addition to the protein backbone of the AGP molecule, each AGP molecule is made up of glycan chains, these form the carbohydrate portion of the molecule. Each glycan chain is made up of monosaccharide molecules, bonded together by glycosidic bonds. The actual monosaccharides of which can appear within the glycan chain are typically mannose, galactose and glucosamine; with some chains being terminated by either the monosaccharide fucose, or neuraminic acid. These molecules are then arranged into glycan chains like those seen in Figure 1.

The glycan chains are bound to the molecule through a process called glycosylation; this is the post-translational addition of carbohydrate units to a protein molecule. This process, literally, makes AGP a glycoprotein. A glycan chain is bound to the protein backbone at one of the five bonding sites stated above. However, there is a degree of specificity within the AGP molecule as to what type of branch can bond to a particular bonding site; tetra-antennary chains cannot bind at site one, site two cannot bond chains with a high presence of the monosaccharide fucose, bi-antennary branches cannot bind to site 4, and chains with a high presence of fucose within them can be found on sites 4 and 5 of the protein backbone.

AGP possesses a low isoelectric point (pI) between 2.8-3.8 (Mondal *et al.*, 2009), this is believed to be due to the presence of neuraminic acid as a common terminating sugar on each of the glycan chains and accounts for 10-12% of the carbohydrate moiety of the overall AGP molecule (Fournier *et al.*, 2000). Fucose can be another terminating sugar on the glycan chains, however, Fournier *et al.*, (2000) states that 30% of human plasma does not contain any fucose, and that a high degree of fucosylation is suggestive of a low presence or absence of bi-sialylated branches, and an increased presence of tri- and tetra-sialylated branches (Fournier *et al.*, 2000). The structure of the AGP protein has been shown to contain 15% α -helices, 41% β -sheets, 12% β -turns, and 24% of unordered structure (Ceciliani and Pocaqua, 2007), which has led to the belief that AGP belongs to a sub-family of lipocalins called the immunocalins, due their similar structures.

1.1.4 AGP Function

The exact function of AGP remains unknown at this point in time. Numerous studies have taken place to determine the function of AGP, however, there is still disagreement. It is fully possible that AGP is exactly what these studies have shown; a PAPP, that acts as a transportation molecule, an inhibitor of microbial function, that can regulate the body's immune response to a degree by increasing vascular permeability (Haraldsson and Rippe, 1987), and a binder of basic drugs (Hochepped *et al.*, 2003) such as methadone (Behan, 2010) and steroids if need be.

Due to the increased presence of AGP within the human body during the APR, AGP is thought to be involved within this process. The structure of AGP is similar to that of the immunocalins; this is a family of proteins of which are able to exert immunomodulatory effects on the human body, and help to regulate the body's immune system (Logdberg and Wester, 2000). Due to the structure of the glycoprotein being mostly β -Sheets, AGP is thought to be involved within the APR as a transport protein (Ceciliani and Pocaqua, 2007). This theory can also be taken further, as AGP has been shown to bind basic drugs (Ceiliani and Pocaqua, 2007); this is thought to be achievable due to the low pl of the molecule. However, AGP has also been shown to bind neutral drugs and steroid hormones as well (Israeli and Dayton, 2001). AGP has also been shown to bind toxic molecules from micro-organisms (Ceciliani and Pocacqua, 2007), further strengthening the theory that AGP is a transport molecule during the APR.

Furthermore, there is a possibility that AGP may encourage vascular permeability within the APR (Fournier, 2000). This supports the theory that AGP may be involved within this process as there is an increase in vascular permeability within the area of injury leading to an increase in swelling and heat/redness around the affected area in question.

However, it should also be stated that as there is an increase in the level of AGP within the serum during numerous different conditions; the rise in AGP is itself not diagnostic of a disease or other physiological state. There are numerous different states that could affect the level of AGP, thus, it would not

be reliable to use this rise as a biomarker. It is the change in glycosylation pattern that could act as a potential biomarker for a physiological state.

1.2 Glycosylation Patterns of AGP

1.2.1 The Production of Glycosylation Patterns

The glycosylation pattern of AGP is the post translationally added monosaccharide units to the protein backbone of the AGP protein, making AGP a glycoprotein. The glycosylation pattern is then determined by the arrangements of these monosaccharides as oligosaccharide structures, also known as branches. As previously stated, there are three genes which produce AGP; AGP-A, AGP-B and AGP-B'. While the AGP-A variant is produced in greater quantities than AGP-B/B' (Dente *et al.*, 1987), it was found that the concentrations of each variant of AGP would increase during the APR, and that the glycosylation of each of these variants is not dependant on their genetic expression (van Dijk *et al.*, 1991).

Glycosylation is a complex process driven forward by enzyme catalysation. The process of glycosylation typically occurs in two stages; the synthetic pathway (the production of the necessary glycan chains) takes place within the cytosol and the endoplasmic reticulum (ER), and the processing pathway (the quality checking of the glycan chains) taking place within the ER and the golgi apparatus. Each glycan chain is made up of monosaccharide units, these mono units are converted into high sugar nucleotides (such as uridine diphosphate (UDP)-GlcNAc and guanine diphosphate (GDP)-mannose) and are transferred to membrane bound lipid dolichylphosphate (Dol-P) on the cytosolic side of the ER to form Man₅GlcNAc₂-Dol-P. GlcNAc-1-phosphotransferase, GlcNAc-transferase and mannosyltransferases are then required to catalyse the transfer from their donor sugar nucleotide to the Dol-P. This donor sugar nucleotide then translocates to the luminal side of the ER, through the use of a flippase. A further seven monos are then donated in this way, binding together through glycosidic bonds to produce the fourteen Glc₃Mann₉(GlcNAc)₂ oligosaccharide precursor.

After the production of the oligosaccharide precursor, synthesis of glycosylation patterns occurs in four stages, this is summarised in Figure 2. below;

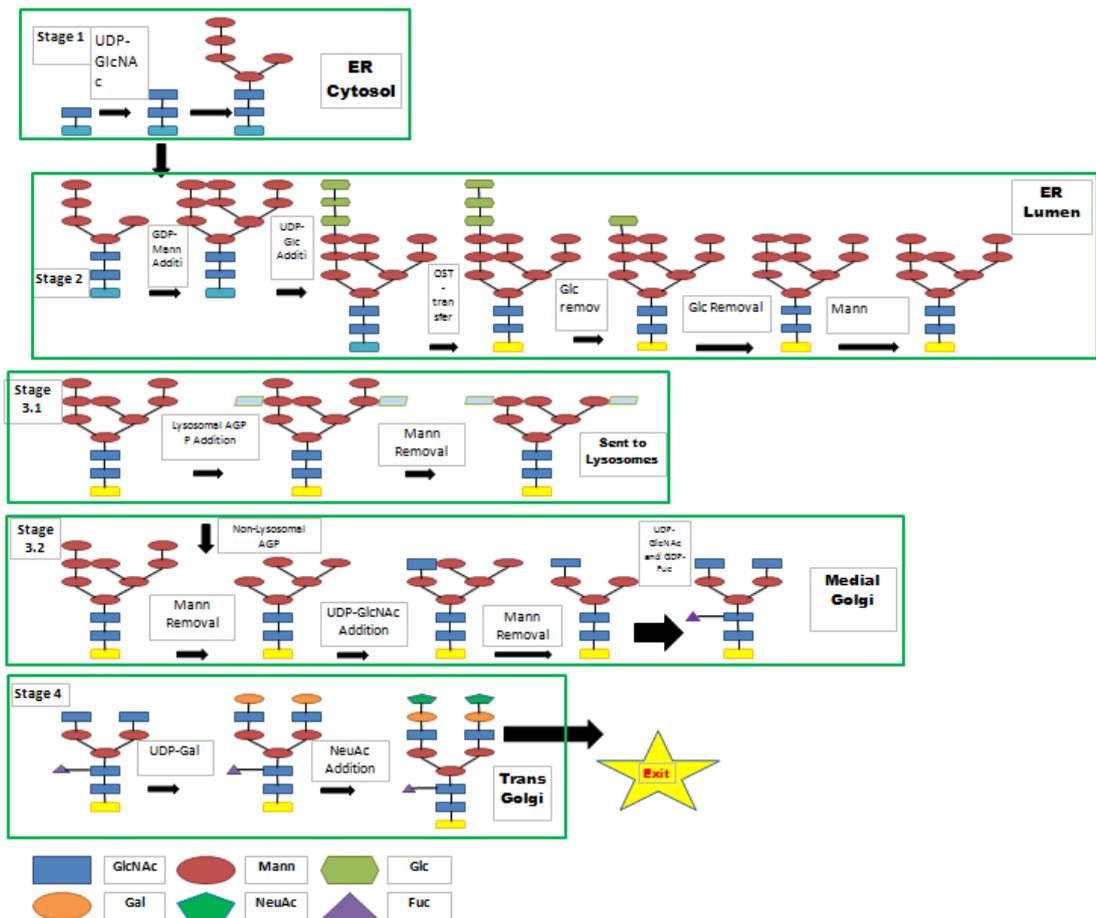


Figure 2. Glycosylation Pathway Diagram

- Initially, stage 1 is the transfer of the precursor from Dol-P donor to nascent glycoproteins occurs within the rough endoplasmic reticulum (RER); this process requires a multimeric oligosaccharyltransferase complex.
- Stage 2 then involves glycosidases trimming the precursor while being transported from the RER to the golgi.
- Stage 3 involves the substitution of the precursor, which takes place within the medial golgi by GlcNAc-transferases.
- Finally stage 4 of glycosylation pattern production involves the elongation of the precursor which takes place within the trans-golgi network, resulting in the addition of glycan chains to the protein backbone.

Furthermore, the protein structure of β -sheets and loops are formed through the bonding between the anomeric –OH group of the terminal monosaccharide (GlcNAc) and the –NH group of the Asn residue. However, possible interactions may occur between N-linked glycan chains and aromatic, uncharged amino acid side chains within the three amino acids on either side of the glycosylated Asn residue. The glycans attached to the Asn are then exposed to the competitive action of glycosidases (which catalyse the “trimming” of the glycan chains), such as glucosidase and mannosidase, and glycosyltransferases (which catalyse the addition of the monosaccharide donors to the donor protein). It is this competition between enzymes for substrates that increases the diversity within the microheterogeneity.

There are several regulatory steps in place to prevent the synthesis of incorrect glycoproteins within the body; upon trimming of two glucose molecules by α 1,2-glucosidase I and α 1,3-glucosidase II within the ER, the nascent glycoproteins then enter the calnexin/calreticulin cycle which ensures only correct folded proteins can continue within the pathway. If the folding is incorrect, a luminal glycosyltransferase adds a glucose residue onto the misfolded protein, forcing it to re-enter the calnexin/calreticulin cycle. This process would be repeated until the protein is correctly folded and any remaining glucose is removed by glucosidase II.

1.2.2 The Effect of the APR on Glycosylation Patterns

It has been seen previously, that the APR can produce an increase in the level of AGP when the body is experiencing a stressful stimuli (Kremer *et al.*, 1988); however, not only can the APR affect the concentration of AGP within the body, it can also affect the types of monosaccharides and the arrangements of these monosaccharides as glycan chains; these are the glycosylation patterns. As mentioned previously, when the body is physiologically “healthy”, only 12-20 glycoforms of AGP exist within the body. It has been noted in previous studies however, that during the APR, an increased number of glycoforms can exist and appear within an individual. Within human AGP, three types of glycan branching are of interest within this study. These are bi-

antennary branching (a chain ending in two branches), tri-antennary branching (chain ending in three branches) and tetra-antennary branching (chain ending in four branches). The increased presence of tri and tetra branching is indicative of more complex branching types within the AGP molecule. An increased presence of fucose monosaccharide units may also be indicative of tri and tetra-antennary chains within the AGP molecule (Fournier *et al.*, 2000), however, evidence from Higai *et al.*, (2005) has since found the degree of branching and the degree of fucosylation to be independent of each other, i.e. samples were found to contain an increased number of bi-sialylated glycans (indicative of decreasing glycan complexity) with an increased level of fucosylation (a possible indicator of branch complexity). It's been found that glycosylation patterns exhibit decreased branching during acute inflammation (Brinkmann-van der Linden *et al.*, 1996), while chronic inflammation states, such as rheumatoid arthritis, have been found to exhibit increased branching patterns (De Graaf *et al.*, 1993) and an increase in fucosylation (Pawlowski *et al.*, 1989). This evidence is further supported by Higai *et al.*, (2005), who found increased instances of bi-sialylated glycans within the sera of patients suffering from acute inflammation, however, increases in fucosylation were also found within these samples; conversely, chronic inflammation sufferers were found to possess increased fucosylation within their tri- and tetra-sialylated glycans. It has been found by Fujimura *et al.*, (2008) that there was also an increase in fucosylation in glycans obtained from those suffering from prostate cancer.

Furthermore, it has also been seen previously, that the degree of fucosylation can change throughout an APR event. In a study by Orczyk-Pawilowicz *et al.*, (2009), it was found that the degree of fucosylation was altered between the 2nd and 3rd trimesters of normal pregnancies. The plasma collected during the 2nd trimester lacked any fucose, however, a dramatic increase in the levels of fucose was seen within the plasma during the 3rd trimester, leading to the suggestion that this increase in fucose could be a regulatory step in the gestation period, and the potential use of AGP as a monitor for successful development. It has been suggested by Gauldie *et al.*, (1985) and Kushner and Mackiewicz (1987) that the qualitative patterns of APPs (of which AGP is included) is not influenced to a significant degree by the type of injury sustained; however, it is suggested by Goldberger *et al.*, (1987)

and Kageyama *et al.*, (1985) that there is a limited linear relationship between the severity of the injury suffered and the degree of plasma protein expression.

1.2.3 Previous Studies of the Glycosylation Patterns of AGP in Disease

It has been seen in previous studies, that AGP glycosylation patterns can be altered by the APR when afflicted by disease. A study was carried out by Gallacher (2009) into the possible usage of AGP glycosylation patterns as a marker in early breast cancer. The research was a twofold analysis in which both the monosaccharide composition and oligosaccharide composition of AGP samples from patients with invasive cancer were compared to commercial AGP, age matched healthy AGP samples, benign samples, and non-invasive cancer samples. Results of the monosaccharide analysis showed that the invasive cancer samples possessed the highest concentration of fucose when compared to all other samples within the study. The hexosamine N-acetyl-galactosamine (GalNAc), a monosaccharide unit not found within the normal populations tested, was also found within the AGP collected from the invasive breast cancer group; showing that the APR can affect the types of monosaccharide units that can appear within AGP molecules. Within the oligosaccharide analysis portion of this study, there was an overall decrease in the complexity of the glycan chains with the increasing severity of the disease, i.e. more bi-antennary chains were seen within the invasive cancer sample group as opposed to more complex chains. As seen here, within breast cancer, there was an obvious difference between invasive cancer AGP samples when compared to “healthy” samples, and commercial AGP samples; thus, showing that AGP glycosylation patterns could be used as a potential biomarker in the detection of early stages of breast cancer. This study also showed a correlation between the levels of galactose, a main component of the branches of AGP, and the complexity (an increase in the number of peaks) of the branches.

Another study carried out by Anderson (2002) has shown the ability of AGP glycosylation patterns to be used to distinguish between different types of liver disease. Again the study used a twofold approach of analysis, by analysing the monosaccharides units and these molecules arrangements as oligosaccharide chains. The sample groups within this study were alcoholic liver

disease, hepatitis B, acute hepatitis C, chronic hepatitis C, cirrhosis, primary sclerosing cholangitis (PSC), and secondary biliary cirrhosis (SBC); these were then compared to “healthy AGP samples”. Within each sample group, an increase in the presence of fucose (hyperfucosylation) was found; with the hepatitis sample groups showing the highest levels of fucose within the study. The presence of N-acetyl-galactosamine (GalNAc) was also found within this sample group; GalNAc is not typically found within the monosaccharide composition of “healthy” AGP. The progression of hepatitis infections to cirrhosis would also show a decrease in the level of fucose as the hepatitis infection progresses. When analysing the oligosaccharide structure of the AGP within each of the sample groups, the study found that as each liver disease progressed in severity, the oligosaccharide make-up would increase in complexity, specifically showing an increase in the number of tri- and tetra-antennary branches. Again, the most promising results were within the hepatitis sample groups, with acute hepatitis C samples showing an increased number of bi-antennary branches when compared to controls, before showing an increase in the number of tri- and tetra-antennary branches when then the infection shifted to chronic hepatitis C; with chronic hepatitis C infection showing an increased level of these branching types as well. The glycosylation patterns of the AGP used within this study had shown to be able to successfully distinguish between different types of liver diseases, with the most success being found within the hepatitis sample groups and their progression to cirrhosis.

As seen in the above studies, the glycosylation patterns of AGP have shown the potential to be used as a biomarker for breast cancer and liver disease. It is this information that forms the basis for this study. Within this study, AGP glycosylation patterns shall be used to determine whether or not it is possible if a physical injury event can affect the AGP glycosylation pattern of AGP. The APR when brought on by physical injuries has shown to produce an increase in the serum concentration of AGP; however, could sports injuries produce an alteration in the glycosylation patterns of the AGP within the sample population of this project? Would there perhaps be individual glycosylation patterns seen between different sports injury types? By analysing the monosaccharide units and their arrangements within the AGP molecule as

glycan chains, by developing a “Glycosylation Fingerprint” as it were, this project hopes to answer these questions.

1.3 Mountain Biking

1.3.1 Mountain Biking History

Mountain Biking is a multi-disciplinary sport, of which consists of riding specially made mountain bikes over a selected distance or course. Mountain biking is split into different disciplines of which includes races and events recognised and officiated by the Union Cycliste Internationale (UCI); road cycling (road racing, and time trials in individual and team disciplines), track racing (containing three different disciplines, sprint events, endurance events and combined events), cyclocross, bicycle motocross (BMX) (involving supercross and freestyle events), and mountain biking, of which involves cross country (XC), cross country marathon, downhill (DH) and four cross (4X) events. Each discipline has their own specific rules and competitions. For example, XC racing involves competitors racing each other over a predetermined track of varying heights and lengths en masse, while dirt jumping practitioners compete on a specially designed track where the focus of the competition is the satisfactory completion of airborne jumps. Competitors within any discipline must possess great strength, speed, endurance and skill when on their respective course. Most disciplines require the use of protective equipment, which includes at least helmet and gloves, but body armour and a full face helmet can be used to provide, and is recommended, for further protection (UCI Official Guidelines); some disciplines even require the rider to carry their own first aid kit (UCI Official Guidelines). XC is perhaps the “classic” form of mountain biking known the public. As previously mentioned, XC MTB involves the riders racing each other over a predetermined length of track (typically 3-6km in length following UCI Guidelines) with varying turns, climbs and descents. The first rider to cross the finish line is the winner of the event; the winner of the event scores 250 points, of which goes towards their collective score for the entire season. The winner of the world cup is the rider whom at the end of the season possesses the most points within their discipline. There is also a single world championship race held every season, the winner of which is crowned the world champion within their discipline. Both the world cup and world championship events are sanctioned by the UCI. As can be seen here, the sport of XC MTB possesses a focus on the endurance of the athlete to compete over the distances seen within a XC race, as opposed to the

showmanship skill needed by those competing within the dirt jumping circuit, or the speedy reaction times necessary for DH mountain biking. Those who want more difficulty than what XC MTB can provide can then move onto the sport of DH mountain biking should they wish. DH mountain biking will be the focus of this study, and will be covered next.

1.3.2 Downhill Mountain Biking History

The sport of DH mountain biking is a time based sport in which participants ride down outdoor trails and race tracks in the quickest time possible. Most courses tend to involve mountainous terrain (hence the name), of which the rider would have to navigate steep drops, large jumps and sharp turns from the starting post to the finish line. Often the rider would be travelling at increasing speed along the course which could result in serious injury should they fall. The governing body for professional DH mountain biking is the UCI based in Switzerland. Due to the inherent dangers involved within the sport, competitors are required to wear full face helmets to minimise the possibility of serious injury. Other protective equipment such as gloves, knee pads, body armour, are not required to be worn during world cup events, however, the use of such equipment is strongly recommended by the UCI. Local race rules are also in place with organisers enforcing stricter equipment checks-this situation of minimum protection levels is currently under review within the UCI.

The sport of DH mountain biking was first developed as a sport in the late 1970's in the U.S.A., steadily growing in popularity on each side of the Atlantic, until it first became a recognised championship sport in 1990 in the UCI Mountain Biking Championships held in Colorado; the World Cup Series has taken place annually since 1993. Seven world cup races take place between the months of March and September every year on UCI approved world championship courses. Qualified riders take part within these races, gaining points based on their time based performance within these races; i.e. the rider who places first in a world cup race will gain 250 points which will go towards their score within the overall world cup standings. The winner of the world cup is the rider, who at the end of the seven event races has the most points in the world cup standings. In addition to the world cup, there is also the world

championship race every year as well. This is one race of which the competitor who possesses the fastest completed run once all competitors have raced will be crowned the DH MTB world champion.

However, DH mountain biking is not only a sport for professionals; there are many outdoor parks of which possess trails and race tracks for amateurs looking to sharpen their skills or even just for fun. Typically these trails are graded based on the perceived difficulty of the course; Glentress Forest Mountain Biking Trails (located in the Scottish borders) possesses four trail difficulties, **green** (easy, 3.5-4.5km in length, typical completion time of 0.5-1 hour), **blue** (moderate, 16km, 2-4 hours), **red** (difficult, 18km, 1.5-3 hours) and **black** (severe, 29km, 3-5hours); information taken from 7stanes Glentress and Innerleithen webpage. It is also possible for riders to partake on free style routes where they can freely ride the terrain rather than staying on a particular trail. There is also a freeride park within Glentress of which possesses a series of jumps, table-tops and assorted obstacles of which allow the rider to perform tricks on their bike as they see fit. The nature of these obstacles can result in injury should a rider fall off of them. Again, it is recommended by the Forestry Commission that each rider wear an appropriate cycle helmet and gloves, in addition to any other protective clothing they may wish to wear in order to minimise injury. It has been seen that by wearing a helmet, a rider can reduce the risk of head injury by 85% and brain injury by 88% (Schwellnus and Derman, 2005).

As previously mentioned, the sport of DH mountain biking was chosen within this project due to the inherent challenges of the sport to even the most seasoned rider. The nature of the sport, the ever increasing speed while riding downhill, the drops, the jumps and sharp turns can lead to serious injuries as a result of a momentary lapse in concentration while riding or executing a jump. The natural terrain of the trail may not afford the rider a soft landing on impact, and not all riders wear all the safety equipment available to them; injuries can happen to any rider while they're riding the trail.

A study carried out into recreational mountain biking injuries by Aitken *et al.*, (2011), using mountain bikers from Glentress Mountain Biking Trails and five local medical facilities in the surrounding area during a 12 month period of

study. During the period of study, 130,900 cyclists visited the park, and of these cyclists, 202 were treated at one of the five local medical facilities for an injury, resulting in 1.54 injuries per 1000 cyclists. Of these cyclists, 88% (178) of the injured cyclists were male, while 12% (24) were female. The greater participation of the sport by the male gender can also be seen within the members of the National Off-Road Bicycle Association (NORBA), with 87% of its members being male (USA Cycling, 2011). 16% of the injured cyclists required hospital admission. Of the 202 cyclists treated at the local medical facilities, 182 individuals were available for a further interview. Within the group of 182 injured individuals, 289 injuries were suffered, which resulted in 1.6 injuries per biker. Injury types were classified as wounding (affecting 63% of cyclists), soft tissue injury (37% of cyclists), fracture (37% of cyclists), head injury (13%) and dislocation (8%). Within Glentress, 45% of the injuries studied were suffered on the freeride trail, while 30% of injuries were suffered on the **red** route, 11% were suffered on the **blue** route and the **black** route respectively, while 1% of injuries were suffered on the **green** route, thus, showing that injuries can happen on any trail difficulty.

A review into mountain biking injuries by Krosnich *et al.*, (1996) found that for every 100 hours of mountain biking, 4.34 riders will be injured as a result of DH mountain biking, higher than the study carried out by Aitken *et al.*, (2011), and higher when compared to 0.37 riders who partake in XC mountain biking. Additionally, another 1996 study by Krosnich *et al.*, found that the most common injury to riders when falling off of their bikes was that of abrasions; what is also interesting to note is that during this multi-disciplinary study, 81.4% of injuries that occurred within the scope of the study happened during DH mountain biking. A further study by Chow and Krosnich (2002) supports these findings, they found that 70.5% of injuries present within the study were minor injuries and involved the extremities as opposed to the head and the abdominal cavity, while the incidences of concussions were found to be not uncommon either, with several studies reporting the injury incidence to be from 3-13% of injuries studied (Krosnich *et al.*, 1996, Krosnich *et al.*, 2002).

It is also worth noting that while the majority of injuries that happen to riders may be minor, this is typical when the rider is falling off of their bike to the side, rather than heading over the handlebars. Krosnich and Chow (2002) found

that riders who fell over their handlebars would be more likely to result in head and neck injuries (Apsingi *et al.*, 2006), while falling off to the side of the bike would result in the abrasions to the legs seen above. One study of trauma registries by Kim *et al.*, (2006) showed that orthopaedic injuries were the most common trauma admission as a result of mountain biking crashes, with 46.5%. Of these admissions, 66% of patients required surgery, with one patient even dying from his injuries. Fatalities occurring during riding are rare, in a study by Rivara *et al.*, 1997 a cyclist died after crashing without wearing a helmet, while another cyclist died after suffering from a ruptured diaphragm (Alvarez-Segui *et al.*, 2001). Interestingly, however, a study by Gaulrapp *et al.*, (2001), found that the most common site for injury was that of the leg, specifically, the calf and knee; Gaulrapp *et al.*, then go on to state that the most common fracture within their study was that of the shoulder, as opposed to the most common site of injury. A study by Jeys *et al.*, (1999) further supports this result.

However, it has also been reported that some cyclists have suffered from overuse injuries throughout the course of their riding. Overuse injuries are defined as injuries having been caused by repetitive actions leading to muscle, tendon and soft tissue damage. It was found in a 1998 study by Dingerkus *et al.*, that 45% of the 208 mountain bikers interviewed during their study were suffering from an overuse injury. The most common overuse injury areas within the body are saddle area, neck, hands, lower back, knee joints, and wrists (Froböse *et al.*, 2001).

Perhaps, what is most interesting of all to note, is that it is possible that riders who are competing within a DH mountain biking race, are four times more likely to become injured than those who are riding a trail for fun (Krosnich and Rubin, 1994); with the main contributing factors for injury found to be loss of control, the high speed descents present within a trail and the competitive atmosphere itself. This is understandable as every racer wants to win the race they are taking part in; it is interesting to note that within Britain's only UCI World Cup approved course (Fort William), that speeds of 60+km/h are possible on the course (reference data from our own lab). Should a rider fall while travelling at these speeds down the descending course, it is fully possible that an injury could be very severe. As it can be seen here, a great deal of

experience and skill is necessary to not only navigate the trail, but to do so in as fast a time as possible and in the safest way possible.

In summary, it is seen that while those with experience may not become injured very often, it is fully possible for the most seasoned rider to injure themselves while riding downhill. The potential for injuries due to the terrain of the trails and the speeds at which the riders can reach and Edinburgh Napier University's own strong links with DH mountain biking riders make DH mountain biking an ideal choice within this project into looking at the glycosylation patterns of physical injuries. The awareness that Edinburgh Napier University possesses within the mountain biking community and the steps taken within the recruitment of this project means that time between injury occurrence and venepuncture will be as short as possible as is convenient for the injured volunteer; which in turn, will provide a more accurate picture of the glycosylation patterns of the AGP within the injured volunteer in the time since their accident. Full ethical approval for this project was granted by the Edinburgh Napier University Faculty of Health, Life and Social Sciences Research Ethics and Governance Committee.

1.4 Project Aims

The main aim of the project was to determine whether or not the acute phase response altered the glycosylation patterns of the AGP of injured mountain bikers who have suffered a physical injury as a result of DH mountain biking. Furthermore, the project sought to determine whether or not different injury types resulted in the development of different glycoforms of AGP; i.e. will a shoulder injury produce different types of altered AGP as opposed to a leg injury. The final aim of this project was to determine whether or not the altered glycoforms of AGP could be used to diagnose a potential physical injury. The information gathered within this project would then be used as the basis for further study within this area. Figure 3, below, shows the plan of action for this project.

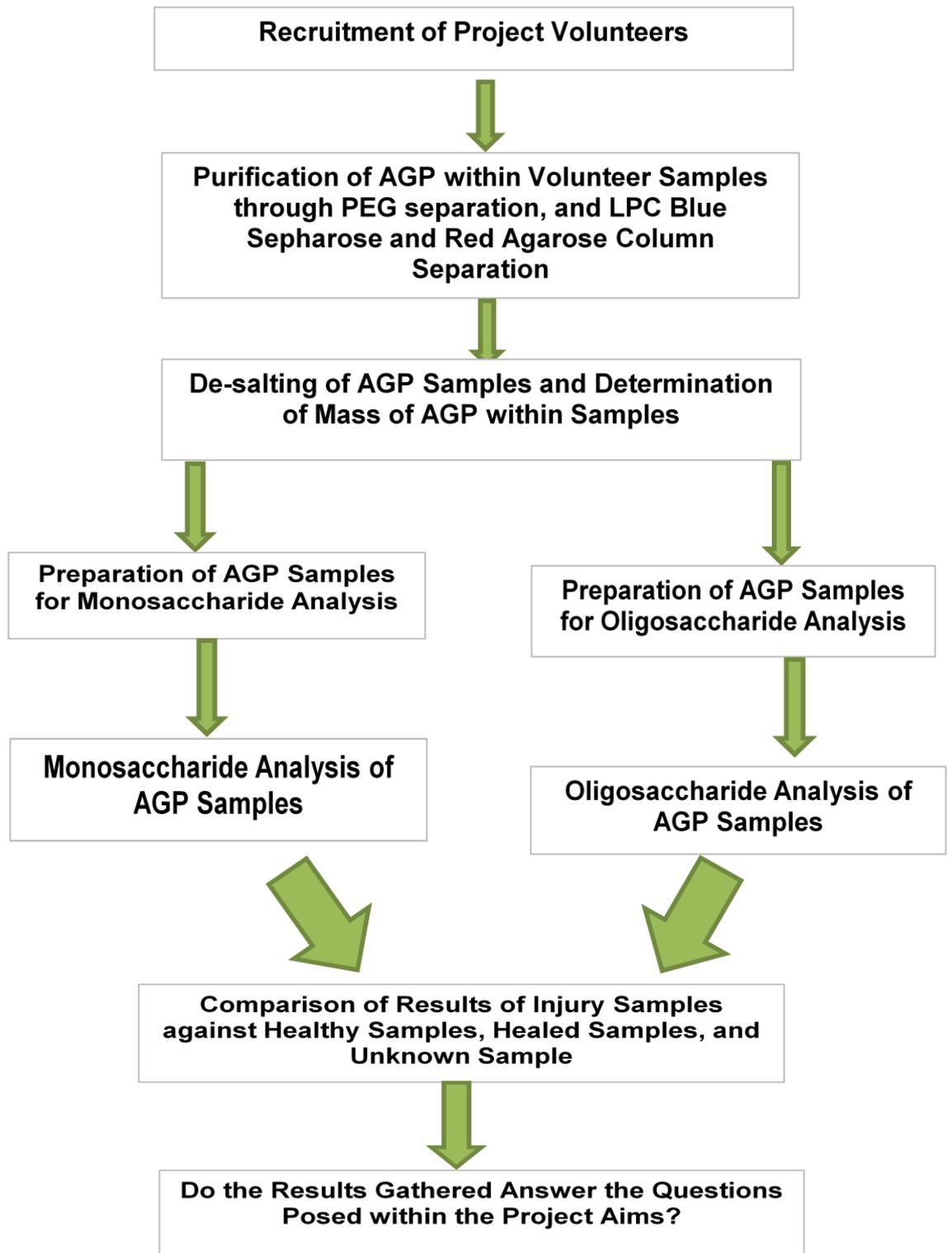


Figure 3. Project Action Plan

2.1 Materials

2.1.1 Volunteer Samples

The Edinburgh Napier University Ethics Committee approved the acquisition of ~5ml blood samples from volunteers who have been injured while taking part in DH mountain biking. In this preliminary study, any injury which occurred while taking part in DH mountain biking was eligible for use within this study. Volunteers were recruited via word of mouth, email, and the distribution of project descriptive leaflets to mountain biking centres and trails. An initial ~5ml blood sample was taken from project volunteers and placed into EDTA, anti-coagulant lined tubes, and the volunteer filled in an Napier University ethically approved questionnaire to categorise the injury they experienced. Should the volunteer wish, they were invited to provide a second ~5ml blood sample after their injury had healed to provide a “healthy control” to act as a comparison of healthy AGP to injury AGP. One sample remained as an “unknown” throughout the study, to determine the possibility of AGP being used as a diagnostic marker for sports injury. This sample, XX001 ??/, was purified, its glycosylation pattern analysed and compared to the known injury AGP samples to determine if there are any similarities or differences between the unknown sample and the known injury samples.

<u>Sample Number</u>	<u>Injury Area (Type)</u>	<u>M/F</u>	<u>Age</u>
SH001 18/3	Shoulder(Bruising and Cuts)	M	22
GI001 7/6/10	General Injuries(Bruising and Cuts)	M	41
EW001 20/3	Elbow(Abrasions)	M	34
LG001 12/9	Leg(Abrasions)	F	30
SH002 7/6/10	Shoulder(Break)	M	42
WR001 11/16	Wrist(Fracture)	M	21
XX001 ??/?	<i>Unknown</i>	<i>F</i>	
GI001 7/6/10	Healed	M	42
EW001 20/3	Healed	M	34
LG001 12/9	Healed	F	30
WR001 11/16	Healed	M	21

Table 1. Patient Demographics

2.1.2 AGP Purification

HPLC grade water was purchased from Rathburn Chemicals Ltd (Walkerburn, UK). 10ml Bio-rad columns were purchased from Bio-rad (Hemel Hempstead, UK). Glacial acetic acid, Polyethylene glycol (PEG) 3350, Potassium chloride, Potassium thiocyanate, Reactive blue 2 sepharose (Cibacron blue 3GA), Red sepharose CL-6B, Sodium acetate, Sodium azide, Sodium chloride, and Trizima were purchased from Sigma-Aldrich (Poole, UK). Blood samples were PEG separated through the use of an eppendorf centrifuge 5415C, and samples were concentrated using a Christ RVC 2-18 concentrator purchased from SciQuip Ltd, Shrewsbury, UK. Ethanol was purchased from Bamford Laboratories Ltd, before being diluted to 10% (v/v). A Pharmacia LKB Pump P-1 was used to load buffers and samples to the columns, a single path optical UV-1 monitor and control unit was used to measure the absorbance of the running samples at 280nm before recording the samples absorbance using a Servogor 120 chart recorder; all of which were purchased from Pharmacia, UK.

2.1.3 De-salting of Injury AGP Samples

HPLC grade water was purchased from Rathburn Chemicals Ltd (Walkerburn, UK) and Amicon centrifugal filter tubes with a MW cut off of 10,000 were used and purchased from Millipore Ltd (Hertfordshire, UK). A Universal 320 (large scale) centrifuge (Sartorius, UK) was used to spin the NaCl out of the injury AGP samples. A Christ RVC 2-18 concentrator (from SciQuip Ltd, Shrewsbury, UK) was used to concentrate samples down.

2.1.4 Determination of level of AGP in samples

Calibration curves for reference were produced through the use of commercial AGP purchased from Sigma-Aldrich; and HPLC grade water was used (Rathburn Chemicals Ltd, Walkerburn, UK). The absorbance of commercial and injury AGP samples were read using a BioMate 3

spectrophotometer purchased from Thermo Fischer Scientific (Loughborough, UK).

2.1.5 High pH Anion Exchange Chromatography

2.1.5.1 Monosaccharide Analysis

The necessary monosaccharide units for the monomix standards, 2-deoxy-D-galactose, fucose, galactose, glucosamine, mannose; in addition to the commercial AGP, 2ml hydrolysis reaction aluminium lined vials, Dowex - 50WX8-100 cation exchange resin (hydrogen form, 8% carbon loading, dry mesh 50-100) (which replaced the now out of stock Dowex- 50X12-100 cation resin) and 4M hydrochloric acid was purchased from Sigma-Aldrich (Poole, UK). HPLC grade 2M trifluoroacetic acid (TFA) was purchased from Thermo-Fischer Scientific (Loughborough, UK). HPLC grade water was purchased from Rathburn Chemicals Ltd, Walkerburn, UK. 50% w/v sodium hydroxide was purchased from VWR International Ltd (Lutterworth, UK). Solutions were degassed through the use of a helium cylinder and a Dionex degas module, to perform chromatography, a CarboPac PA-100 analytical (4x250mm) column and a (4x50mm) guard column were used on a Dionex 600 system with pulsed electrochemical detection (PED) and Chromeleon 6 software to record analytical results, all purchased from Dionex, Camberley, UK (now owned by Thermo-Fischer Scientific, Loughborough, UK).

2.1.5.2 Oligosaccharide Analysis

The necessary peptide-N-glycosidase F (PNGase F) (purified from *Flavobacterium meningosepticum*), 10% NP-40 buffer and NE G7 buffer were purchased from New England Biolabs Inc. (Hertfordshire, UK). HPLC grade water was purchased from Rathburn Chemicals Ltd, Walkerburn, UK. Ethanol was purchased from Bamford Laboratories Ltd (Rochdale, UK), the AGP N-linked glycan library was purchased from Prozyme (Europa Bioproducts Ltd, Cambridgeshire, UK). The 50% w/v sodium hydroxide was purchased from VWR International Ltd (Lutterworth, UK). sodium acetate, a CarboPac PA-100

analytical (4x250mm) column and a (4x50mm) guard column were used on a Dionex 600 system with a GP50 gradient pump, ED40 electrochemical and pulsed electrochemical detection to carry out analytical analysis of samples using Chromeleon 6 software to record analytical results, all purchased from Dionex, Camberley, UK (now owned by Thermo-Fischer Scientific, Loughborough, UK).

2.2 Methods

2.2.1 Sample Acquisition

As this is a preliminary project, there was no bias shown towards favouring a certain type of injury within the volunteer selection process. The procurement of samples was solely dependant on the willingness of volunteers to take part. Volunteers were recruited for this project through word of mouth, email correspondence between project organisers and cycling clubs, and through the use of project descriptive leaflets being handed out at Mountain Biking events and displays. Each project volunteer must have been injured while riding a Mountain Bike, and must have been injured at the time the venepuncture was carried out by the qualified phlebotomist. Volunteers were asked to sign a consent form to take part within the project (or have their parent's written consent if they were under 18) before the venepuncture could take place where they would then provide a 5ml blood sample, and afterwards, they were then asked to fill out a short questionnaire with which categorisation of their injury could then take place for project records. Should the volunteer then wish, once they had healed from their injury they could then come back to the University at a later date to provide a second 5ml blood sample to provide a reference comparison of their own healthy AGP of which could then be compared to their own injured AGP glycosylation patterns. At this time of writing (May, 2012), seven volunteers have provided a blood sample, with which further analysis has then progressed, while four of those volunteers have also provided a second blood sample for further healthy comparisons.

Within these samples, one sample has purposely remained as an "unknown"; this sample was classified as XX001 ?? to prevent the identification of the sample and the injury the rider suffered while riding downhill. This sample was then purified normally according to protocols, and the AGP within the sample was analysed by HPAEC as normal along with all of the known samples. The aim of this experiment was to determine whether or not there would be any diagnostic capabilities within the glycosylation patterns of the AGP. The resultant chromatographs of the analysis will be compared to the other known samples to determine if there are any similarities or differences between the unknown sample and the known injury samples.

2.2.2 Removal of Red Blood Cells from Samples

Once the samples were taken from the project volunteer, they were prepared before use within the HPAEC. To begin, samples were removed from the venepuncture collection tubes, and transferred into clean eppendorf tubes. Polyethylene glycol (PEG) 3350 was added to the blood samples (40% w/v), 0.4g of PEG was added to each 1ml sample. The contents of the eppendorf were lightly vortexed and then placed in a fridge at 4°C over night. The sample eppendorf was then placed in an eppendorf centrifuge at 14,000rpm for 30 minutes. The clear supernatant of the sample was then transferred into another fresh eppendorf, the pellets were then discarded; samples would be spun again at 14,000rpm for 30 minutes should they require. Samples were then placed within the freezer at -20°C until use within the project.

2.2.3 Low Pressure Chromatography- Blue Column

Three proteins were left within the sample once they have been through the PEG 3350 preparation; these proteins are albumin, α -anti-trypsin(AAT), and the desired alpha-1-acid glycoprotein (AGP). In order to procure pure AGP, the albumin and AAT had to be removed from each sample. Firstly, albumin was removed from the sample, which was achieved by using a low pressure chromatography column filled with reactive blue sepharose beads. When a sample was run through the column, albumin would bind to the sepharose within the column, allowing AAT and AGP to pass through the column and the detector.

The necessary buffers needed for this stage were prepared first. Elution buffer (Blue) was prepared by dissolving 7.45g of potassium chloride, 6.05g of Trizima, and 0.2g of sodium azide in distilled water using a magnetic stirrer, before being made up to one litre using more distilled water. The pH of the solution should be that of pH7; if not, 2M HCl should be added to the buffer until it reads pH7. Elution buffer (Blue) is necessary to move the samples through the column and the detector.

As albumin was bound to the sepharose following the passage of AAT and AGP through the column, albumin was removed from the column by the

addition of a description buffer to the column before another sample could be loaded. Description buffer was made by dissolving 12.15g of potassium thiocyanate in some elution buffer (Blue) using a magnetic stirrer, and making the solution up to 250mls by the addition of more elution buffer (Blue). The description buffer would remove the bound albumin from the sepharose within the column, to prevent clogging of the column with albumin and sample contamination between samples.

The column was then made up by adding 5mls of reactive blue sepharose beads to a bio-rad column, allowing the beads to settle and allowing the ethanol to elute out into a waste jar. More sepharose can be added to the column until it reads 5mls of sepharose beads. The column can then be stored in elution buffer (Blue) until use.

The necessary equipment should then be set up in the following orientation:

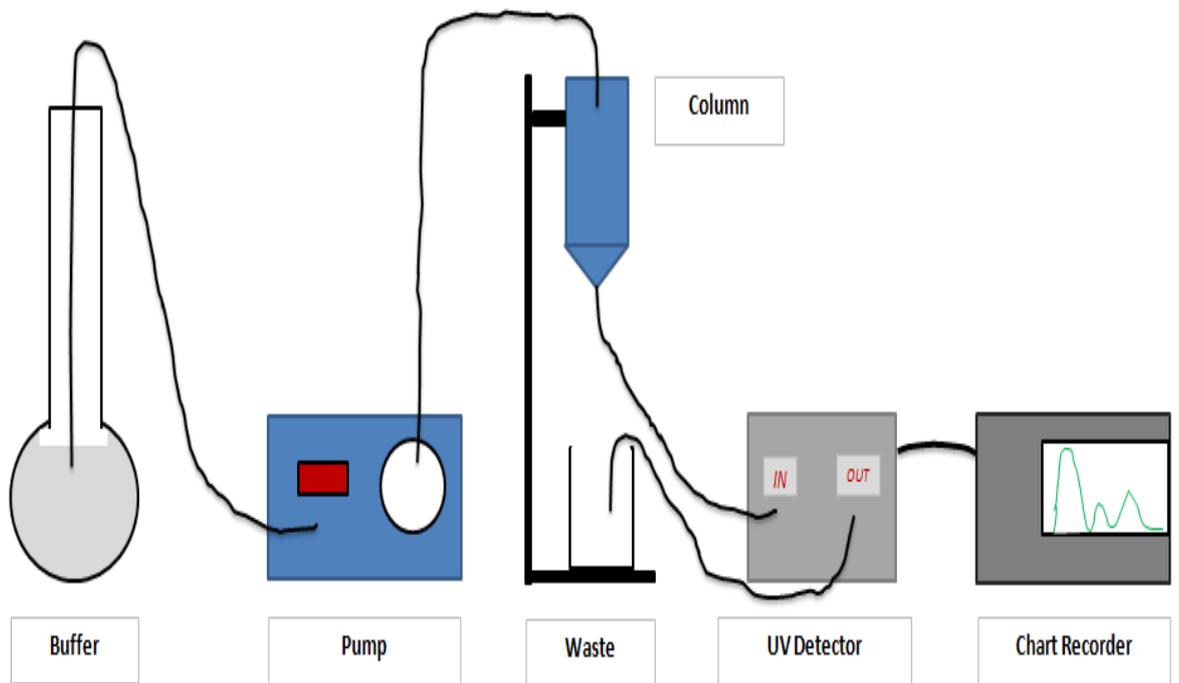


Figure 4. Equipment Orientation for Low Pressure Chromatography

Elution buffer (Blue) was then run through the column and the detector to equilibrate the column; the buffer was allowed to pass into the waste jar. Once a “flat base line” was achieved on the detector, the column was equilibrated and a sample was then added to the column. The sample was added into the column by removing the pump tubing from the elution buffer (Blue) bottle and placing the tubing into the eppendorf. Once the eppendorf was empty, the pump tubing could then be placed back into the elution buffer (Blue) bottle.

Once the chart recorder showed a line appearing above the baseline, the sample was collected by removing the waste jar tubing from the waste jar and placing it into a centrifuge tube. A single peak was being detected at 280nm, of which would contain AGP and AAT, with albumin being bound to the column. Once the peak had returned to baseline, all the AGP/AAT had passed through the column and the detector, thus, the waste tubing was placed back into the waste jar.

Description buffer was then passed through the column to remove albumin from the sepharose beads into the waste jar. A small peak appeared on the chart recorder, this is the albumin passing through the chart recorder. Again, once the chart recorder was back at baseline, all albumin had been removed from the column. Should another sample have been applied to the column, the column was re-equilibrated by addition of more elution buffer (Blue), to remove all traces of description buffer from the column. Once a “flat base line” had been achieved again, another sample could then be added to the column. Should the column have been needed again at a later date, then addition of 10% ethanol to the column and storage in the fridge at 4°C until next use was necessary.

2.2.4 Drying Down AAT/AGP Mixture-Interim Step Between Blue and Red Columns

The gyrovap was turned on and given time to come down to the necessary temperature of -60°C; typically around 20 minutes. Once ready, the centrifuge tubes containing the AAT/AGP mixtures were placed within the appropriately sized rotor, care being taken to make sure the samples were balanced within the gyrovap. The centrifuge was then set to spin for two hours;

in terms of regular practice, samples tend to evaporate roughly around 1ml per hour within the centrifuge tubes, samples within smaller vessels such as eppendorf tubes could take longer. Samples continued to be spun until each sample reached 2ml of solution. Samples could then be stored in the freezer at -20°C until next use.

2.2.5 Low Pressure Chromatography- Red Column

There was a further need to remove AAT from the AAT/AGP mixture within each sample. This was achieved by passing each sample through a column filled with reactive red 120-agarose type 3000 Cl beads. The red agarose beads bound AAT to the column, allowing the desired AGP to pass through the column. The buffers needed for this step of AGP purification were prepared before low pressure chromatography could begin.

Elution buffer (Red), the mobile phase for this step of AGP purification, was prepared by dissolving 4.102g of sodium acetate in 50ml of distilled water, before being made up to 250ml using more distilled water. 1.15ml of glacial acetic acid was then added to a 100ml volumetric flask, which was then filled to 100ml using distilled water. Elution buffer (Red) was then made up by adding 136ml of sodium acetate solution and 13.5ml of glacial acetic acid solution to a 1l volumetric flask, which was further made up to 1l using distilled water.

The cleaning buffer needed for this purification stage was prepared by dissolving 5.84g of NaCl in 25ml of elution buffer (Red), before making the solution up to 100ml using more elution buffer (Red). This buffer would remove the bound AAT from the column, allowing it to pass into the waste jar, preventing build up of AAT within the agarose beads within the column, and any possible contamination of further samples with leftover trace material from previous samples.

As before, a bio-rad column was filled up to 5ml with the reactive red 120-agarose type 3000 Cl beads, allowing the 0.5M NaCl elute to run out into the waste jar, and compacting the red agarose beads within the column, topping up the column further to read 5ml when filled with red agarose beads.

Furthermore, the necessary equipment was set up in the same orientation as seen in figure 4. Elution buffer (Red) was run through the column to achieve equilibration, with equilibration being shown with a “flat base line” on the chart recorder. Once equilibration was achieved, the sample was added to the column by taking the buffer tube out of the buffer bottle and placed into the centrifuge tube containing the AAT/AGP mixture. Once the entire sample was pumped into the column, the pump tubing was then placed back into the elution buffer (Red) bottle. Again, when a peak appeared above the baseline, the waste jar tubing was taken out of the waste jar and placed into a fresh centrifuge tube for collection of isolated AGP.

When the peak fell below baseline, collection was stopped and the waste tubing placed back into the waste jar. The column was then cleaned using cleaning buffer, this removed the bound AAT from the column while the elute was allowed to run into the waste jar; a small peak showing on the chart recorder, this was the AAT removed from the column. Once this peak decreased below baseline, cleaning had completed and should another sample have been needed to run through the column, then the column was further re-equilibrated before the addition of another sample. The column could then be stored in 10% Ethanol at 4°C should no further samples be run through the column. Again, samples were then dried down to 2ml each within the gyrovap before further use in the project.

2.2.6 De-salting Centrifugation

Before the mass of AGP within each injury sample could be determined using the spectrophotometer, it was necessary to remove the NaCl molecules introduced into the AGP sample during the red agarose AAT removal, this NaCl can interfere in the reading of the mass of AGP within the spectrophotometer. This was achieved by placing each 2ml sample within a clean centricon filtered centrifuge tube (each possessing an MW of 10,000), placing each sample within the top compartment of the centrifuge tube. Each sample was then topped up with a little HPLC grade water until the solution reached the rim of the top of the tube. Each centrifuge tube was then placed within a centrifuge, making sure to balance the centrifuge before spinning the samples at 4000rpm for 30 minutes

at 23°C. Removal of the NaCl molecules was achieved by using centrifugal force as the centrifuge forced all the smaller NaCl and water molecules out of the top layer through the 10,000 MW filter out to the bottom layer of the tube; allowing the larger AGP molecules to stay in the top layer of the centrifuge tube.

After 30 minutes had passed, each sample was taken and the bottom layer of elute (this is NaCl water) was emptied into a sink. Furthermore, the top layer of elute was then topped up with more HPLC water, and placed back within the centrifuge for another cycle of 30 minutes. This process was repeated, until the top layer of elute passed near completely through the filter when topped up with HPLC water; this indicated that the sample was then pure AGP without the NaCl molecules within; this process would roughly take around 6 cycles. Each AGP sample was then be topped up with 1ml of HPLC water and lightly shaken to encourage re-suspension before being transferred into fresh eppendorf tubes and dried down to completion (removal of all liquid) within the gyrovap. Once completely dried down, each sample was re-suspended in 1ml of HPLC water and lightly vortexed to encourage re-suspension. Each sample could then be placed within the spectrophotometer to determine the mass of AGP within each sample.

2.2.7 Determination of Mass of AGP within Injury Samples using Absorbance at 280nm

The mass of AGP within the injury samples was too small to measure accurately with scales, this was achieved by measuring the absorbance of the samples at 280nm within the spectrophotometer before comparing this to a standard curve of AGP absorbance which was constructed using a commercial AGP standard through the use of the Beer Lambert Law. The Beer Lambert Law states that there is a linear relationship between absorbance and the concentration of an absorber of electromagnetic radiation. Firstly, a stock solution of AGP was made up by dissolving 6mg of commercial AGP within 2ml of HPLC water. Once the absorbance of this sample had been determined, then another standard 0.5mg/ml less in quantity was made up from the preceding sample using the formula $C_1V_1=C_2V_2$. For example, the standards within this study were calculated using the following; in all calculations, V1 is the value to

be determined, while all standards were to be made up to 1ml (V2) using HPLC water:

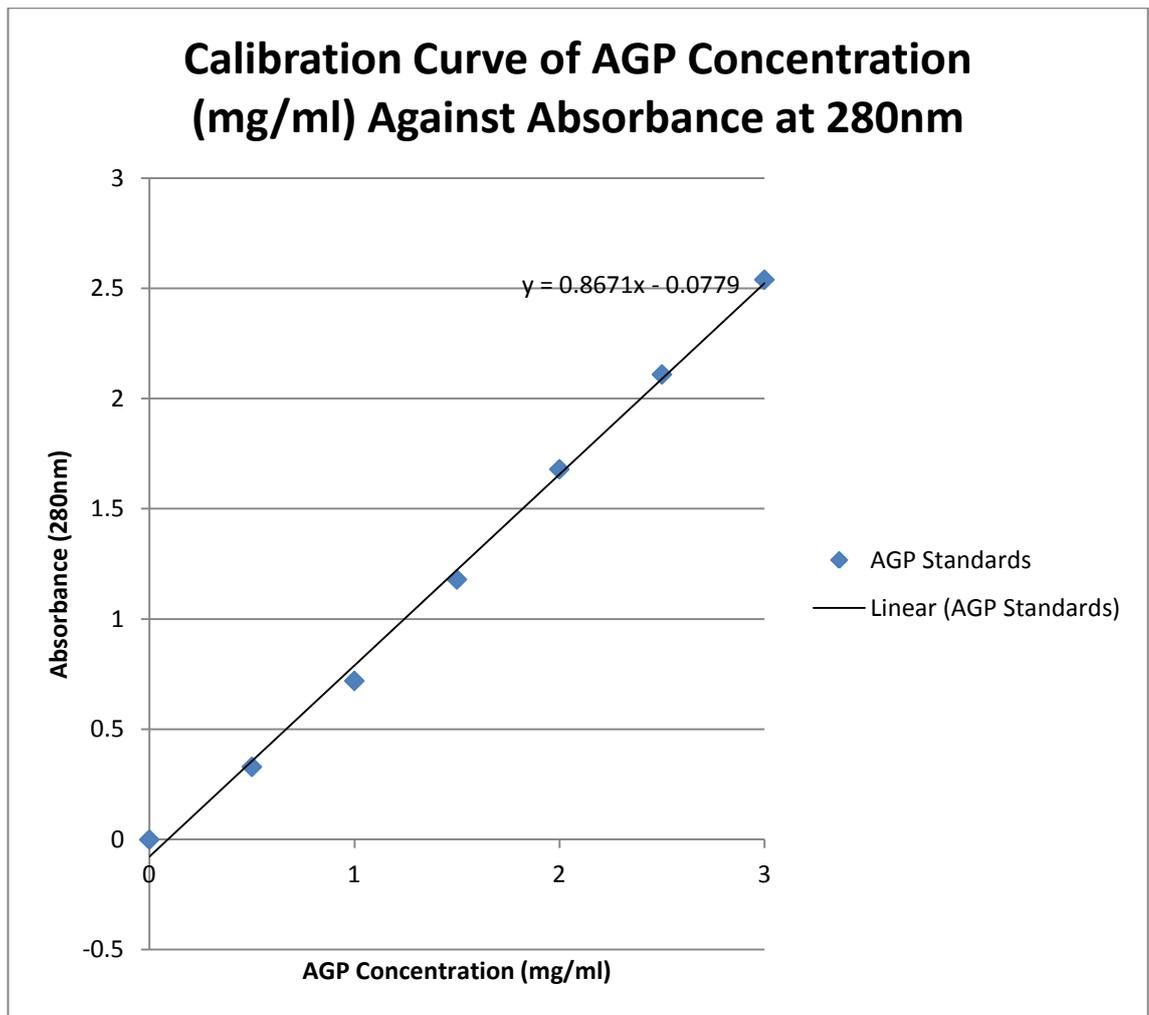
- 3mg/ml standard= 6mg of commercial AGP in 2ml of HPLC water.
- 2.5mg/ml standard, C1= 3mg/ml, C2= 2.5mg/ml
 $V1 = (C2V2)/C1 = (2.5 \times 1)/3 = 0.833\text{ml}$ of 3mg/ml
 $V2 = 1\text{ml} = 1 - 0.833\text{ml} = 0.167\text{ml}$ of HPLC water.
- 2.0mg/ml standard, C1= 2.5mg/ml, C2=2mg/ml
 $V1 = (2 \times 1)/2.5 = 0.8\text{ml}$ of 2.5mg/ml standard
 $V2 = 1 - 0.8 = 0.2\text{ml}$ of HPLC water.
- 1.5mg/ml standard, C1= 2.0mg/ml, C2=1.5mg/ml
 $V1 = (1.5 \times 1)/2 = 0.75\text{ml}$ of 2.0mg/ml standard
 $V2 = 1 - 0.75 = 0.25\text{ml}$ of HPLC water.
- 1mg/ml standard, C1=1.5mg/ml, C2= 1mg/ml
 $V1 = (1 \times 1)/1.5 = 0.667\text{ml}$ of 1.5mg/ml standard
 $V2 = 1 - 0.667 = 0.33\text{ml}$ of HPLC water.
- 0.5mg/ml standard, C1=1mg/ml, C2=0.5mg/ml
 $V1 = (0.5 \times 1)/1 = 0.5\text{ml}$ of 1mg/ml standard
 $V2 = 1 - 0.5 = 0.5\text{ml}$ of HPLC water.
- 0mg/ml standard= 0mg of AGP, 1ml of HPLC water.

<u>Quantity (mg)</u>	<u>AGP Vol. (ml)</u>	<u>HPLC Water Vol. (ml)</u>	<u>Standard Total Volume (ml)</u>
3.0	1.00	0.00	1.0
2.5	0.83 from 3mg/ml std	0.17	1.0
2.0	0.80 from 2.5mg/ml std	0.20	1.0
1.5	0.75 from 2mg/ml std	0.25	1.0
1.0	0.67 from 1.5mg/ml std	0.33	1.0
0.5	0.50 from 1mg/ml std	0.50	1.0
0.0	0.00	1.00	1.0

Table 2. AGP Dilution Chart

Once these solutions had been made up within fresh eppendorf tubes, each sample was then placed within a quartz cuvette and placed within the spectrophotometer at 280nm, making sure to clean the quartz cuvette between samples; results were recorded for each of the commercial AGP samples, before placing the injury samples within the spectrophotometer and reading those results.

The results (minus the injury samples) were placed within an excel table, creating a scatter graph with AGP mass on the X-Axis and Absorbance on the Y-Axis, adding a trendline to the graph and displaying the equation on the graph; a gradient was then gained as “ $y=.....X$ ”. In practice this was shown as $y=0.8671X$, which was then rearranged to $X=y/0.8671$, where X is the mass of AGP to be determined, while y is the samples absorbance, this information is found within the graph below.



Graph 1. Calibration Curve of AGP Concentration (mg/ml) Against Absorbance at 280nm

Once the mass of AGP within the samples had been determined, it was then necessary to determine how much AGP was needed for the next stages of the project. This was determined using $C_1V_1=C_2V_2$, where the concentration of the AGP within the purified sample was multiplied by its volume, which was equal to the concentration of the AGP within a second sample when multiplied by its volume. In this case the equation was rearranged to determine the volume needed for the next steps of preparation (V_2), which required specific amounts of AGP to complete. The rearranged equation was $V_2= (C_2/C_1) \times V_1$; using LG001 12/9 (which was determined to possess an AGP concentration of 1.727mg/ml) as an example:

$V_2= (50\mu\text{g}/1727\mu\text{g}) \times 1000\mu\text{l}= 29\mu\text{l}$ of LG001 12/9 was needed for acid hydrolysis breakdown (monosaccharide preparation).

$V_2= (100\mu\text{g}/1727\mu\text{g}) \times 1000\mu\text{l}= 58\mu\text{l}$ of LG001 12/9 was needed for enzyme digestion breakdown (oligosaccharide preparation).

Using this information, it was then possible to move on with further preparation for testing within this project.

2.2.8 Further Preparation for Monosaccharide Analysis

2.2.8.1 Acid Hydrolysis of Injury AGP samples (Monosaccharide Analysis Preparation)

In order to determine the glycosylation pattern for each injury sample, it was necessary to determine the specific monosaccharide units that make up the carbohydrate portion of the AGP molecule, before analyzing the arrangement of each of these monosaccharide units as glycan (oligosaccharide) branches. To analyze the monosaccharide units within the HPAEC, it was necessary to first break the glycosidic bonds within the AGP molecule that bond every monosaccharide unit to each other and to the AGP protein backbone. This was accomplished by acid hydrolysis, which breaks down the bonds between monosaccharides through a combination of acidic application and heat.

Firstly, a heat block was set up and time was given to allow it to reach the necessary temperature of 100°C for acid hydrolysis. From here, the

calculated volumes of injury sample AGP were transferred into fresh aluminium lined glass vials; the aluminium lining would prevent any escaping evaporate. Should the calculated volumes of AGP have equated to more than 100µl, then these volumes were dried down to completion and then reconstituted in 100µl of HPLC water. Once the samples were in the aluminium vials, then 100µl of 2M trifluoroacetic acetic acid and 50µl of 4M hydrochloric acid were added to each sample before screwing the black lid on each sample tightly and before placing them into the heat block for 4 hours. Note that the lids must be checked after 2 hours as they can become loose due to the expanding glass and plastic of the lids, if they are loose, then they must be tightened. Once the allotted time had passed, samples were taken off of the heat block and placed onto the bench to cool to a safe temperature before being handled within the next stage of the project.

2.2.8.2 Dowex Separation of Injury AGP samples (Monosaccharide Analysis Preparation)

Now that the monosaccharides were now free within the glass vials, it was then necessary to separate the free monosaccharides from the leftovers of the protein backbone within the sample. This was accomplished by passing the hydrolysed AGP sample through a column filled with Dowex resin. A Dowex column is simply a pasteur pipette plugged with half a centimetre of glass wool, before placing the pipette into a retention stand above a waste beaker, then filled with a centimetre of Dowex resin and allowing the elute to run out into the waste beaker. The Dowex resin should be pH4 before being used in this procedure; if not, then the beads should be washed with HPLC water until the required pH is reached.

Once the Dowex resin had been added to the column, then six 1ml fractions of HPLC water were ran through the column, one at a time, to achieve column equilibration. Then an AGP injury sample was run through the column, collecting the elute in a fresh eppendorf. A further two 1ml fractions of HPLC water were added to the column, again collecting the elute in an eppendorf tube; resulting in two eppendorf tubes filled with 1ml of a mixture of free monosaccharides suspended within HPLC water. Should anymore samples had required processing through a Dowex column, then a new column was made

and equilibrated for every sample. Each sample's two eppendorf tubes were then dried down to 0.5ml before being added together to make one 1ml eppendorf tube for each sample, which was then further dried down to completion and reconstituted in 195 μ l of HPLC water and 5 μ l of internal standard (2-deoxy-D-galactose); these reconstituted samples were then be transferred into fresh 1.5ml Dionex vials for analysis within the HPAEC.

2.2.8.3 Preparation of Monosaccharide Control (Monomix) for use in the HPAEC

The monomix was this project's control solution for monosaccharide analysis; it is a five sugar unit solution that was ran through the HPAEC prior to an analytical run to show that the HPAEC was working as expected, and allow the appropriate comparison and quantification of any monosaccharide sugars detected within the injury samples tested to take place. The monomix contained the four sugars that are typically found to make up AGP; these are fucose, glucosamine, galactose and mannose. Also present within the solution was an internal standard (2-deoxy-D-galactose) which allowed quantification of the other carbohydrates in the monomix.

Firstly, a stock of each carbohydrate was made up by dissolving 10mg of each of the sugars in 10ml of HPLC grade water, creating a carbohydrate concentration of 1mg/ml. From this, 200 μ l of each sugar was transferred into a fresh eppendorf tube, creating a 1ml solution containing equal amounts of each of the sugars. Once the 1ml monomix was complete, 20 μ l of the monomix solution was transferred into a fresh 1.5ml Dionex vial; then 180 μ l of HPLC grade water was added to the solution bringing the total volume within the vial to 200 μ l, creating a 1 in 10 dilution of the original monomix. This dilution was a necessary step as the sugars, undiluted, could clog the column over time; it also allows a clearer separation of the five peaks that the five carbohydrates are expected to produce in a working analysis.

2.2.8.4. Monosaccharide Analysis by HPAEC

High pH Anion Exchange Chromatography (HPAEC) separates a substance out via the degree of negative charge produced by substances basic components. In this instance, HPAEC was used to separate out a sample of AGP based on the degree of negative charge produced by the monosaccharides found within the tested samples. Within chromatography, a solid phase and a mobile phase are needed; a Carbo-Pac 100 column was the solid phase and a solution of 3%/97% sodium hydroxide(NaOH)/HPLC grade water was used as the mobile phase to move the samples through the column at a rate of 0.5ml a minute on an isocratic gradient. The samples' base sugar components were then detected using a PED electrochemical detection system, which would highlight the negative charges within each of the sugar components, resulting in a chromatograph which would show the user which sugar has come off the column at a precise time and order.

In this case, the prepared injury AGP samples were placed within the HPAEC, alongside a monomix control; care being taken to place the monomix first, as this would show the HPAEC was working properly, and, therefore, could prevent sample wastage should a problem arise within the HPAEC. Once the all the samples were ready, the analytical run could begin, starting off with the monomix before automatically proceeding onto each sample. A typical running time for one sample's separation run was 30 minutes (45 minutes when including a regeneration run to clean the column), thus, if a lot of samples needed processed, appropriate time management measures were taken. Once finished, samples were kept within the freezer for reference, and the injury AGP sample chromatographs were printed off and used for future analysis.

2.2.9 Further Preparation for Oligosaccharide/Glycan Branching Analysis

2.2.9.1 Heat Denaturation of Injury Sample AGP (Oligosaccharide Preparation)

Similarly to monosaccharide preparation of injury AGP samples, there was a need within this project to study the arrangements of the monosaccharide units (the glycan branching) within the AGP molecule. To accomplish this, acid

hydrolysis was not possible; in this instance, there was a need to maintain the glycosidic bonds between the monosaccharide molecules so that determination of the actual arrangements of the branches could take place.

Firstly, there was a need to determine the volume of injury AGP sample needed to provide 100µg worth of AGP for oligosaccharide preparation; an example of such was shown on page 34. As with monosaccharide preparation, should the volume have been more than 100µl, then the sample was dried down to completion before being reconstituted in 100µl of HPLC water. Each sample was then placed within a fresh aluminium lined 1.5ml glass vial, before being placed into the heat block at 100°C for 3 hours. This would denature the protein backbone of the AGP molecule, while leaving the monosaccharide arrangements within the molecule intact. Once the three hour period was complete, addition of 100µl of HPLC water and a light vortex was used to encourage re-suspension of the AGP sample; as the heat block had been at 100°C, it is possible that the majority of AGP sample would have evaporated within the glass vial, the addition of HPLC water can help to encourage this re-suspension. Once re-suspended, each sample was then dried down to completion before the next stage of oligosaccharide preparation.

2.2.9.2 Enzyme Digestion of Injury AGP Samples (Oligosaccharide Preparation)

While heat denaturation denatures the AGP protein molecule, it does not separate the desired glycan branches from the protein backbone; this was achieved by the addition of PNGase F enzyme. This cleaves the desired glycan branches from their five bonding sites on the surface of the AGP protein backbone.

Firstly, an incubator was set up to allow time to reach the optimum temperature of the PNGase F enzyme, which was 37°C. From here, a stock solution of PNGase F solution was made; only five units of the enzyme were needed for each sample at this stage of the preparation. To accomplish this, 1µl of a 500 unit/µl stock of the enzyme was taken and diluted in 99µl of HPLC grade water within a fresh eppendorf; this diluted the enzyme, and allowed for

1µl of the PNGase F dilution to contain the desired 5 units of enzyme needed for digestion of the AGP samples.

To each eppendorf of dried down AGP, 10µl of NE G7 Buffer was added, along with 10µl of 10% NP-40 buffer, and 79µl of HPLC grade water. The mixture was light vortexed to encourage re-suspension of the dried down AGP. Finally, 1µl of PNGase F (5 units) from the prepared enzyme dilution was added to each eppendorf tube, thus beginning the enzymatic digestion of the AGP. Each sample was taken and placed within the prepared incubator at 37°C for 24 hours. After 24 hours had passed, a further 1µl (5 units) of PNGase dilution was added to the solution, before beginning another 24 hour incubation. While this incubation was carried out, 300µl of ethanol per sample was transferred into fresh eppendorf tubes and placed within the freezer to chill overnight for the next step of the preparation.

2.2.9.3 Ethanol Precipitation of Injury AGP Samples (Oligosaccharide Preparation)

At this stage of the preparation, the PNGase enzyme had cleaved the desired AGP glycan branches from the protein backbone; as a result, it was necessary to separate the glycan branches from the digested protein molecule. This was achieved through precipitation of the injury AGP samples using ice cold ethanol and centrifugation. To stop the enzymatic digestion reaction, 300µl of ice cold ethanol was added to the reaction mixture; the volume of ethanol added should be three times that of the enzymatic solution (in a ratio of 3 parts ethanol:1 part enzymatic solution), hence 300µl of ethanol added to 100µl of enzyme solution.

The resultant 400µl AGP samples were then placed within a freezer at -20°C overnight for incubation. After the incubation was complete, the injury AGP samples were centrifuged at 14000rpm for 30 minutes within an eppendorf centrifuge; this would separate the glycan chains within the ethanol from the protein backbone, leaving the protein backbone molecules as a pellet at the bottom of the centrifuge. The supernatant was transferred to a fresh eppendorf tube, while the pellet was discarded. Each AGP sample was then dried down to

completion using the gyrovap. Once dried down, the samples were then reconstituted in 200µl of HPLC grade water before being transferred to fresh 1.5ml Dionex vials for use within the HPAEC for oligosaccharide analysis.

2.2.9.4 Oligosaccharide Analysis by HPAEC

Oligosaccharide analysis by HPAEC utilises the same principle as monosaccharide analysis; each glycan branch is separated out on the basis of the degree of negative charge within the branches, effectively, separating each type of branch out on the basis of size as the isocratic gradient increases, i.e. bi-antennary branches will separate off the column first (typically between 20-30 minutes), tri-antennary branches would be next (30-40 minutes) with tetra-antennary branches coming off last (40-50 minutes). The specific protocol itself, however, is slightly different, in that sodium acetate was also used alongside NaOH and HPLC grade water as the mobile phase within this type of separation; the length of the separation run itself was also longer (50 minutes) to accommodate the retention times of the glycan branches.

The newly reconstituted injury AGP samples were placed within the autosampler of the HPAEC alongside a commercial AGP standard, and an oligosaccharide library as a control to show the HPAEC was working to specifications and to allow comparison of injured AGP to “normal AGP”. Once complete, samples were kept within the freezer and the necessary chromatographs were printed out and used for further analysis.

3. Results

3.1 Monosaccharide Analysis

3.1.1 Purification Results

The blood samples from participants with injury used within the project were collected via venepuncture, carried out within the University by a qualified phlebotomist. The two normal blood samples were received from the NHS Blood Transfusion Service. These samples were collected into anti-coagulant lined (EDTA) blood tubes before use within the project.

It was necessary to remove the red blood cells and most trace proteins from within the samples before any further purification could be carried out. This was achieved by adding 0.4g of 3350 PolyEthylene Glycol (PEG) per millilitre of sample blood. Any molecule under 3350 Daltons in molecular weight was precipitated off within the PEG. Each sample was stored in the fridge at 4°C overnight, before being spun within the eppendorf centrifuge at 14000rpm for 30 minutes. Once each sample had been spun for 30 minutes, the clear plasma precipitation was collected into a fresh eppendorf tube while the red pellet was discarded. This plasma then contained three proteins of similar molecular weights; the desired AGP protein (43kDa), alongside albumin (67kDa) and α -anti-trypsin (AAT) (52kDa), both of which would be removed by low pressure chromatography, described within the methods section of this thesis. An example of a complete blue column chromatogram can be found in Figure 5 below.

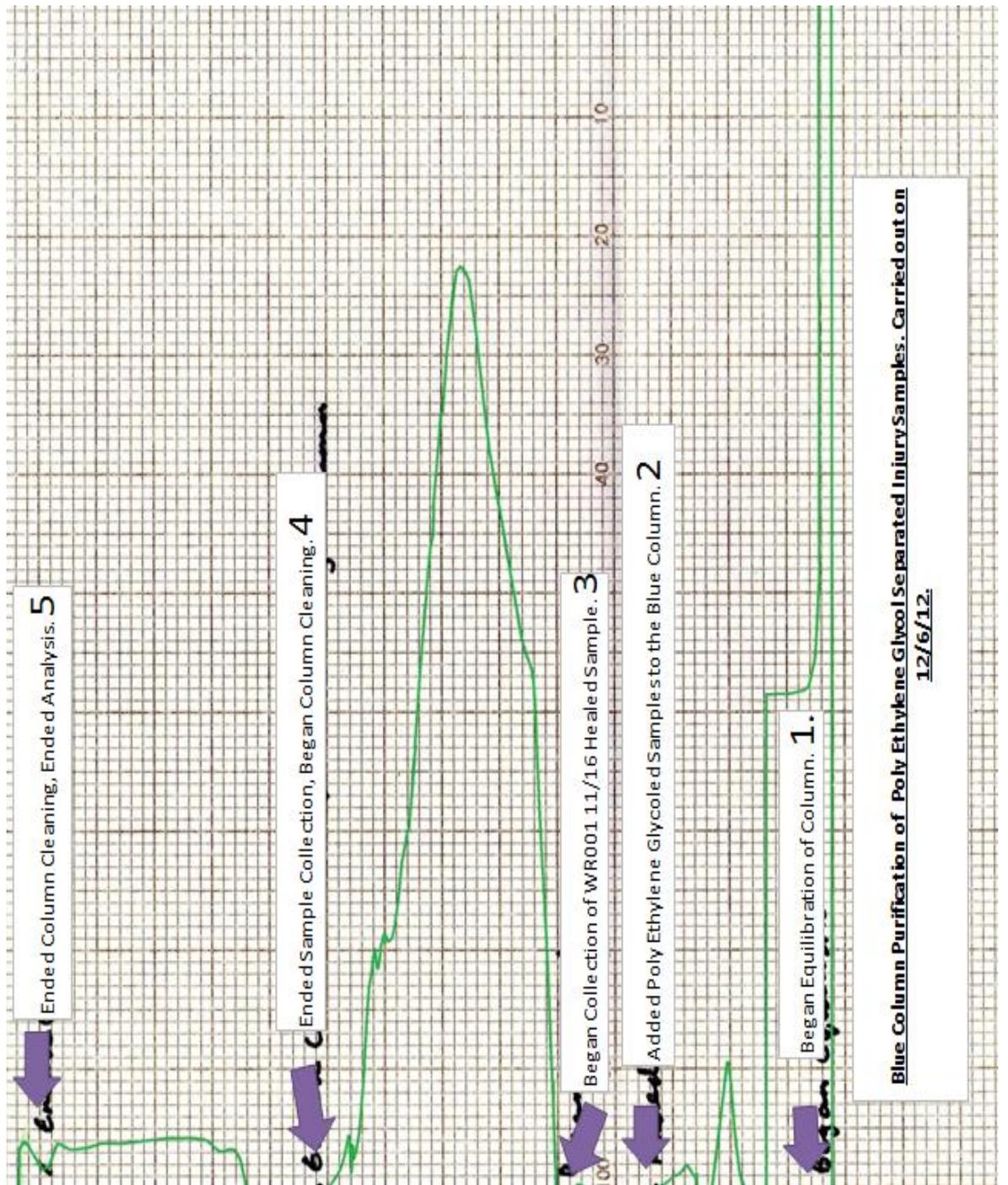


Figure 5. Blue Column Low Pressure Chromatography Chromatograph

Figure 5 shows the transit of a blood sample through the reactive blue sepharose filled Bio-rad column. Point 1 shows the addition of elution buffer (Blue) to the blue column to achieve column equilibration. Point 2 shows the addition of the blood sample to the column, with Point 3 showing the appearance of the AGP/AAT mixture passing through the UV detector, and thus was the collection point within the project. Point 4 shows the moment at which collection was stopped, with all the AGP/AAT having passed through the column; at this point description buffer (Blue) was added to the column in order to remove the albumin bound to the column, with Point 5 showing the point at which column cleaning was completed and blue column purification ended.

Once blue column purification had been carried out, each AGP/AAT sample was dried down to ~2ml of solution before being purified via the action of red column purification. The transit of the protein mixture through the column was then recorded, this can be found below;

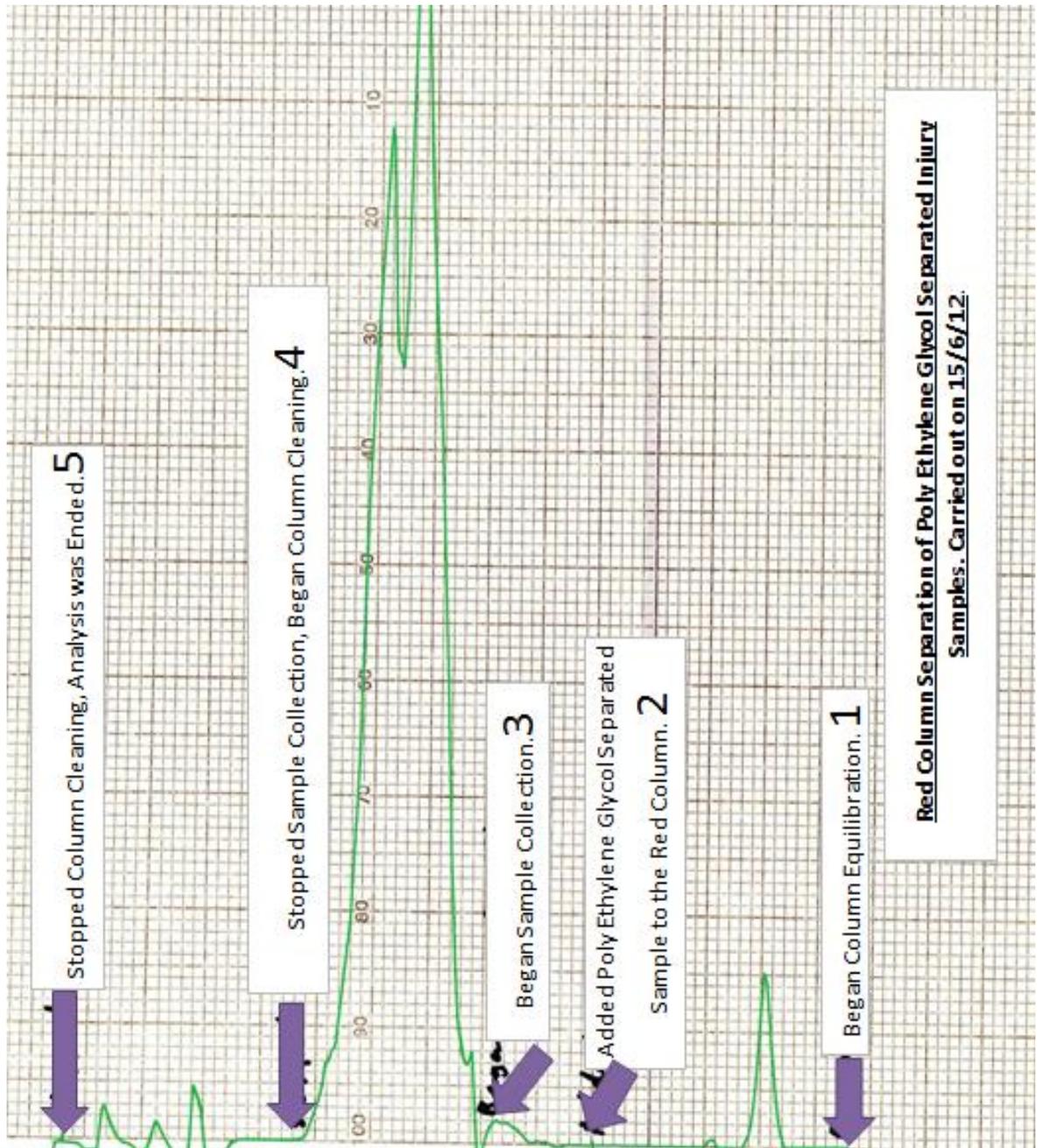


Figure 6. Red Column Low Pressure Chromatography Chromatograph

Figure 6 shows the transit of an AGP/AAT sample through the reactive red agarose filled Bio-rad column. Point 1 shows the addition of elution buffer (Red) to the red column to achieve column equilibration. Point 2 shows the addition of the AGP/AAT sample to the column, with Point 3 showing the appearance of the AGP mixture passing through the UV detector, and thus was the point where collection of the sample began. Point 4 shows the point at which collection was stopped, with all the AGP sample having passed through the column; at this point the cleaning buffer was added to the column in order to remove the AAT bound to the column, with Point 5 showing the point at which column cleaning was completed and red column purification ended.

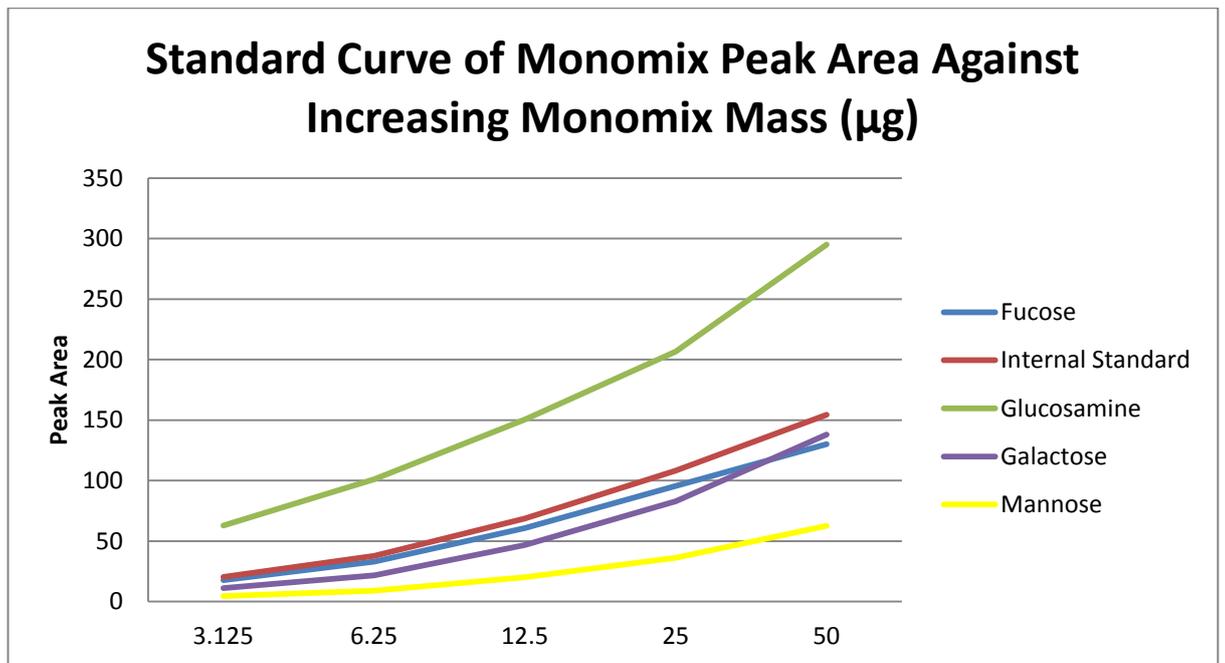
Each sample received for analysis in this project was purified in this manner before being further purified via centrifugation to remove NaCl molecules which were introduced during the red column purification. Once each sample had been purified, they were then read within the spectrophotometer to determine the mass of AGP within each sample. Using the above purification method, the samples were then found to contain the following masses of AGP within them.

<u>Sample Name</u>	<u>Absorbance at 280nm</u>	<u>Concentration of AGP within Sample(mg/ml)</u>
SH001 18/3	2.998	3.450
GI001 7/6/10	3.000	3.460
EW001 20/3	0.991	1.143
LG001 12/9	1.498	1.727
SH002 7/6/10	0.443	0.511
WR001 11/16	0.232	0.268
XX001 ?/?	0.299	0.345
GI001 7/6/10 Healed	1.127	1.300
EW001 20/3 Healed	0.274	0.316
LG001 12/9 Healed	0.632	0.730
WR001 11/16 Healed	0.458	0.530
Normal Blood	0.640	0.738

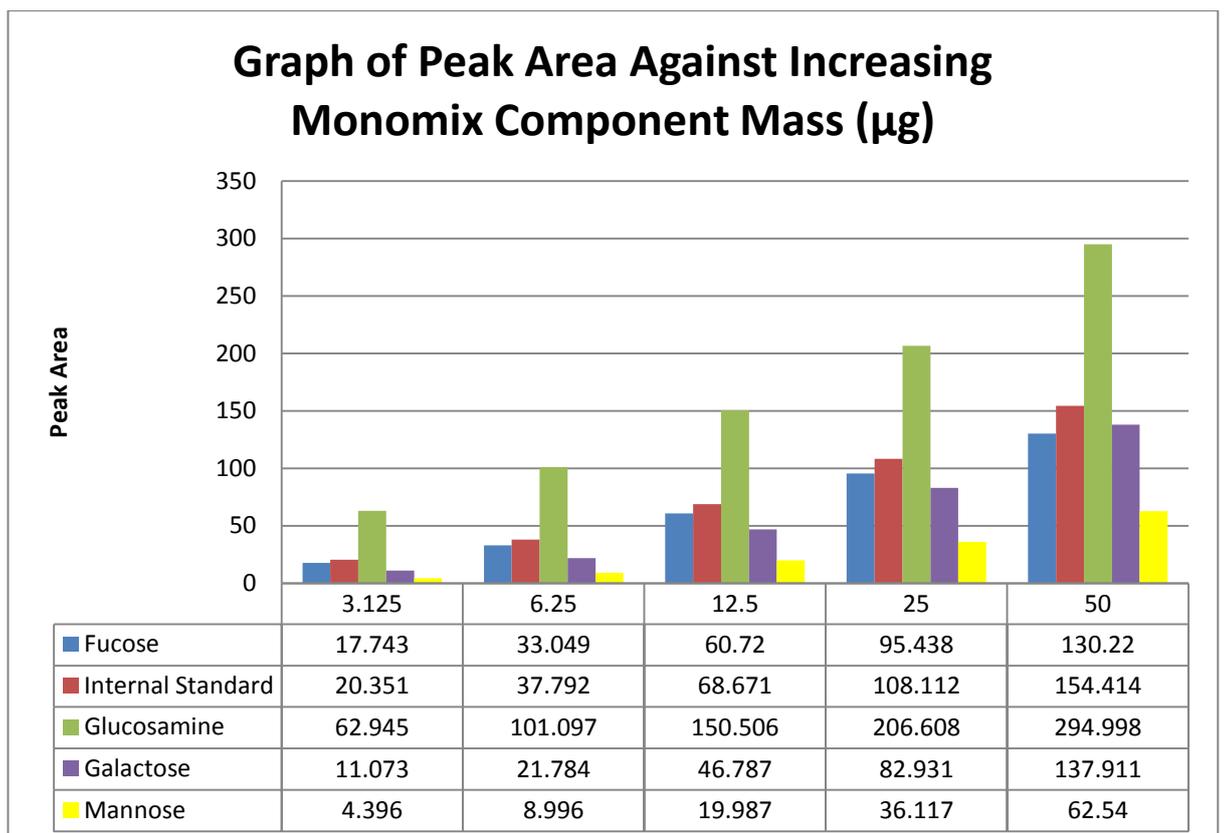
Table 3. Summary of Concentration of AGP and Absorbance when tested at 280nm within Tested Project Samples

3.1.2 Monosaccharide Quantification

Throughout the course of the project, once the monosaccharides within the samples had been detected, it was then necessary to quantify the monosaccharides found within each tested sample. This was achieved by making a monomix solution before making up standard dilutions of the mixture (ranging from 0µg-50µg each within 200µl of HPLC water) which were testing the samples within the HPAEC, and quantifying each of the monosaccharides within each trace over the course of the entire run. Once all the samples had been tested, the results for each individual monosaccharide were collated for the entire run (i.e. the peak areas seen for fucose throughout the five runs were all entered into Excel). Each monosaccharides detected peak area was entered into excel and a standard curve for that monosaccharide over time was produced, and an equation was given; this equation would allow the further determination of a given monosaccharide amount when monosaccharide testing of AGP samples was carried out.



Graph 2. Standard Curve of Individual Monomix Monosaccharide Components



Graph 3. Graph of Peak Area Against Increasing Monomix Component Mass (μg)

The resultant equations for the above monosaccharide components of the Monomix were as follows;

$$\text{Fucose: } y=28.734 x- 18.769$$

$$\text{Internal Standard: } y=33.845 x- 23.666$$

$$\text{Glucosamine: } y= 56.962 x- 7.6543$$

$$\text{Galactose: } y= 31.482 x- 34.35$$

$$\text{Mannose: } y= 14.341 x- 16.616$$

Y is representative of the peak area achieved within monosaccharide analysis, while x is monosaccharide amount. The equations were then rearranged to determine x;

$$\text{Fucose: } x= (Y+18.769)/28.734 = \text{___}\mu\text{g}$$

$$\text{Glucosamine: } x= (Y+7.6543)/56.962= \text{___}\mu\text{g}$$

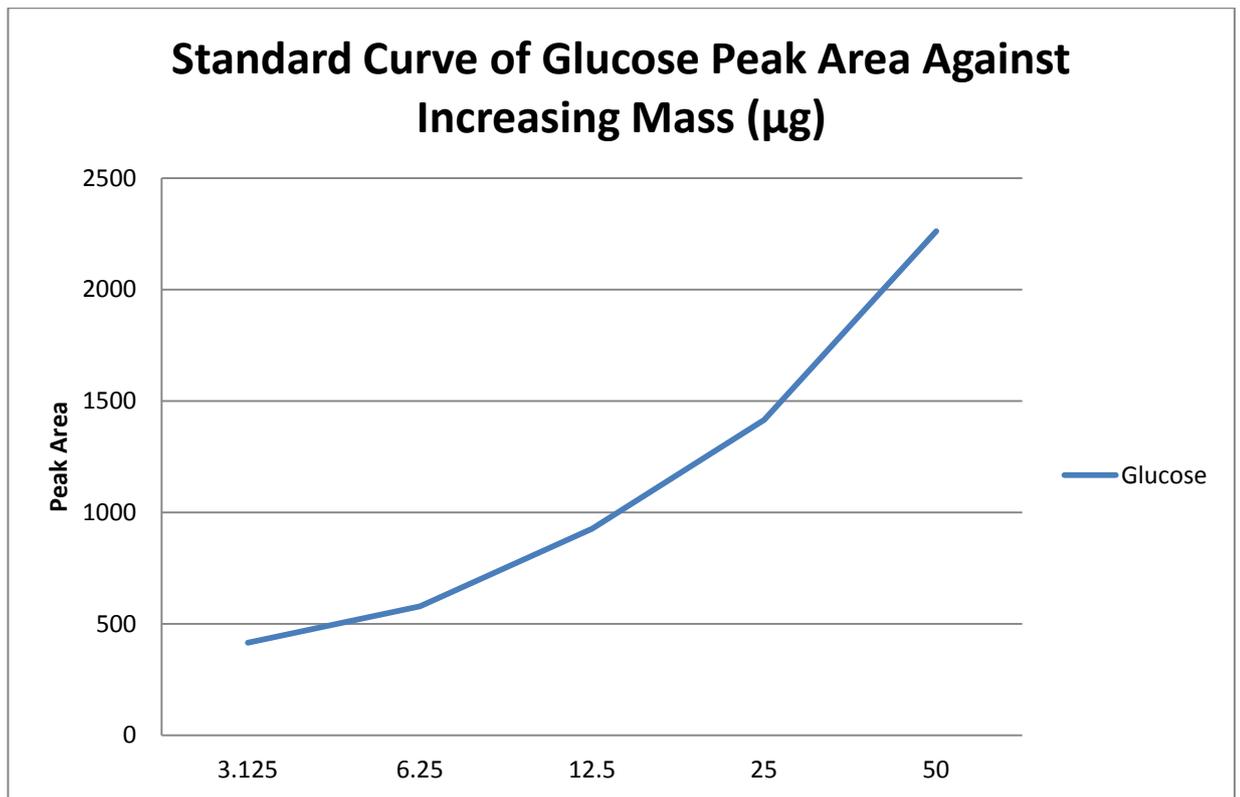
$$\text{Galactose: } x= (Y+34.35)/31.482= \text{___}\mu\text{g}$$

$$\text{Mannose: } x= (Y+16.616)/14.341= \text{___}\mu\text{g}$$

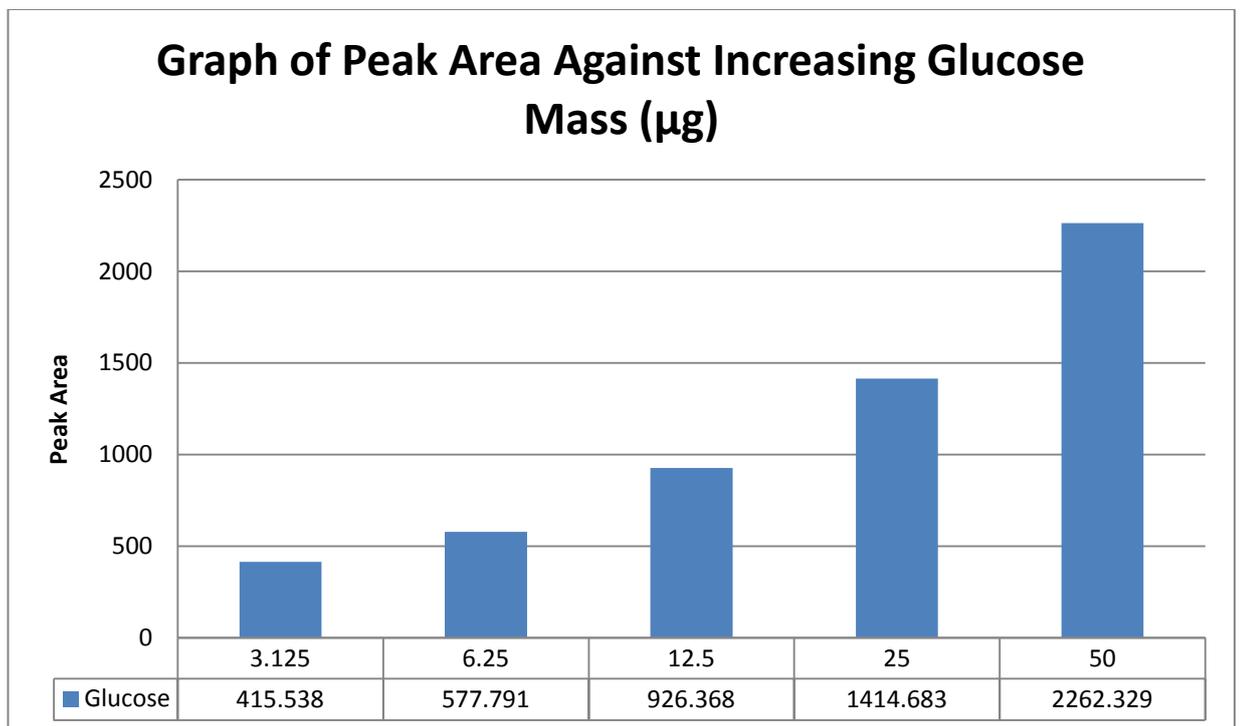
These equations were then used to detect the amounts of the monosaccharides detected within the monosaccharide analysis section of this thesis.

Throughout the course of the investigation, there was an appearance of an unknown monosaccharide within the monosaccharide analysis runs of some tested samples. It was then necessary to determine the identity of the monosaccharide, after which it would be quantified. Due to this monosaccharide appearing between galactose and mannose, it was thought that the monosaccharide was that of glucose; on account of the similar chemical structure between the three molecules. A monomix run with glucose added to the mixture was carried out; glucose was found to appear between the two monosaccharides, as it had been within some of the AGP samples! This evidence was further compounded when glucose was run through the HPAEC on it's own for comparison, with the sugar coming off 19.9 minutes into the run, between the typical retention times for galactose (19.4 minutes) and mannose (21.1 minutes).

Now it was necessary to quantify the amount of glucose found within the necessary injury samples; the protocol followed was akin to that used to quantify the monomix components. Known masses (0 μ g-50 μ g) of glucose were diluted into 200 μ l HPLC water solutions before being tested within the HPAEC. Once the analysis had been completed, the peak areas of glucose within each of the standards were taken and added to excel to create a standard curve for glucose, seen over;



Graph 4. Standard Curve for Glucose



Graph 5. Peak Area Against Increasing Glucose Mass (μg)

The resultant equation for glucose concentration determination was;

$$Y = 453.05x - 239.8$$

This equation was then rearranged so that it may be used to determine the amount of glucose detected within AGP samples;

$$\text{Glucose: } x = (Y + 239.8) / 453.05 = \text{___} \mu\text{g}$$

Using this information, it was then possible to quantify the amounts of glucose detected within the necessary samples.

3.1.3 Monosaccharide Results

Table 4 shows the monosaccharide composition analysis of all the samples, while Table 5 shows the results of monosaccharide analysis in moles of monosaccharide per moles of AGP. Table 6 looks at the percentage differences in the amounts of individual monosaccharides between the physical injury samples and the normal blood sample. Graphs 6-9 show comparisons between all tested samples, and the normal blood received for use within the study.

<u>Monosaccharide Amounts (µg)</u>					
<i>Sample Name</i>	<u>Fucose</u>	<u>Glucosamine</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Mannose</u>
SH001 18/3	0.00	0.14	0.00	0.00	1.30
GI001 7/6/10	0.00	0.17	1.21	0.00	1.18
EW001 20/3	0.66	0.42	1.11	0.00	1.18
LG001 12/9	0.66	0.14	1.95	0.00	1.18
SH002 7/6/10	0.70	0.36	1.65	0.55	1.20
WR001 11/16	0.68	0.14	1.72	0.53	1.60
XX001 ??/?	0.70	0.40	1.66	0.53	1.61
GI001 7/6/10 H	0.00	0.21	1.23	0.00	1.77
EW001 20/3 H	0.66	0.16	1.42	0.53	1.42
LG001 12/9 H	0.66	0.12	1.19	0.00	1.49
WR001 11/16 H	0.68	0.24	1.64	0.53	1.63
Normal	0.66	0.46	1.60	0.00	1.32

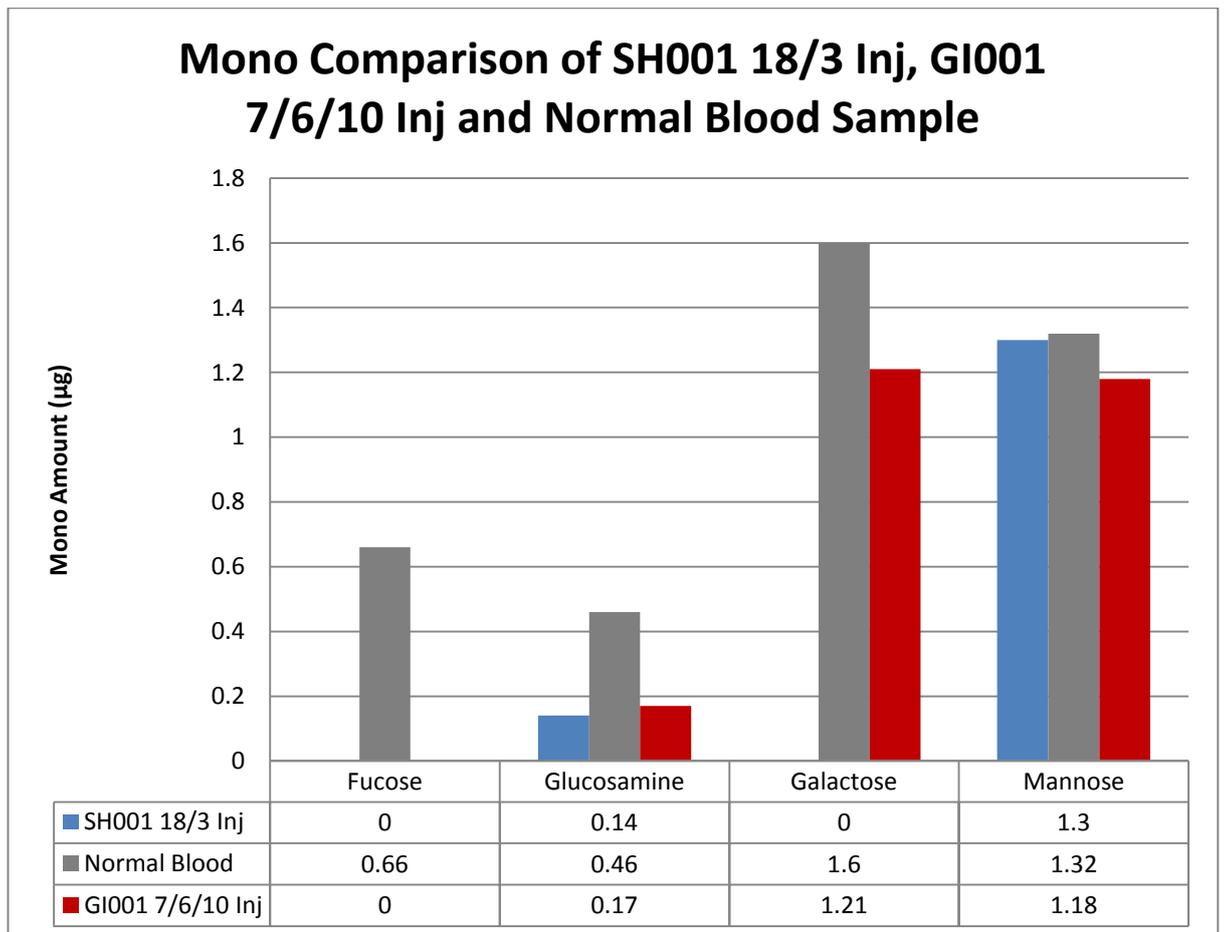
Table 4. Results of Monosaccharide Analysis on Project Samples (µg)

<u>Monosaccharide Amounts (moles of Monosaccharide per mole of AGP)</u>					
<i>Sample Name</i>	<u>Fucose</u>	<u>Glucosamine</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Mannose</u>
SH001 18/3	0.00	11.17	0.00	0.00	124.08
GI001 7/6/10	0.00	13.56	115.49	0.00	112.63
EW001 20/3	69.14	33.51	105.95	0.00	112.63
LG001 12/9	69.14	11.17	186.13	0.00	112.63
SH002 7/6/10	73.33	28.72	157.49	52.5	114.54
WR001 11/16	71.23	31.91	164.17	50.59	152.72
XX001 ?/?	73.33	31.91	158.45	50.59	153.67
GI001 7/6/10 H	0.00	16.75	117.4	0.00	168.95
EW001 20/3 H	69.14	12.76	135.54	50.59	135.54
LG001 12/9 H	69.14	9.57	11.58	0.00	142.23
WR001 11/16 H	71.23	19.15	156.54	50.59	155.58
Normal	69.14	36.7	152.72	0.00	126.99

Table 5. Monosaccharide Analysis of Project Samples (moles mon/mole AGP)

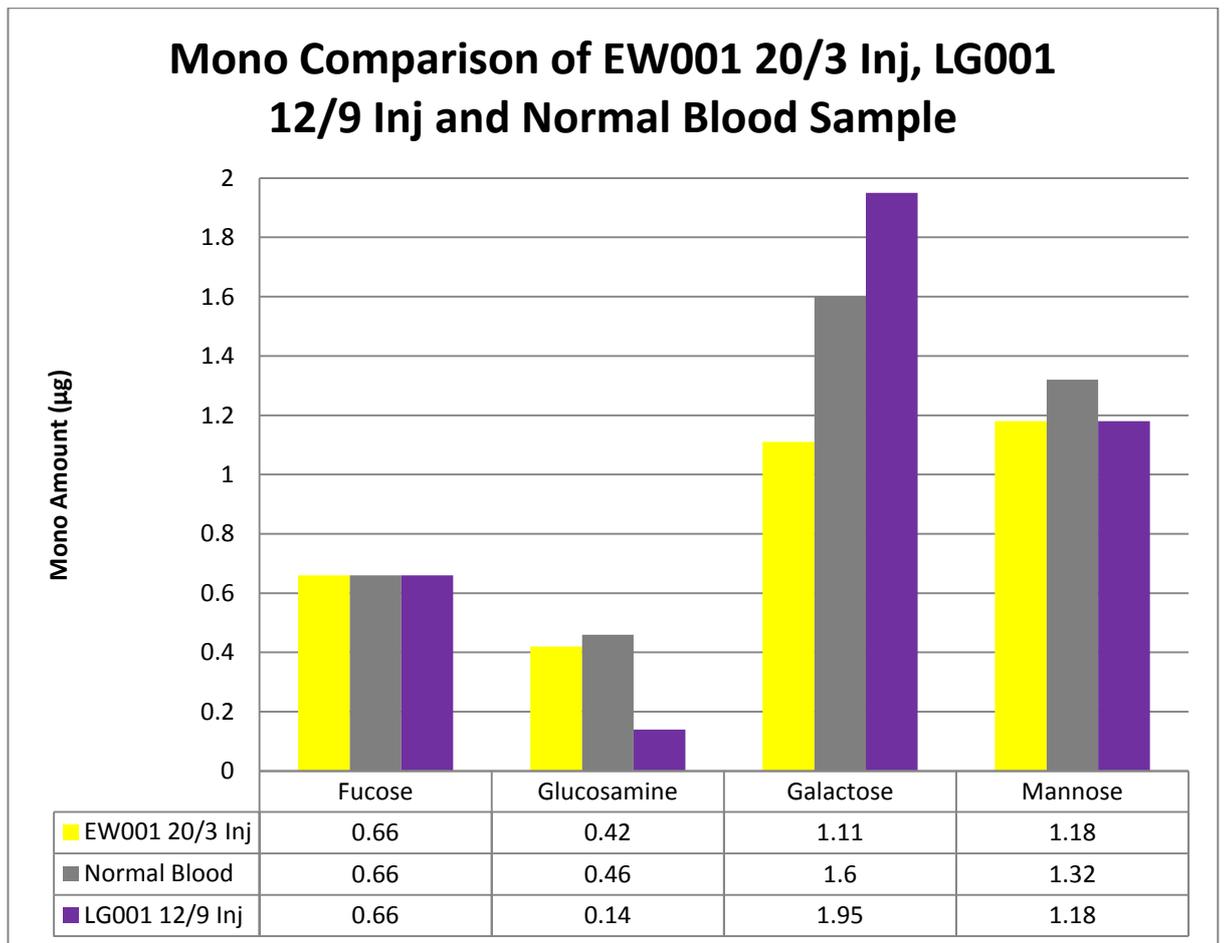
<u>Percentage Differences of Injury Samples against the Normal Blood</u>					
	<u>Sample</u>				
	<u>Fucose</u>	<u>Glucosamine</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Mannose</u>
SH001 18/3	100% decrease	69.7% decrease	100% decrease	Not Present	1.5% decrease
GI001 7/6/10	100% decrease	63% decrease	24.4% decrease	Not Present	10.6% decrease
EW001 20/3	No Change	8.7% decrease	30.6% decrease	Not Present	10.6% decrease
LG001 12/9	No Change	69.6% decrease	21.9% increase	Not Present	10.6% decrease
SH002 7/6/10	6.1% increase	21.7% decrease	3.1% increase	100% increase	9.1% decrease
WR001 11/16	3.1% increase	69.6% decrease	7.5% increase	100% increase	21.2% increase
XX001 ?/?	6.1% increase	13% decrease	3.8% increase	100% increase	22% increase

Table 6. Table of Percentage Differences of Amount of Monosaccharide (µg) within Injury Samples Compared Against the Normal Blood Sample



Graph 6. Comparison of Monosaccharide Composition of SH001 18/3, GI001 7/6/10 and Normal Blood (µg)

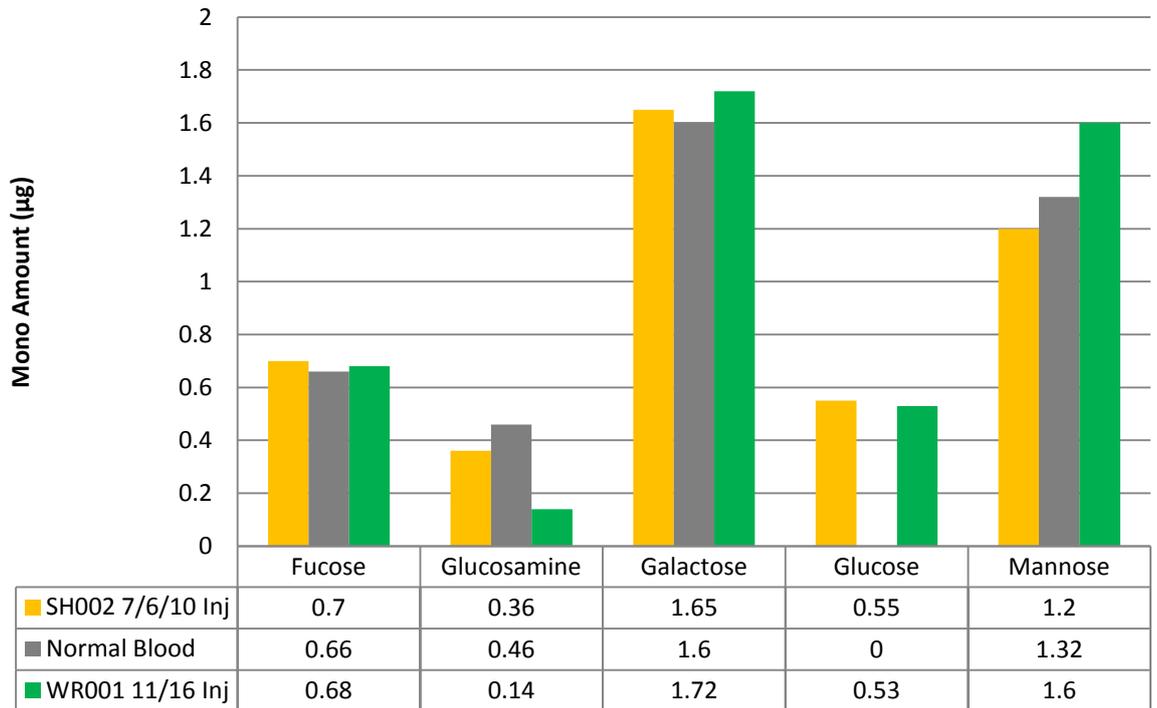
SH001 18/3 showing only two detected monosaccharides out of a possible five when compared to the normal sample. There was found to be a 69.7% decrease in the levels of glucosamine within SH001 18/3 when compared to the normal blood sample, this equates to a 0.3 fold difference between the two samples. There was also found to be a 1.5% decrease in the levels of mannose within SH001 18/3 against the normal blood sample, this itself equates to a 0.98 fold difference. GI001 7/6/10 is showing three detected monosaccharides out of a possible five when compared to the normal sample. Injury sample is showing lower levels of all detected monosaccharides compared to the normal blood sample; there was found to be a 63% decrease in the level of glucosamine (a 0.37 fold difference), a 24.4% decrease in the levels of galactose (a 0.76 fold difference), and a 10.6% decrease in the levels of mannose (a 0.89 fold difference) against the normal.



Graph 7. Comparison of Monosaccharide Composition of EW001 20/3, LG001 12/9 and Normal Blood (µg)

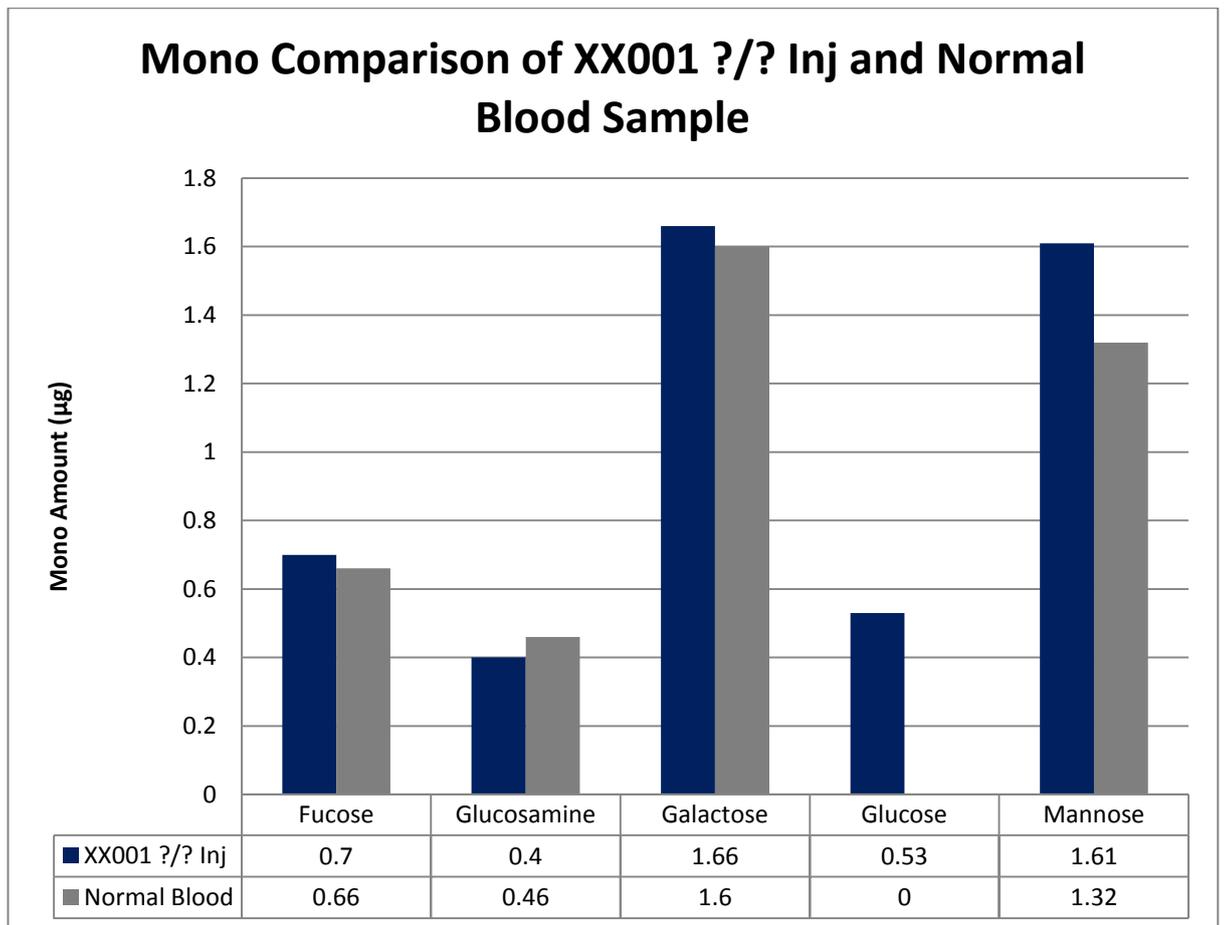
EW001 20/3 showing all four expected monosaccharides. Both samples are showing 0.66µg of fucose. Injury sample is showing an 8.7% decrease in the levels of glucosamine (a 0.91 fold difference), a 30.6% decrease in the levels of galactose (a 0.69 fold difference) and a 10.6% decrease (a 0.89 fold difference) in the levels of mannose when compared to the normal blood sample. LG001 12/9 showing same level of fucose as that within the normal; sample also showing less glucosamine than normal (a 69.6% decrease or a 0.3 fold difference). LG001 12/9's showing more galactose than normal (a 21.9% increase or a 1.21 fold difference). Similar to the previous sample, LG001 12/9 is showing lower levels of mannose when compared to the normal blood sample, showing a 10.6% decrease, or a 0.89 fold difference.

Mono Comparison of SH002 7/6/10 Inj, WR001 11/16 Inj and Normal Blood Sample



Graph 8. Comparison of Monosaccharide Composition of SH002 7/6/10, WR001 11/16 and Normal Blood (µg)

SH002 7/6/10 is showing more fucose than normal sample (an increase of 6.1%, and a fold difference of 1.1). Injury sample is showing a 21.7% decrease in the levels of glucosamine compared to the normal (a 0.78 fold difference). Conversely, the injury sample is showing slightly more galactose than normal (a 3.1% increase or a 1.03 fold difference). SH002 7/6/10 is also showing the unexpected appearance of glucose. Injury sample is showing less mannose than normal blood (a decrease of 9.1% or a fold difference of 0.91). WR001 11/16 is showing a 3.1% increase in the levels of fucose when compared to the normal sample (a fold difference of 1.03), while showing a decrease of 69.6% in glucosamine levels when compared against the normal sample (a fold difference of 0.3). The injury sample showed a 7.5% decrease in the levels of galactose (a 1.07 fold difference) against the normal blood sample. WR001 11/16 also possesses glucose; while also possessing a 21.2% increase in mannose content against the normal sample (a 1.21 fold difference).



Graph 9. Comparison of Monosaccharide Composition of XX001 ?/? and Normal Blood (µg)

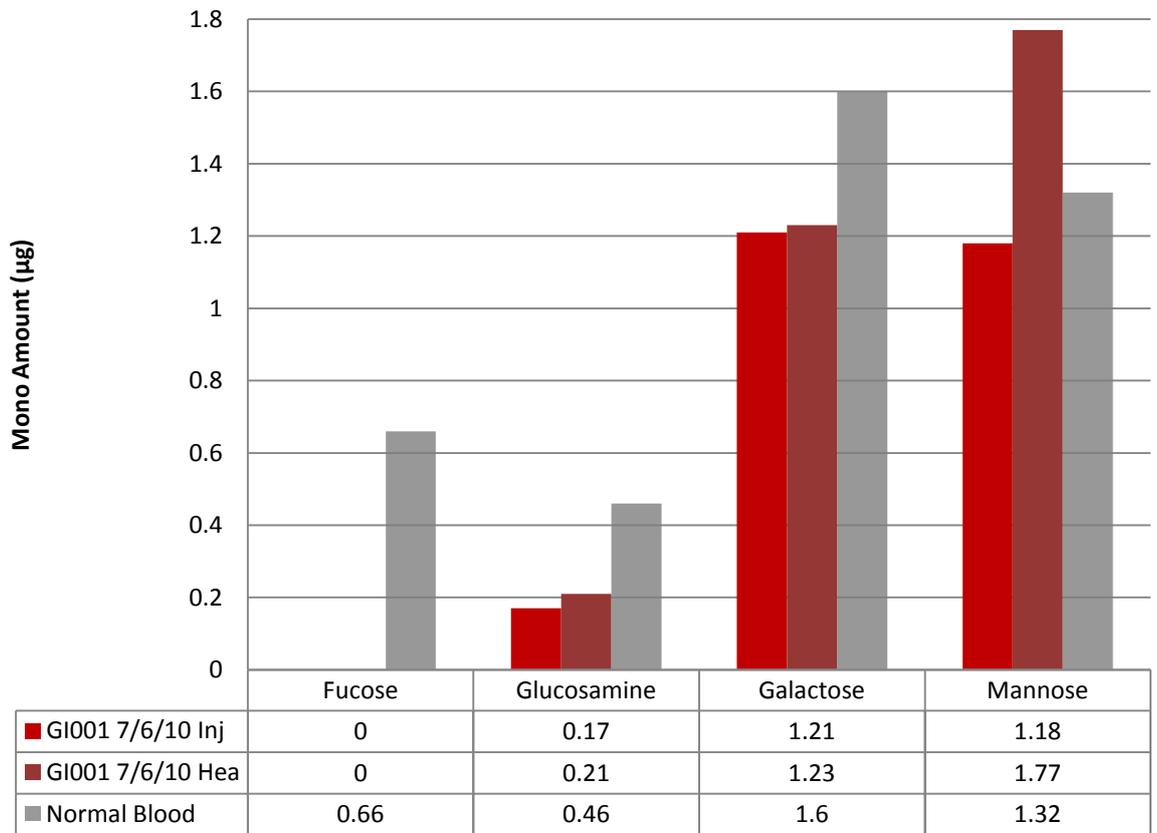
XX001 ?/? possesses 6.1% more fucose (a 1.1 fold difference) than normal sample, while possessing 13% less glucosamine than normal (a fold difference of 0.87). The unknown sample possesses 3.8% more galactose than normal (1.04 fold difference). The unknown sample is also showing the presence of glucose of which is not present in the normal. XX001 ?/? also possesses 22% more mannose than the normal blood sample, which is a fold difference of 1.22.

The following table (Table 7) shows the percentage differences in the amounts of monosaccharides from physical injury samples as compared to their respective healed samples; graphs (Graphs 10-13) show the comparisons of monosaccharide composition between MTB injury samples, and those samples received from willing volunteers once they had recovered from their respective injuries.

<u>Percentage Differences of Injury Samples against their Respective Healed Blood Samples</u>					
	<u>Fucose</u>	<u>Glucosamine</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Mannose</u>
GI001 7/6/10	No Change	19% decrease	1.6% decrease	Not Present	33.3% decrease
EW001 20/3	No Change	65.2% increase	21.8% decrease	100% decrease	16.9% decrease
LG001 12/9	No Change	16.7% increase	63.9% increase	Not Present	20.8% decrease
WR001 11/16	No Change	41.7% increase	4.7% increase	No Change	1.8% decrease

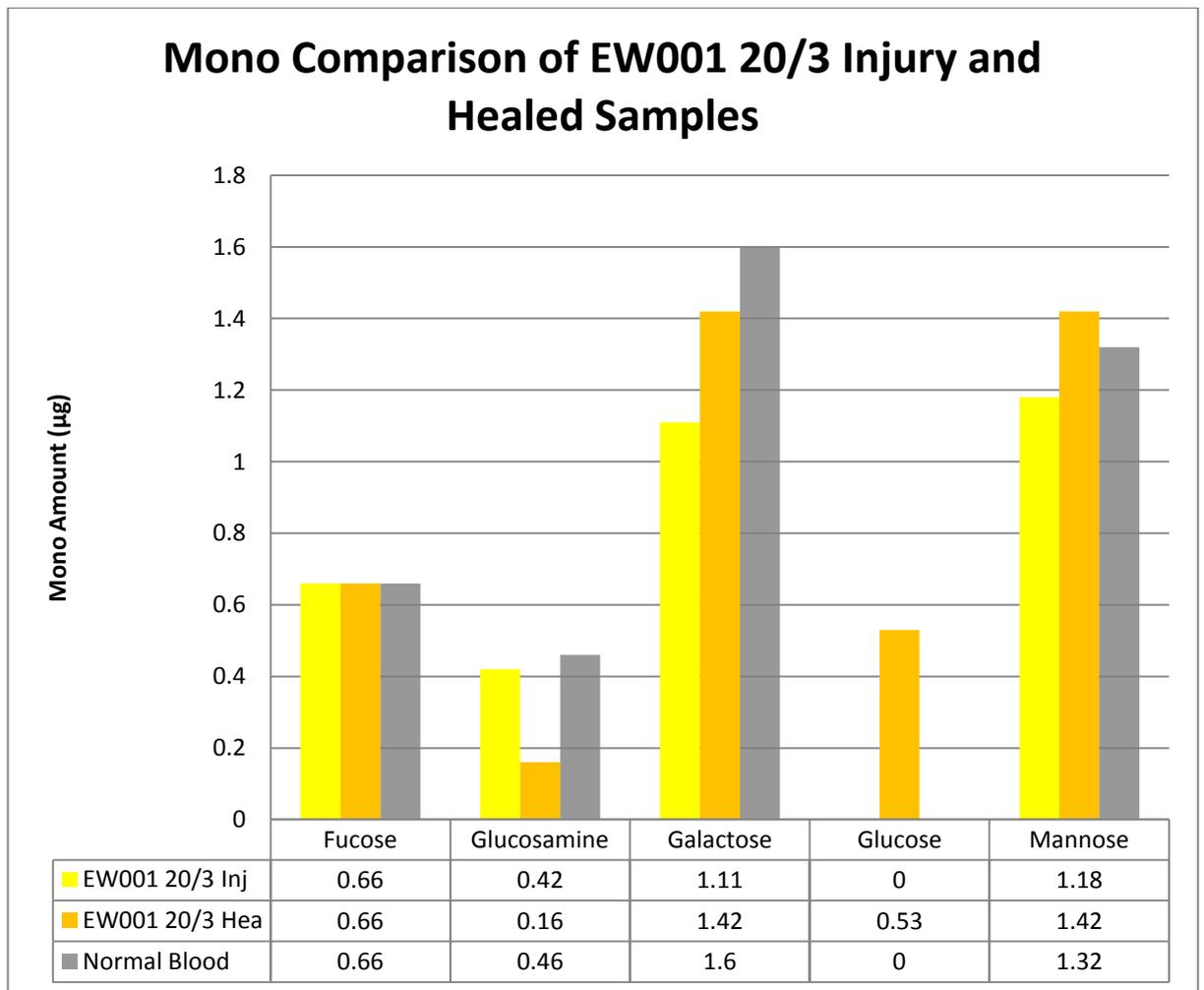
Table 7. Table of Percentage Differences of Amounts of Monosaccharide (μg) within Injury Samples Compared Against their Respective Healed Blood Samples

Mono Comparison of GI001 7/6/10 Injury and Healed Samples



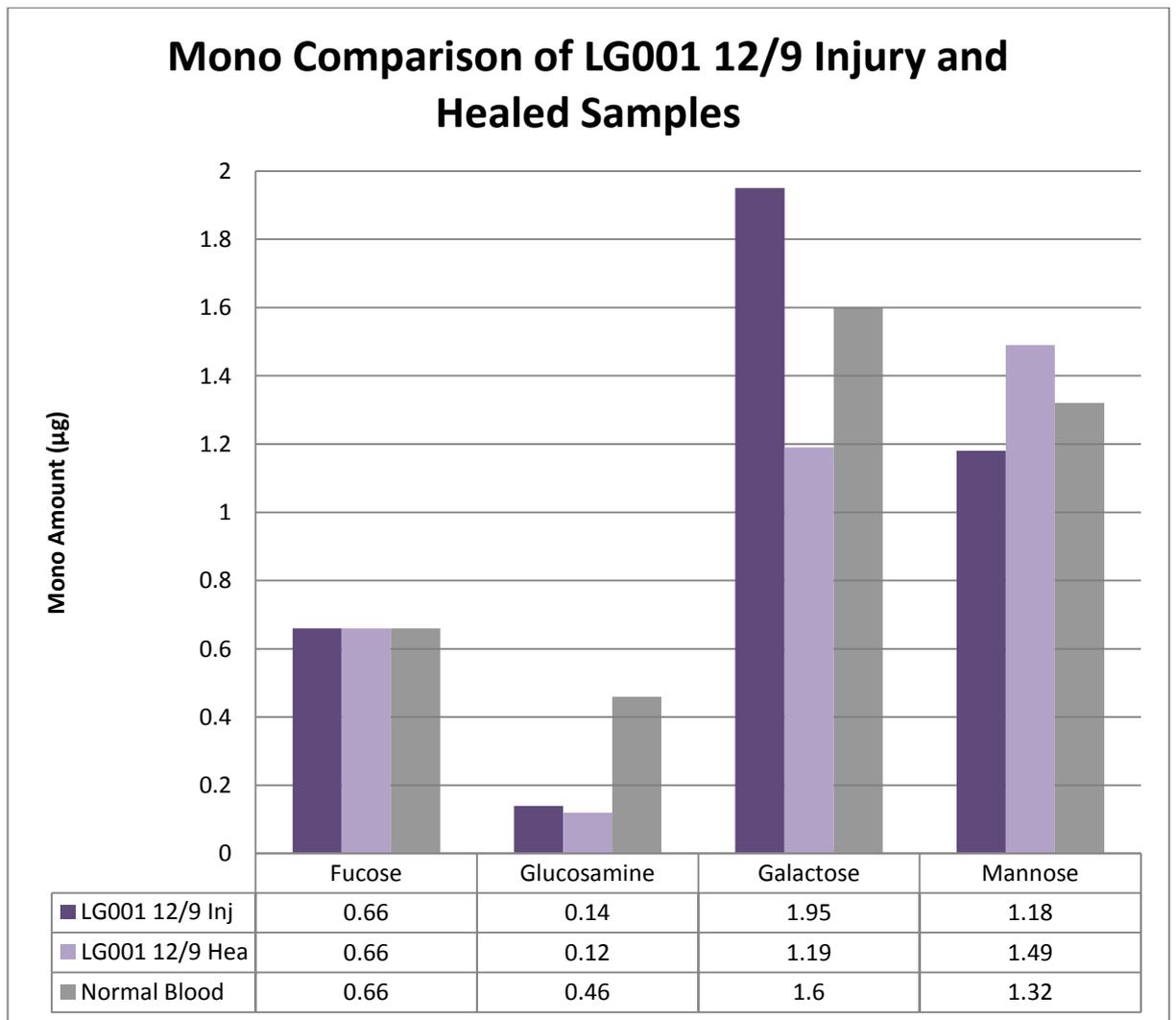
Graph 10. Comparison of Monosaccharide Composition of GI001 7/6/10 Injury and Healed Samples (µg)

The sample is showing three of the four expected monosaccharides; no fucose was detected within the sample. The injury sample is showing 0.17µg of glucosamine, 1.21µg of galactose, and 1.18µg of mannose. A follow up sample was available from the volunteer once their injury had healed. This sample, GI001 7/6/10 Healed, showed the same three monosaccharides that was detected within the injury sample. The healed sample contained 0.21µg of glucosamine (a 19% decrease within the injured state, and a 0.81 fold difference), 1.23µg of galactose (a 1.6% decrease within the injured state, and a 0.98 fold difference) and 1.77µg of mannose (a 33.3% decrease within the injured state, and a 0.66 fold difference); all three monosaccharides have decreased in amount between the subject being healthy and becoming injured.



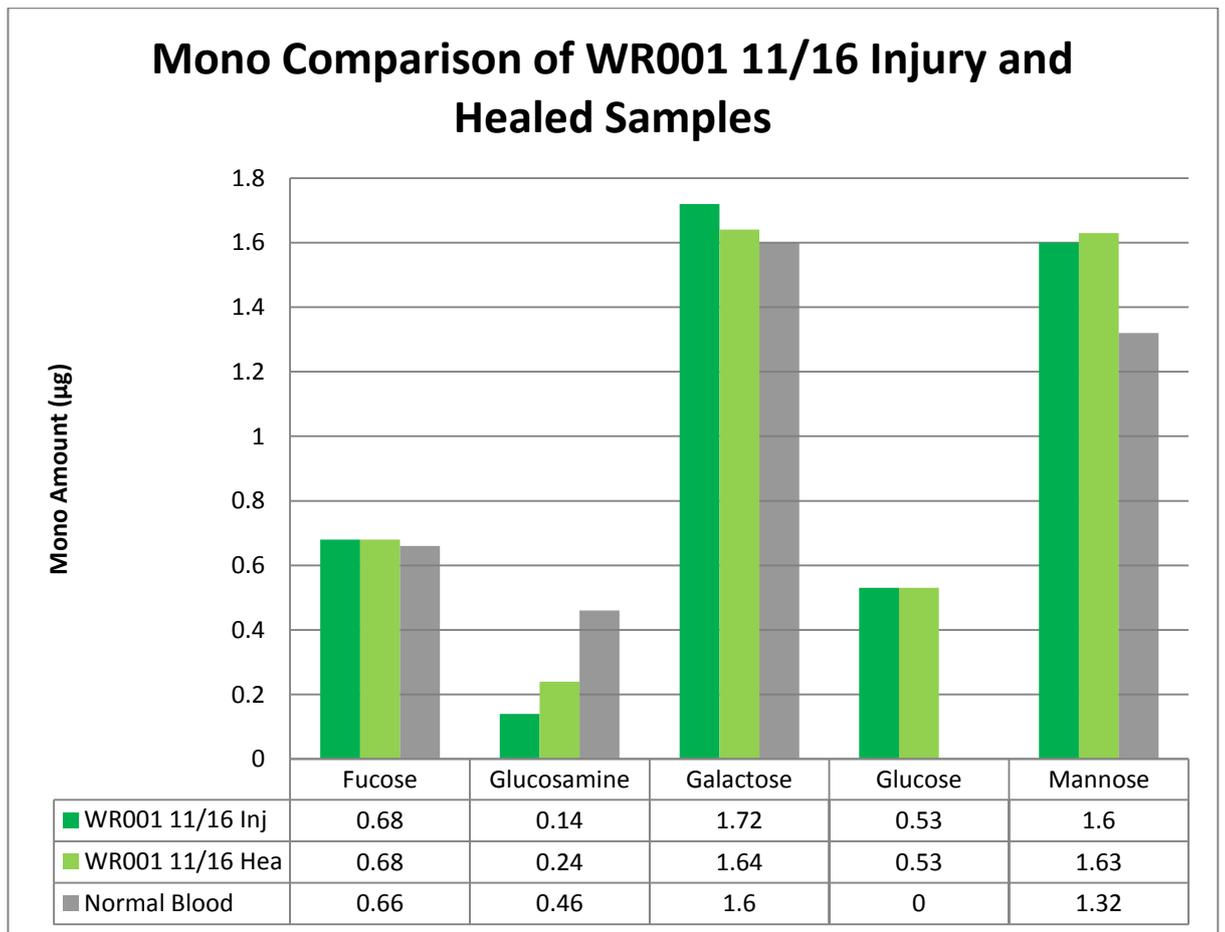
Graph 11. Comparison of Monosaccharide Composition of EW001 20/3 Injury and Healed Samples (µg)

The sample is showing four out of the four expected monosaccharides; with 0.66µg of fucose, 0.42µg of glucosamine, 1.11µg of galactose, and 1.18µg of mannose being detected within the sample. A healed sample was also available from the volunteer, EW001 20/3 Healed. This sample possessed all four expected monosaccharides, plus the unexpected appearance of glucose; with 0.66µg of fucose (no change), 0.16µg of glucosamine (a 65.2% increase within the injured state, and a 2.86 fold difference), 1.42µg of galactose (a 21.8% decrease within the injured state, and a fold difference of 0.78), and 1.42µg of mannose (a 16.9% decrease within the injured state, and a fold difference of 0.83) being detected; 0.53µg of glucose was also detected within the healed sample.



Graph 12. Comparison of Monosaccharide Composition of LG001 12/9 Injury and Healed Samples (µg)

The injury sample shows four of the expected monosaccharides when detected; with 0.66µg of fucose, 0.14µg of glucosamine, 1.95µg of galactose, and 1.18µg of mannose being detected within the sample. A healed sample, LG001 12/9 Healed, was also provided with 0.66µg of fucose (no change), 0.12µg of glucosamine (a 16.7% increase within the injured state, and a fold difference of 1.17), 1.19µg of galactose (a 63.9% increase within the injured state, and a 1.64 fold increase), and 1.49µg of mannose (a 20.8% decrease within the injured state, and a 0.79 fold difference).



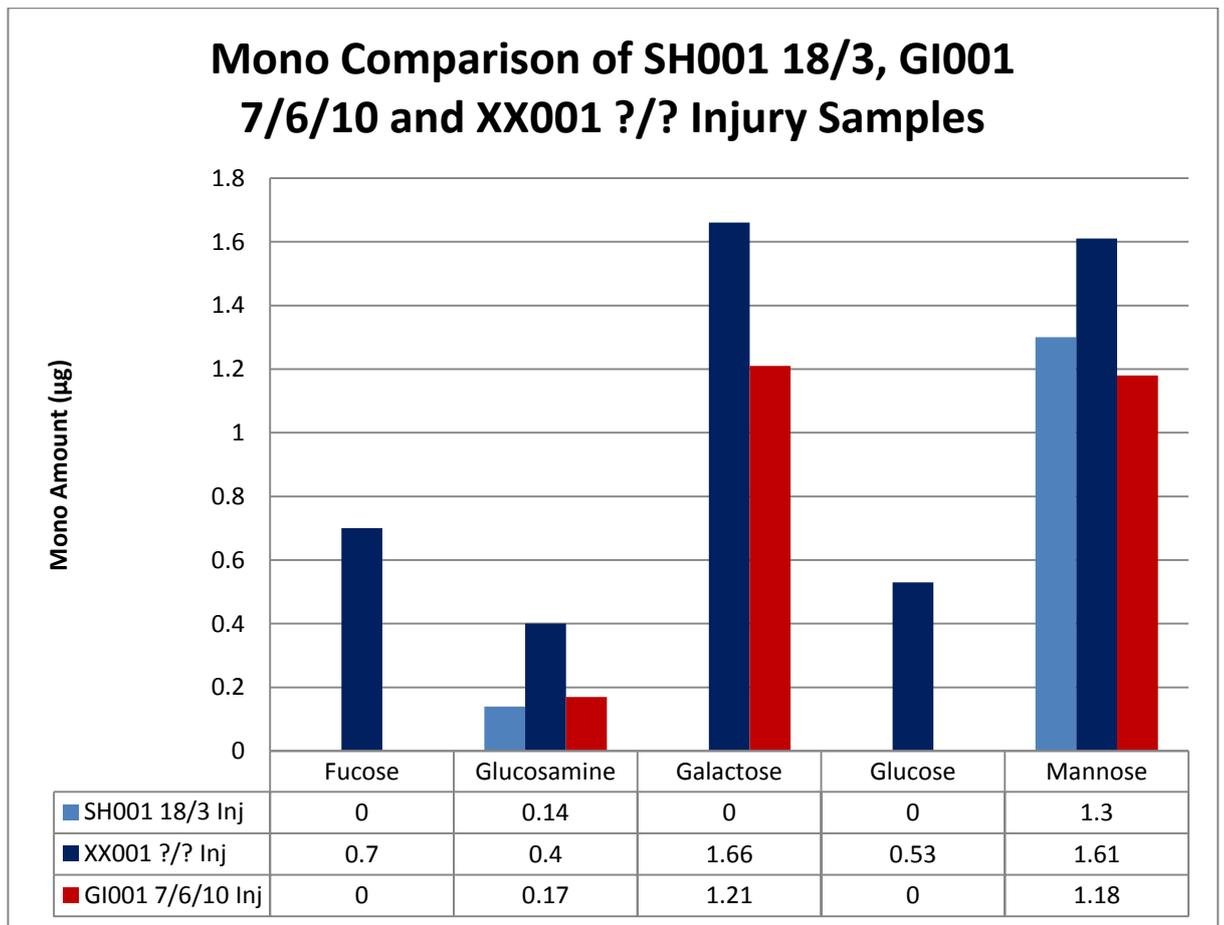
Graph 13. Comparison of Monosaccharide Composition of WR001 11/16 Injury and Healed Samples (µg)

The sample is showing the four expected monosaccharides and glucose again; with 0.68µg of fucose, 0.14µg of glucosamine, 1.72µg of galactose, 1.6µg of mannose, and 0.53µg of glucose were detected during HPAEC analysis. The volunteer also was able to provide a healed sample for analysis; with 0.68µg of fucose (no change), 0.24µg of glucosamine (a 41.7% decrease within the injured state, and a 0.58 fold difference), 1.64µg of galactose (a 4.7% increase within the injured state, and a 1.05 fold difference), 1.63µg of mannose (a 1.8% decrease within the injured state, and a 0.98 fold difference), and 0.53µg of glucose (no change).

Finally, the following table shows the percentage differences in the amount of monosaccharide found within physical injury samples when compared to the unknown injury sample; graphs 14-16 show the comparison of monosaccharide composition between the known injury samples, and the unknown injury sample (XX001 ??/?).

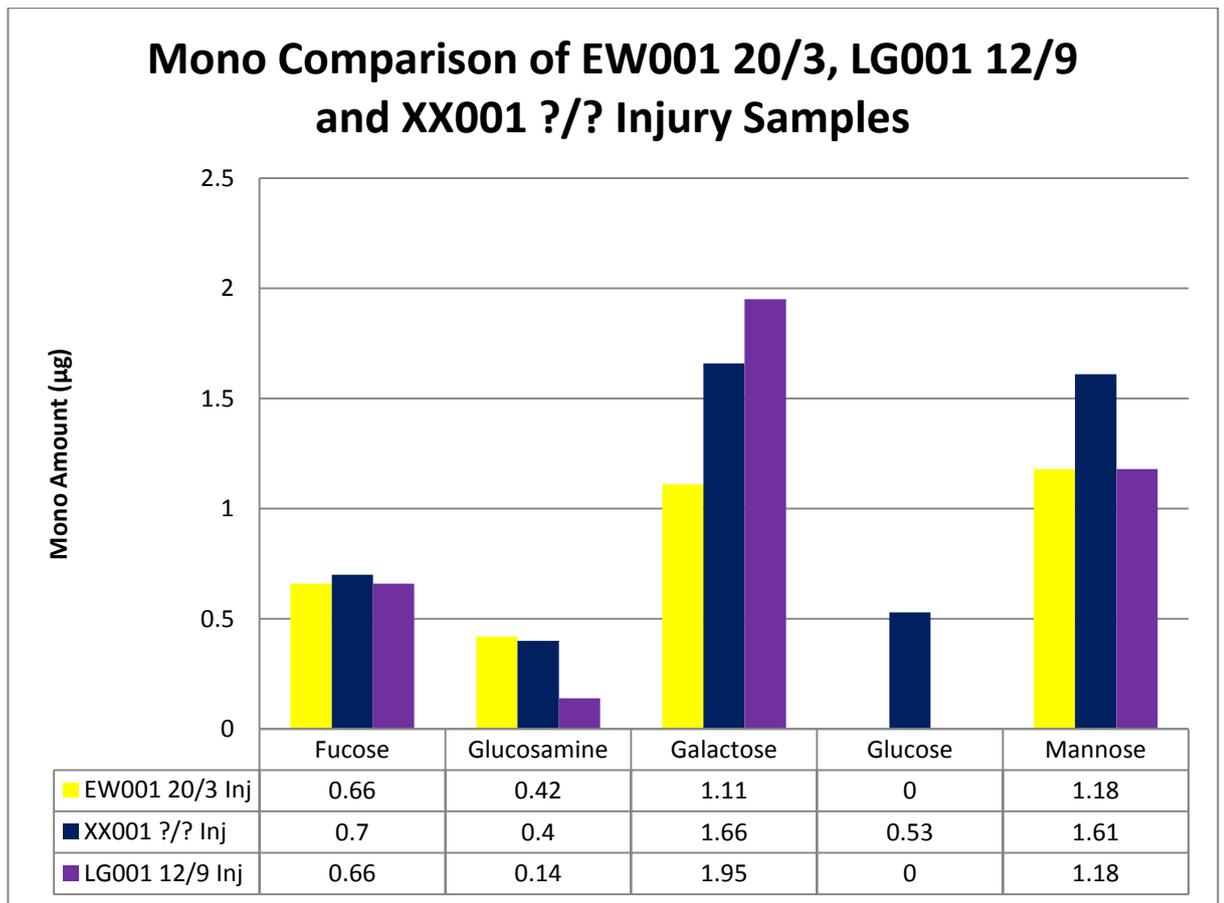
<u>Percentage Differences of Unknown Injury Sample against the Known Injury Samples</u>					
	<u>Fucose</u>	<u>Glucosamine</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Mannose</u>
SH001 18/3	100% increase	185.7% increase	100% increase	100% increase	23.8% increase
GI001 7/6/10	100% increase	135.3% increase	37.2% increase	100% increase	36.4% increase
EW001 20/3	6.1% increase	4.8% decrease	49.5% increase	100% increase	36.4% decrease
LG001 12/9	6.1% increase	185.7% increase	14.9% decrease	100% increase	36.4% increase
SH002 7/6/10	No Change	11.1% increase	0.6% increase	3.6% decrease	34.2% increase
WR001 11/16	2.9% increase	185.7% increase	3.5% decrease	No Change	0.6% increase

Table 8. Percentage Differences of Amount of Monosaccharide (μg) within Unknown Injury Sample Compared Against the Known Injury Samples



Graph 14. Comparison of Monosaccharide Composition between SH001 18/3, GI001 7/6/10 and XX001 ?/? (µg)

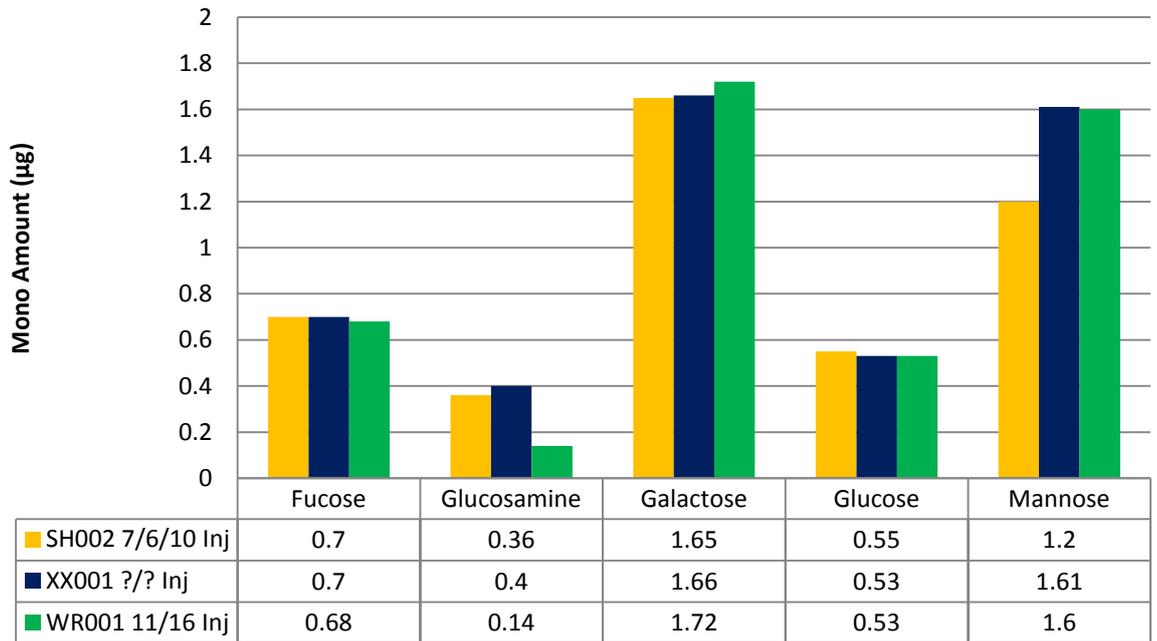
XX001 ?/? showing the presence of five monosaccharides compared to SH001 18/3's two. Despite sharing glucosamine and mannose with XX001 ?/?, SH001 18/3 showing these monosaccharides at lower levels than the unknown, with the unknown possessing 185.7% more glucosamine (a fold difference of 2.86) and 23.8% more mannose (a fold difference of 1.24) respectively. XX001 ?/? showing the previously unexpected monosaccharide, glucose. Again, the unknown is showing five monosaccharides compared to GI001 7/6/10's three. The unknown sample is showing glucosamine (a 135.3% increase against the known sample, and a 2.35 fold difference), galactose (a 37.2% increase, and a 1.37 fold difference) and mannose (a 36.4% increase, and a 1.36 fold difference) at higher levels than the general injuries sample. No fucose has been detected at all in GI001 7/6/10, while it has in XX001 ?/?. Glucose has also been detected within XX001 ?/?.



Graph 15. Comparison of Monosaccharide Composition between EW001 20/3, LG001 12/9 and XX001 ?/? (µg)

The samples share all four expected monosaccharides, with the unknown also showing glucose. There are slight differences in the levels of fucose and glucosamine between EW001 20/3 and the unknown; with the unknown injury possessing 6.1% more fucose (a fold difference of 1.06) and 4.8% less glucosamine (a fold difference of 0.95) than the unknown. More differences are apparent within galactose and mannose levels, with XX001 ?/? showing 49.5% more galactose (a 1.49 fold difference) and 36.4% more mannose (a 1.36 fold difference) than the known sample. XX001 ?/? showing slightly more fucose than has been detected within LG001 12/9 (6.1% more, a 1.06 fold increase), this is also apparent when considering glucosamine (185.7% more, and a fold difference of 2.86) and mannose (36.4% more, and a fold difference of 1.36) levels. However, XX001 ?/? does contain 14.9% less galactose than what has been detected within LG001 12/9, a fold difference of 0.85.

Mono Comparison of SH002 7/6/10, WR001 11/16 and XX001 ?/? Injury Samples



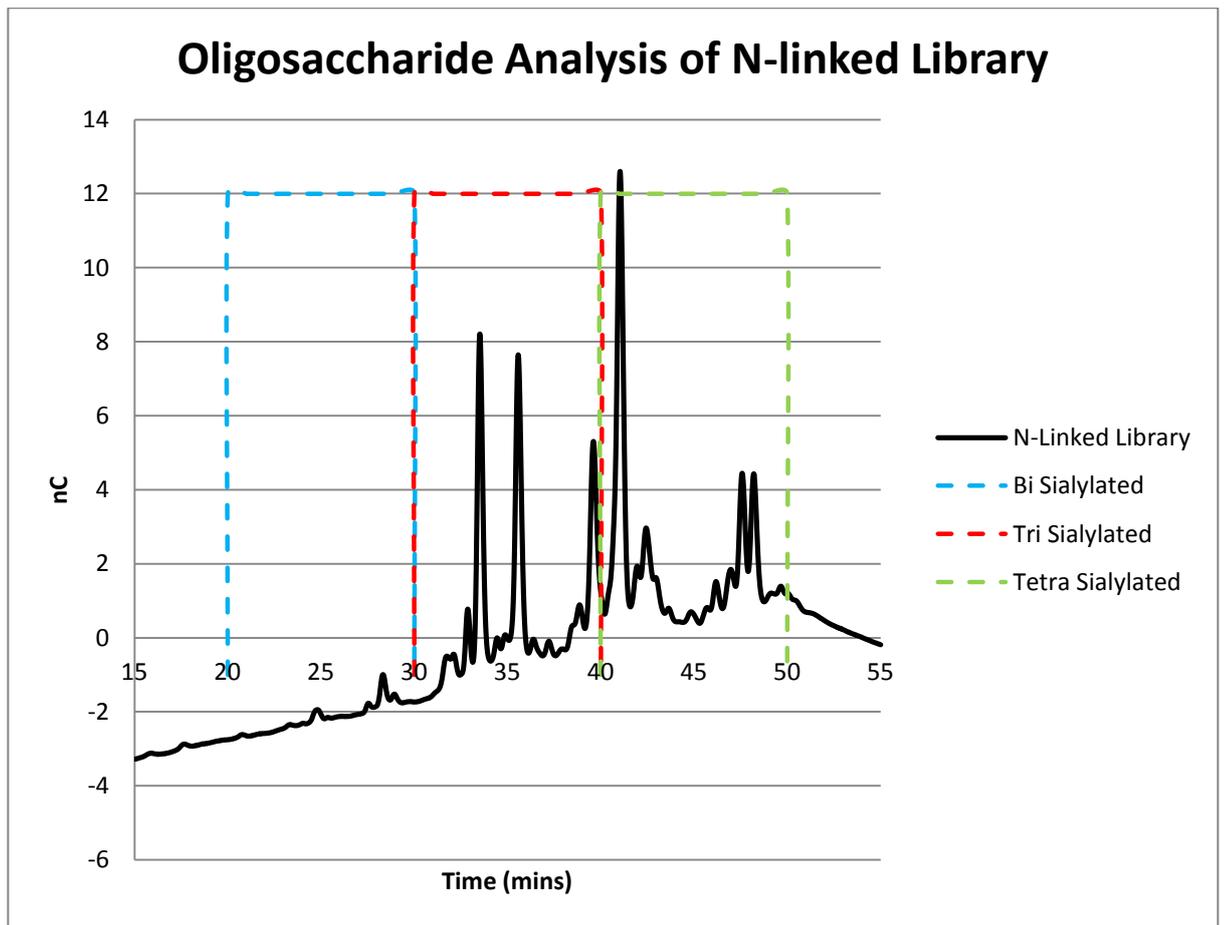
Graph 16. Comparison of Monosaccharide Composition between SH002 7/6/10, WR001 11/16 and XX001 ?/? (µg)

All samples contain the four expected monosaccharides; they also contain the unexpected presence of glucose. The same level of fucose is detected within the unknown and SH002 7/6/10. There are differences in the levels of glucosamine and galactose between the two samples, with 11.1% more glucosamine detected within the unknown (a fold difference of 1.11) and 0.6% more (a fold difference of 1.01) being detected within XX001 ?/?. The unknown possesses 3.6% less glucose than SH002 7/6/10; a resulting fold difference of 0.96. The unknown possesses 34.2% more mannose than SH002 7/6/10 (a fold difference of 1.34). Differences are seen between the unknown and WR001 11/16 with regards to fucose level (with the unknown possessing 2.9% more, a fold difference of 1.03), and galactose levels (with the unknown showing 3.5% less galactose, a fold difference of 0.96). There was a slight 0.6% increase in the level of mannose within the unknown compared to WR001 11/16 (a fold difference of 1.01). The biggest difference between these two samples is in their glucosamine levels, as XX001 ?/? shows 185.7% more of the monosaccharide compared to WR001 11/16; this is a fold difference of 2.86.

3.2 Oligosaccharide Analysis

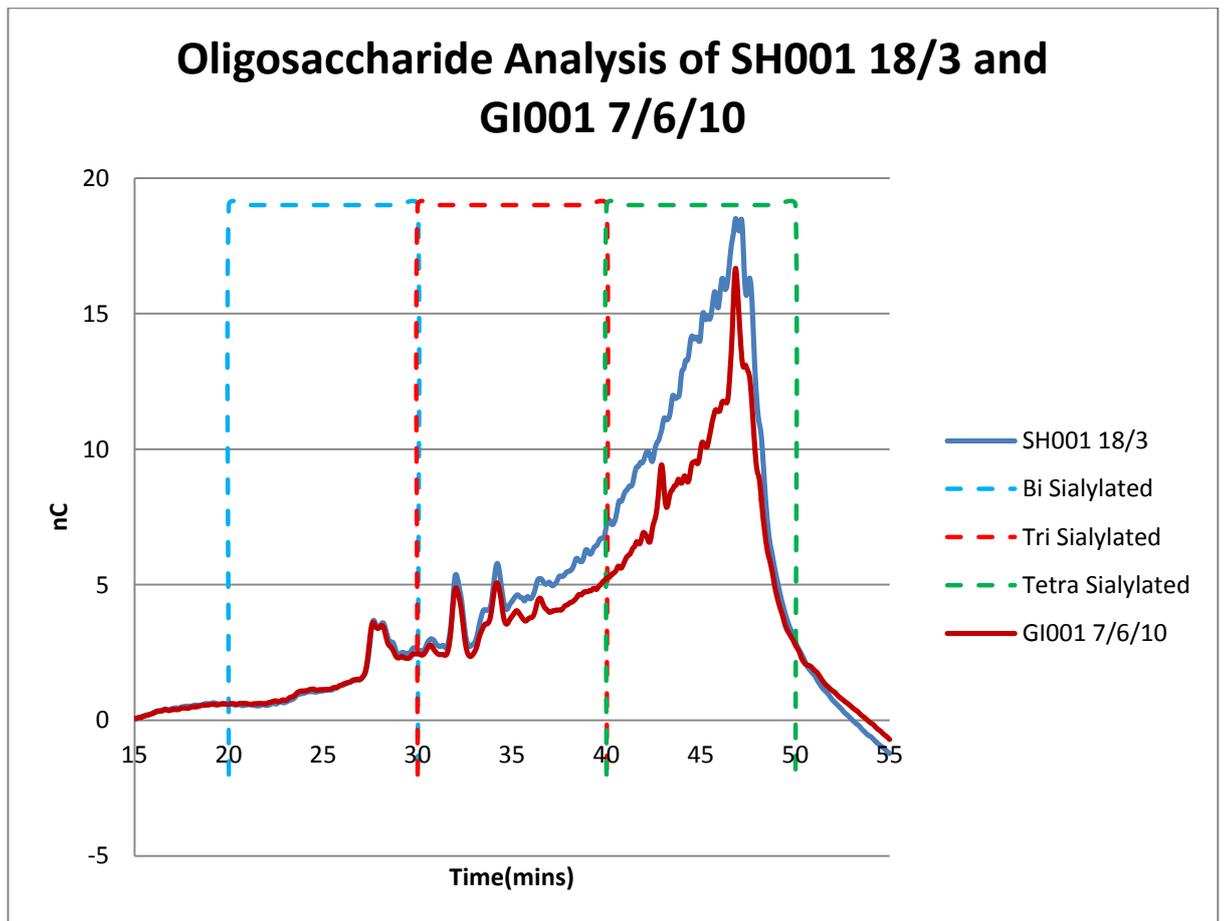
3.2.1 Oligosaccharide Results

The following graphs represent the results of the oligosaccharide analysis carried out on MTB injury samples within the HPAEC. In addition to these samples, a control sample was run through the HPAEC to act as a reference for each of the injury samples. This N-linked library contained oligosaccharide structures within to provide a reference of oligosaccharide structures in vivo, which could then be further used to highlight differences within the structures within the tested samples within this project. Two characteristics that will be analyzed within these results are the peak elution of the tested samples (how high the peaks reach) and the peak activity of the injury samples (how many peaks appear within an area of the graph, whether they appear to increase or decrease in complexity), these will then form the basis of comparisons between samples. Graph 17 shows the N-linked library when analysed on it's own within the HPAEC.



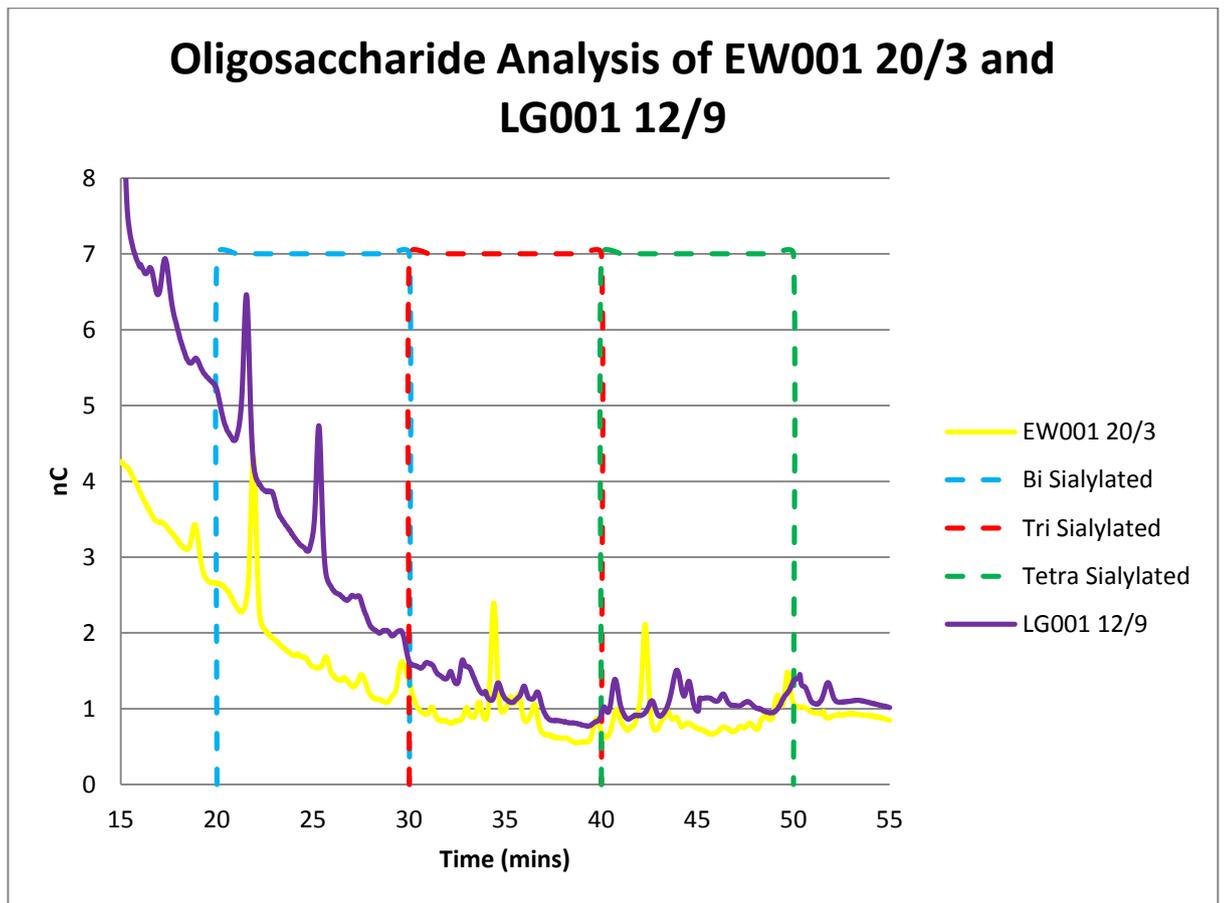
Graph 17. Oligosaccharide Analysis Chromatogram of N-Linked Library

The N-linked library when analysed on its own was shown to contain relatively small peaks within the bi sialylated area; with three being countable. The sample produced two very large peaks within the tri sialylated area (out of nine peaks) and five large (out of eleven) peaks within the tetra sialylated area.



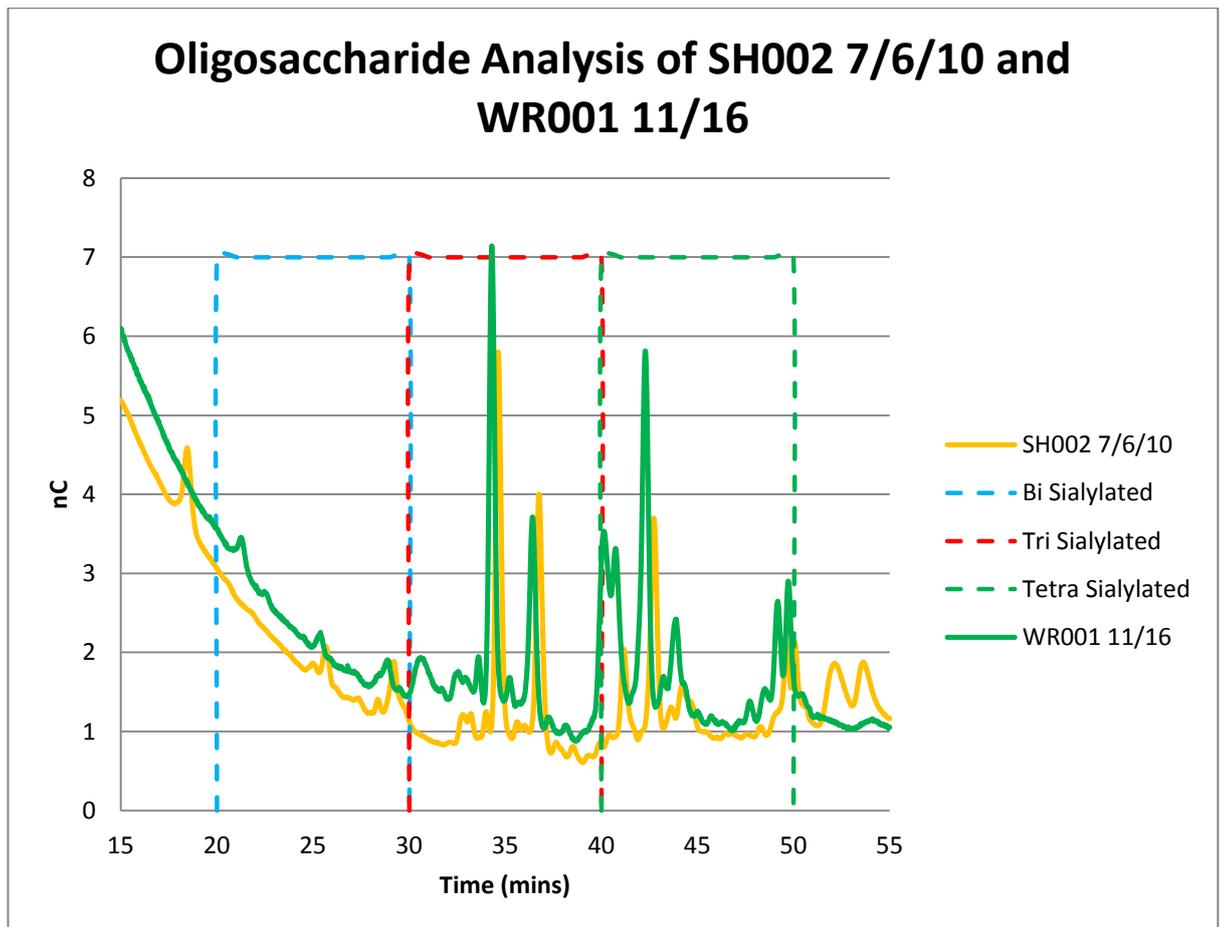
Graph 18. Oligosaccharide Analysis Chromatogram of SH001 18/3 and GI001 7/6/10.

SH001 18/3 showing one visible peak within the “bi” sialylated area (20-30 mins), four peaks within the “tri” area (30-40 mins), and one peak within the “tetra” sialylated area (40-50 mins). GI001 7/6/10 is showing one definite peak within the bi sialylated area, five peaks within the tri sialylated area (two peaks being larger than the others), and two peaks within the tetra sialylated area.



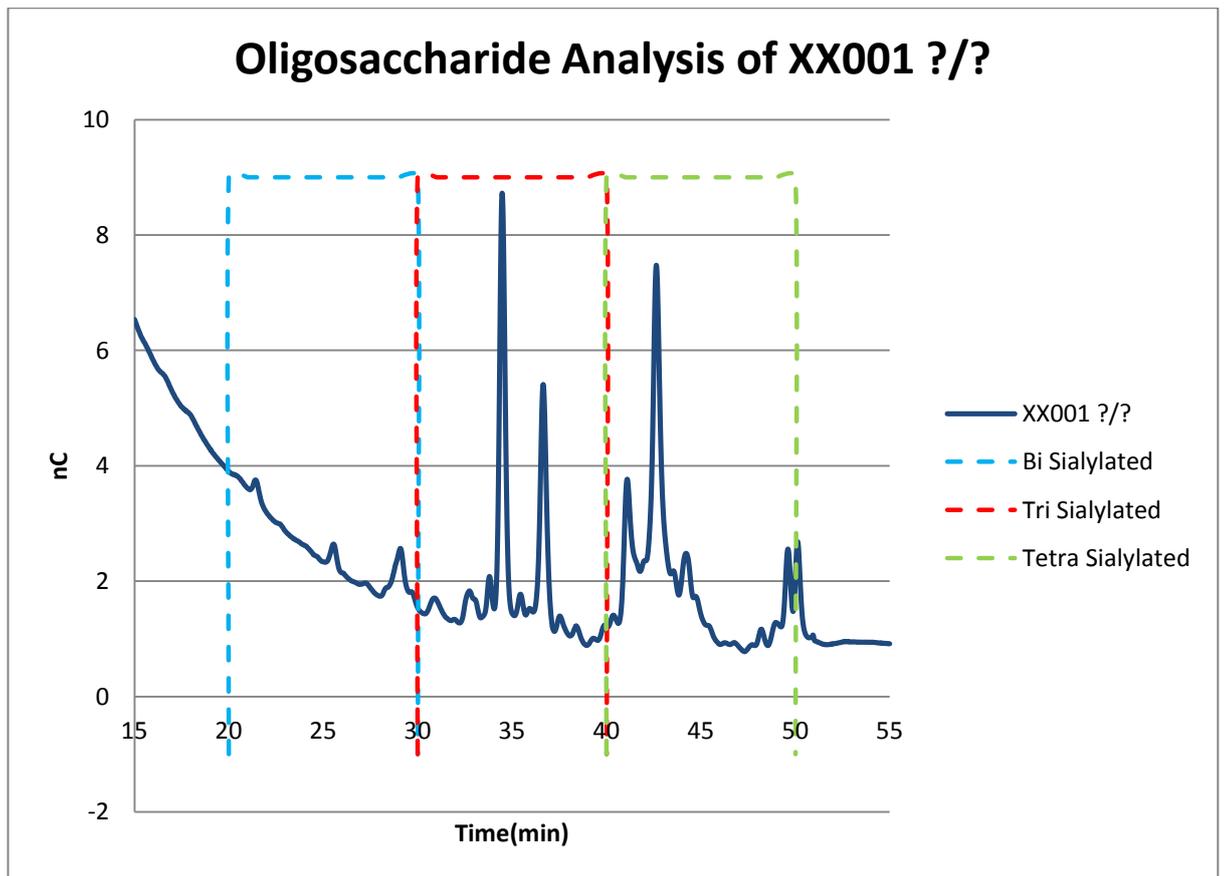
Graph 19. Oligosaccharide Analysis Chromatogram of EW001 20/3 and LG001 12/9

EW001 20/3 possesses one very large bi peak, followed by three smaller peaks, five small and one large peak within the tri sialylated, and eight peaks seen within the tetra sialylated area (one of these peaks being particularly prominent). LG001 12/9 showed a small number of definitive peaks, with two of the most prominent peaks coming within the bi sialylated area of the chromatogram. Following this, a few very small peaks can be seen within the tri and tetra sialylated areas.



Graph 20. Oligosaccharide Analysis Chromatogram of SH002 7/6/10 and WR001 11/16.

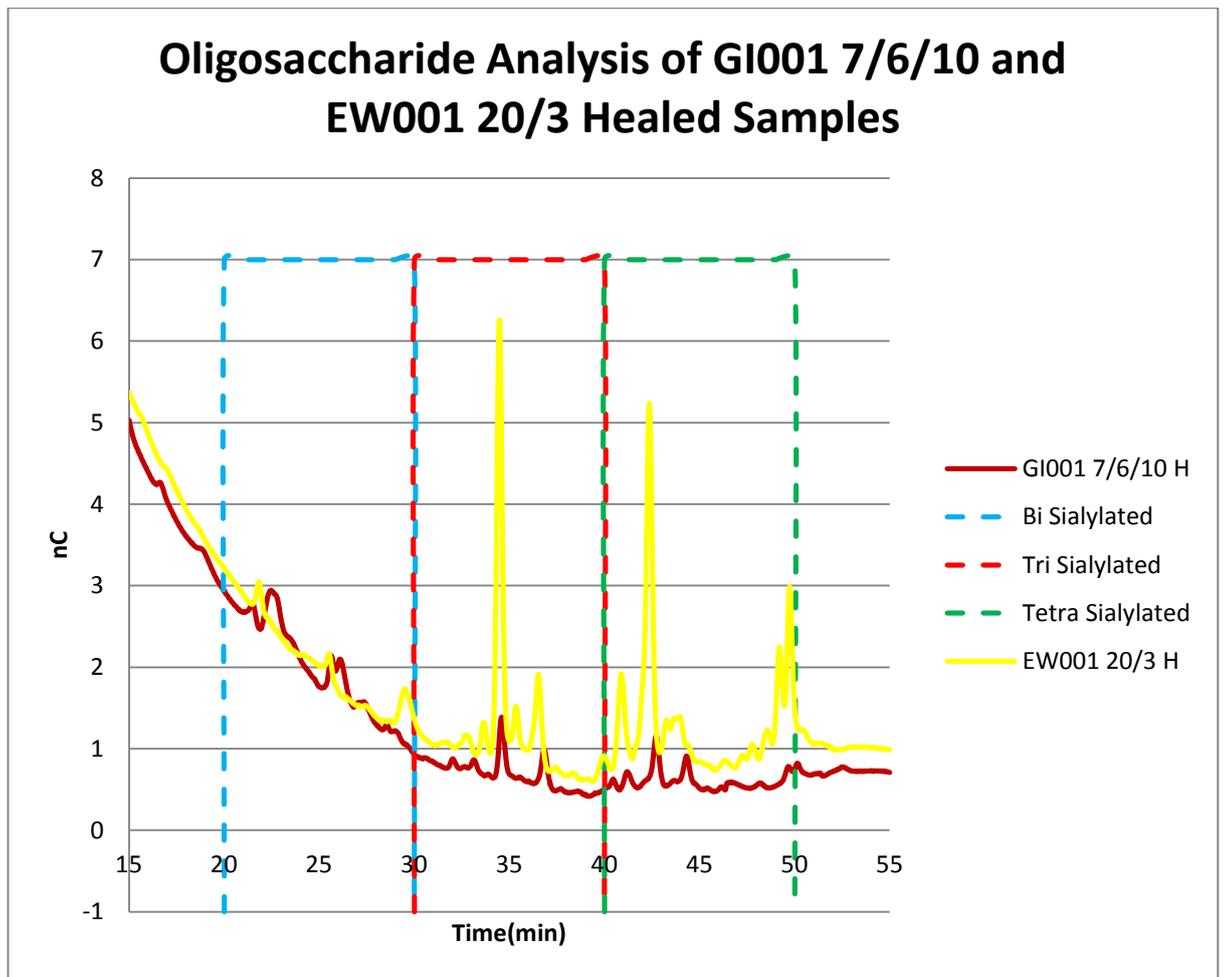
SH002 7/6/10 possesses two small peaks that can be seen within the bi sialylated area, two very prominent peaks can be seen within the tri sialylated area, with three more peaks within the tetra sialylated area and a small double peak coming off towards the end of the tetra portion of the graph. WR001 11/16 is showing a small number of very small peaks within the bi sialylated area. Two prominent peaks appear within the tri sialylated area. There are four peaks that appear within the tetra sialylated area.



Graph 21. Oligosaccharide Analysis Chromatogram of XX001 ?/?.

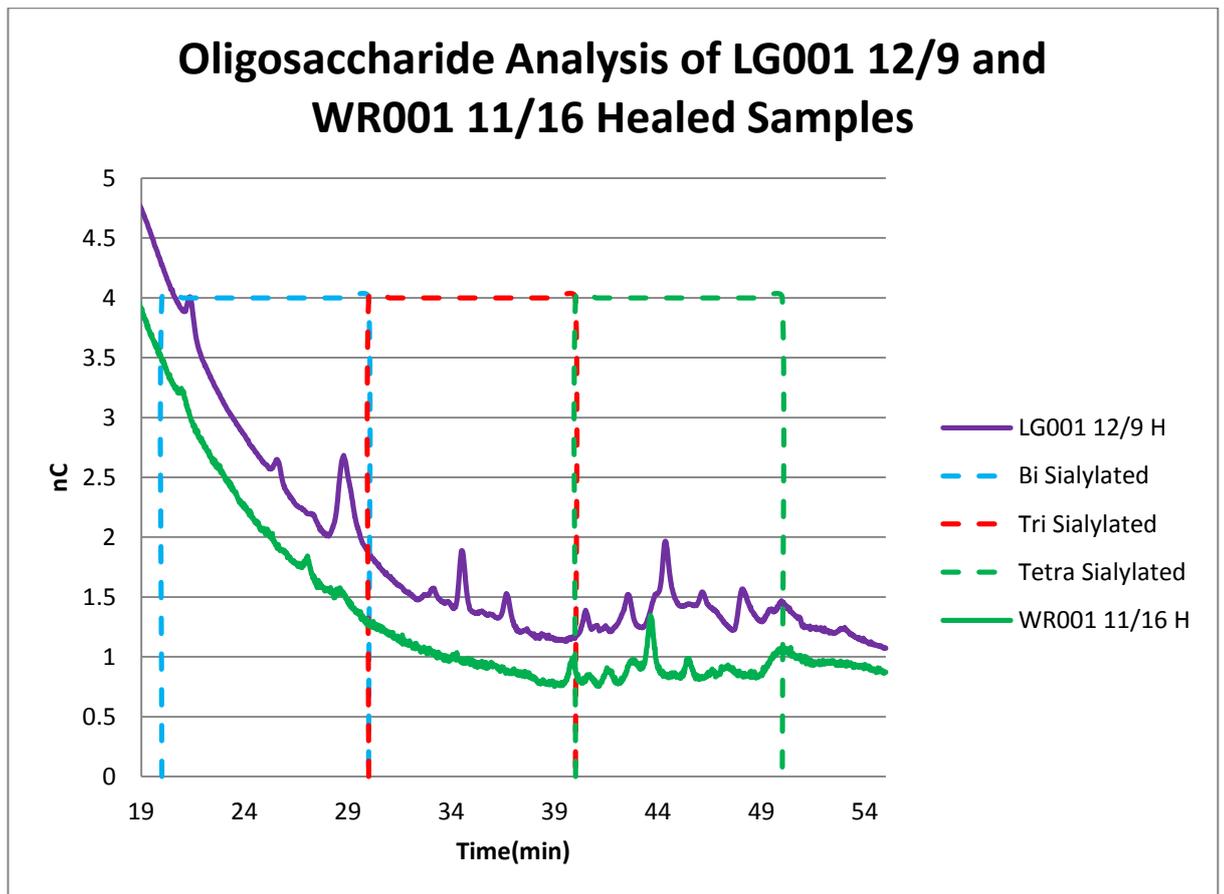
XX001 ?/? showing three small peaks within the bi sialylated area. Five peaks appear within the tri sialylated area of XX001 ?/?. Finally, three large peaks are present within XX001 ?/?'s tetra sialylated area. A small double peak also came off within the tetra portion of XX001 ?/?.

Further chromatograms show the samples that were acquired for the project from previous project volunteers who were able and willing to provide a second sample once they had healed from their injuries; allowing this project a reference point of comparison within the individual of their AGP glycosylation patterns between an injured state and a healed state.



Graph 22. Oligosaccharide Analysis Chromatogram of GI001 7/6/10 and EW001 20/3 Healed Samples.

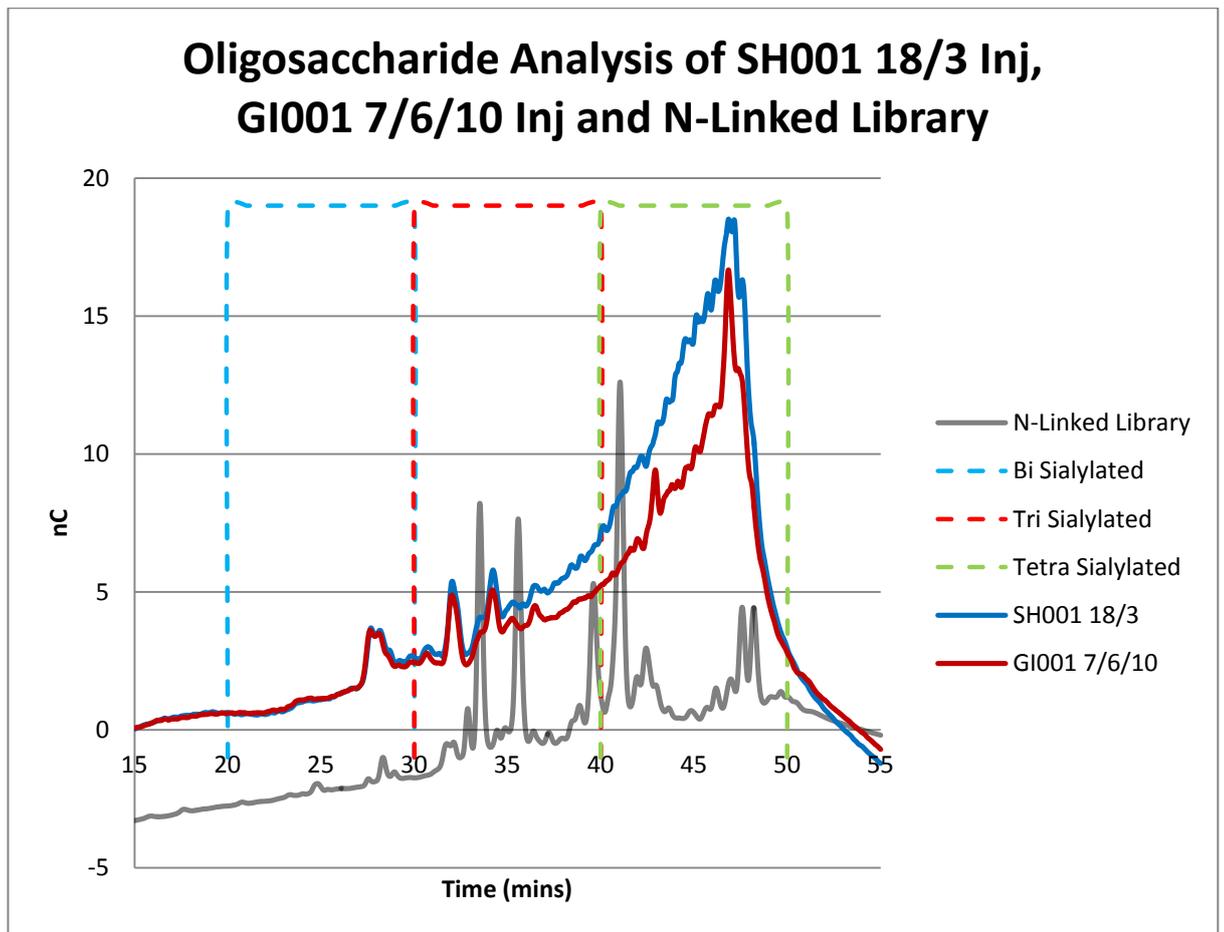
Within this graph, there are two small peaks within the bi area of GI001 7/6/10 H. Two more peaks appear within the tri area of the sample. There are a further two peaks within the tetra area of the sample. There are two small bi peaks within the bi area of EW001 20/3 H. There are also five tri branches and a further eight peaks are seen within the tetra areas of the sample.



Graph 23. Oligosaccharide Analysis of LG001 12/9 and WR001 11/16 Healed Samples.

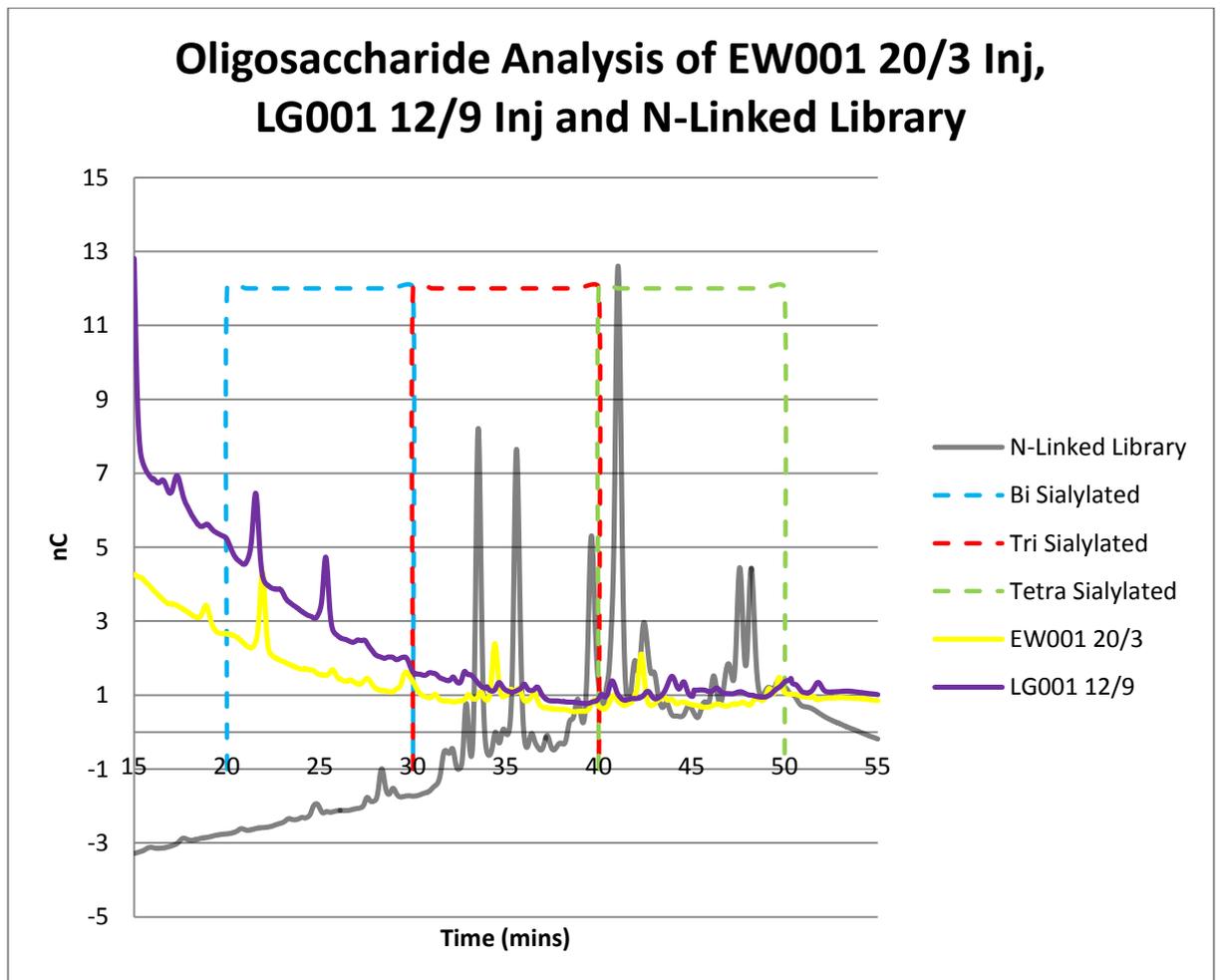
LG001 12/9 Healed sample possesses three small peaks can be seen within the bi sialylated area. Two small peaks can be seen within the tri area while five small peaks can be seen within the tetra areas of the sample. WR001 11/16 Healed sample; the sample possessing three countable small peaks coming off within the bi sialylated area within the sample, however, they are slight. There is one very slight peak within the tri peak area of the chromatogram. There are also three definite tetra peaks within the sample.

Graphs 24-27 show the injury samples when compared to the N-linked library (N-lib).



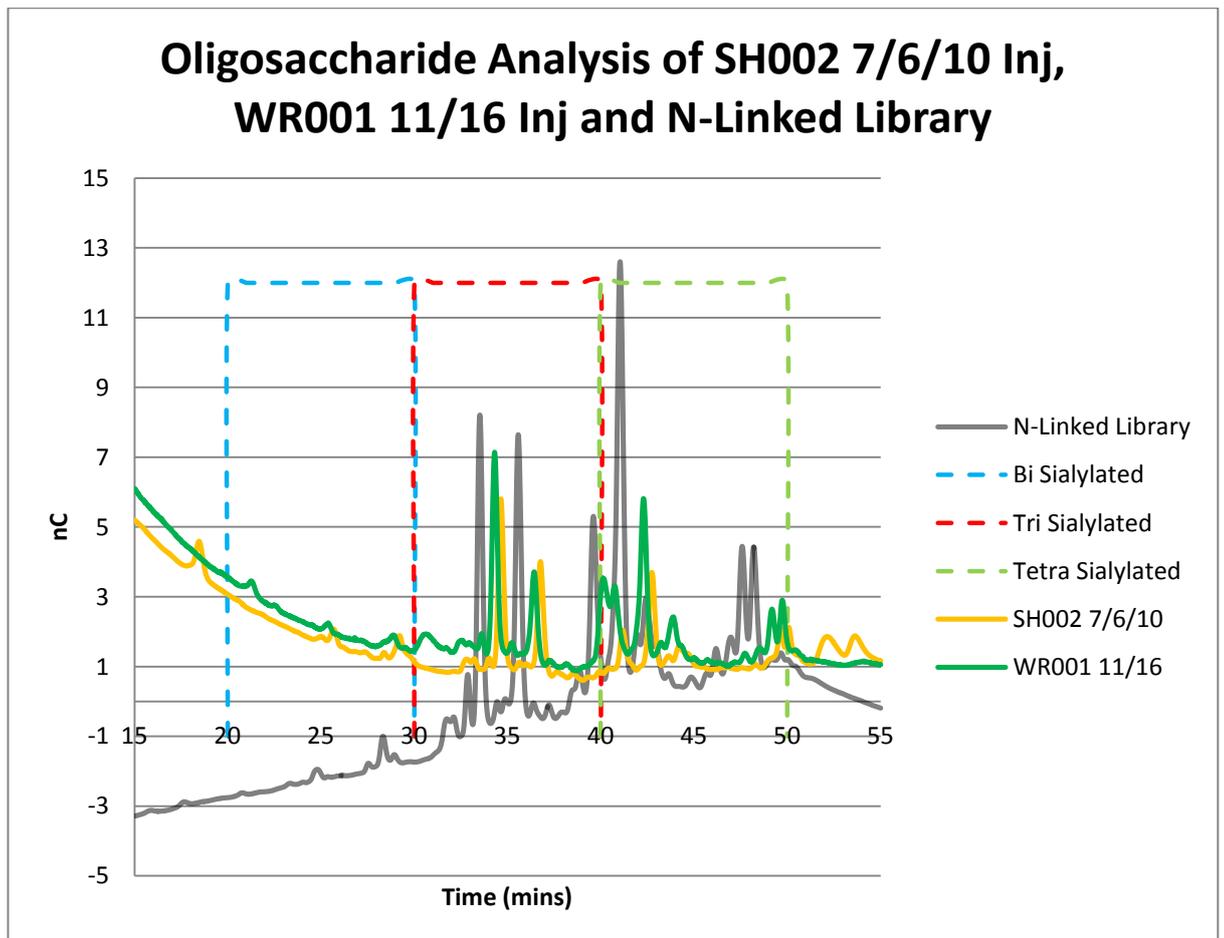
Graph 24. Comparison between SH001 18/3, GI001 7/6/10 and N-Linked Library Oligo Chromatograms.

N-Lib is showing clear peak activity as opposed to the peaks within SH001 18/3 and GI001 7/6/10. Compared to the N-Lib, SH001 18/3 is eluting bi-sialylated peaks to a higher degree, the same is true for GI001 7/6/10, both coming just under 5nC. There is a decrease in peak activity within tri-sialylated area of the graph for both injury samples, with two N-Lib peaks eluting to a higher degree. The peaks within the tetra-sialylated area of the injury samples then elute to a higher degree within both samples, both coming in just under 20nC, however, the peaks appear to show decreasing activity compared to the N-Lib, which produced five clear peaks compared to SH001 18/3's one peak, and GI001 7/6/10's two peaks.



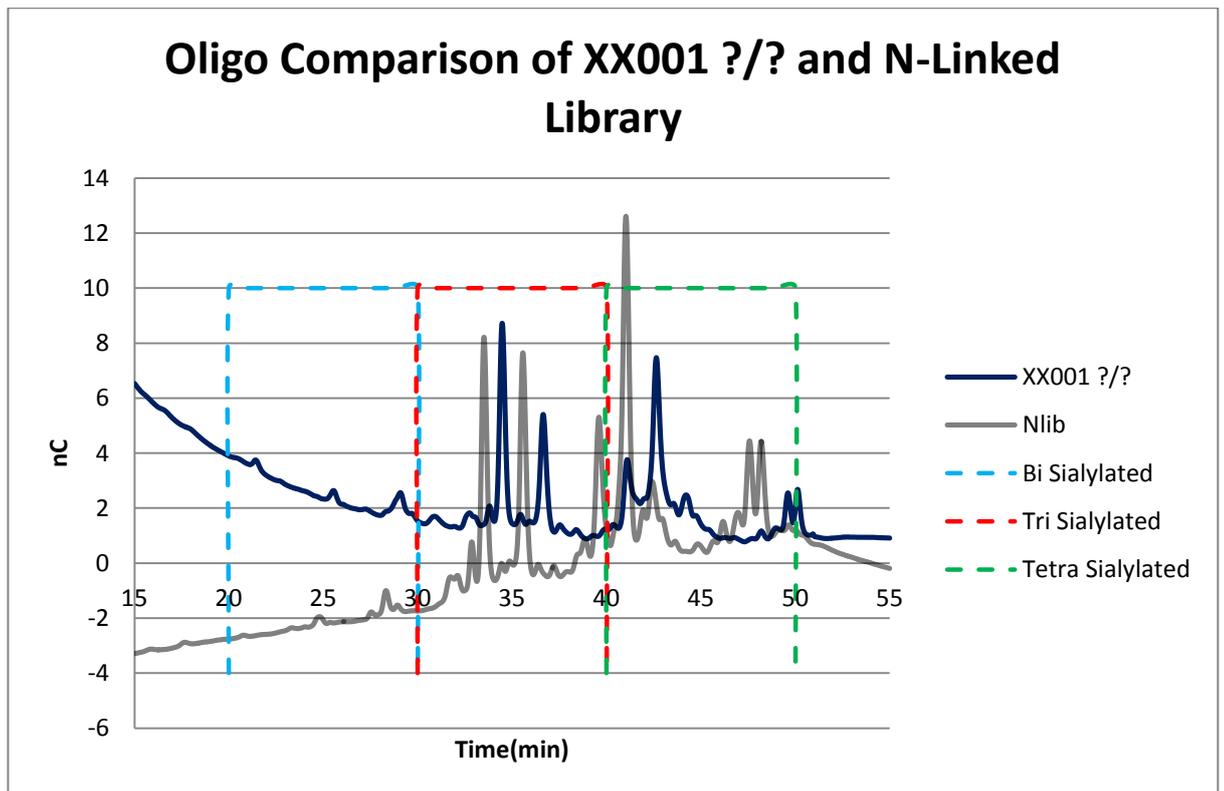
Graph 25. Comparison between EW001 20/3, LG001 12/9 and N-Linked Library Oligo Chromatograms.

Both injury samples are showing an increased elution and peak activity within the bi-sialylated area of the chromatograph when compared to the N-Lib. However, both samples then show a decrease in peak elution and peak activity in both of the tri and tetra-sialylated areas of the chromatograph when compared to the N-Lib. Very different from the N-Lib chromatogram.



Graph 26. Comparison between SH002 7/6/10, WR001 11/16 and N-Linked Library Oligo Chromatograms

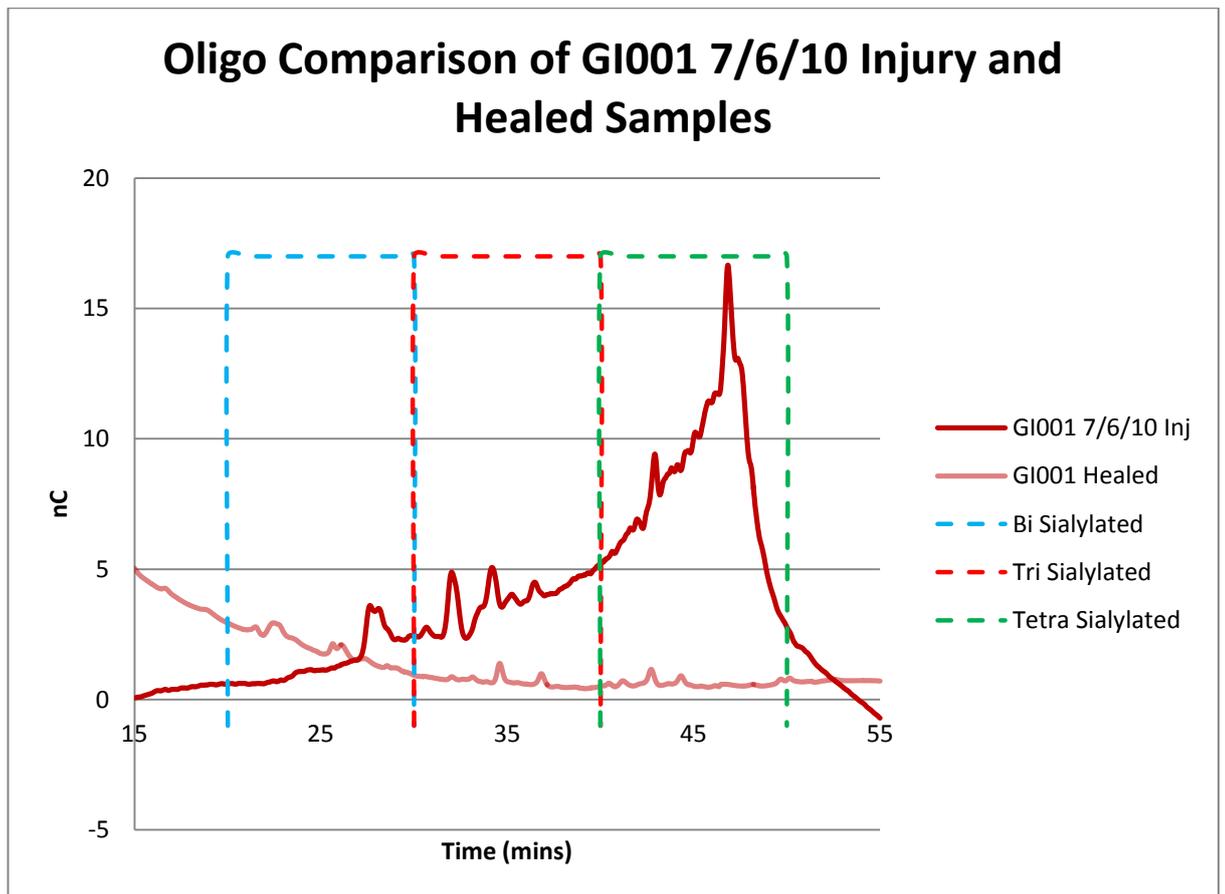
Both injury samples are showing an increase in peak elution within the bi-sialylated area of the chromatogram when compared to the N-Lib, however, both injury samples also show a decrease in peak activity within the same area of the chromatogram. Both injury samples then show a decrease in peak elution and peak activity when compared to the N-Lib within the tri-sialylated areas of the graph. SH002 7/6/10 would then also go on to show a decrease in peak elution and activity within the tetra-sialylated area also, while WR001 11/16 would show a decrease in peak elution compared to the N-Lib, WR001 11/16 would also show an increase in peak activity.



Graph 27. Comparison between XX001 ?/? and N-Linked Library Oligo Chromatograms.

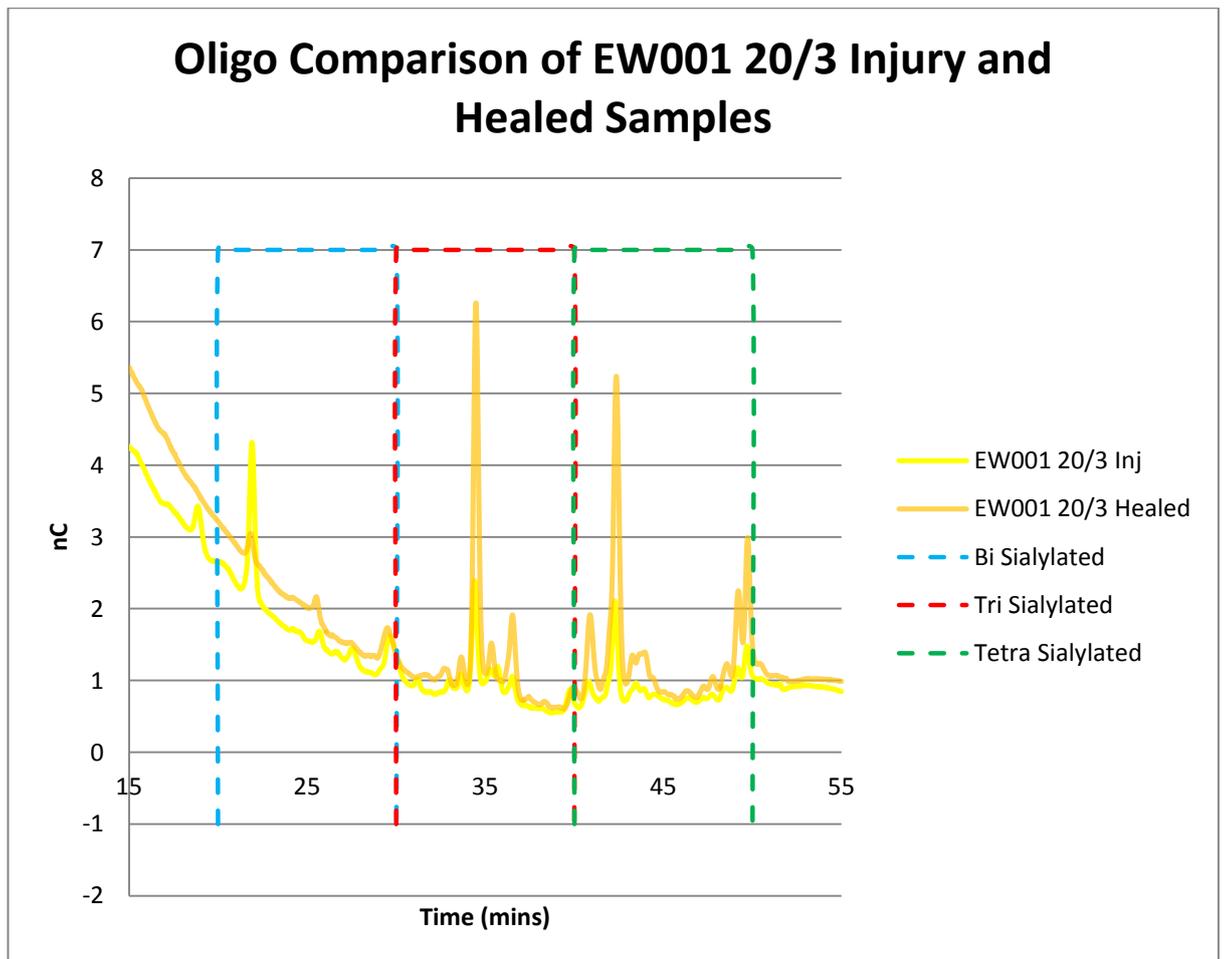
When compared to the N-Lib, the unknown would show an increase in peak elution and activity within the bi-sialylated areas of the chromatogram. The unknown would also then show an increase in tri-sialylated peak elution, while exhibiting a decrease in tri-sialylated peak activity compared to the N-Lib. Finally, XX001 ?/? would also show a decrease in tetra-sialylated peak elution when compared to the N-Lib, however, it would also show an increase in peak activity.

Following on from these comparisons, due to the procurement of blood samples from those who had healed from their injuries, it was also possible to analyze these healed blood samples within the HPAEC. These chromatograms could then be compared to those gathered from their injury samples to assess any potential change of AGP glycosylation patterns within the individual and to measure the extent of these changes when compared to the healed samples; with the injury being the “Injured” state, and the healed sample being the “Resting” state; this was due to the limitations of the project. Graphs 28-31 show the oligo chromatogram comparisons of injury samples against their healed sample counterparts.



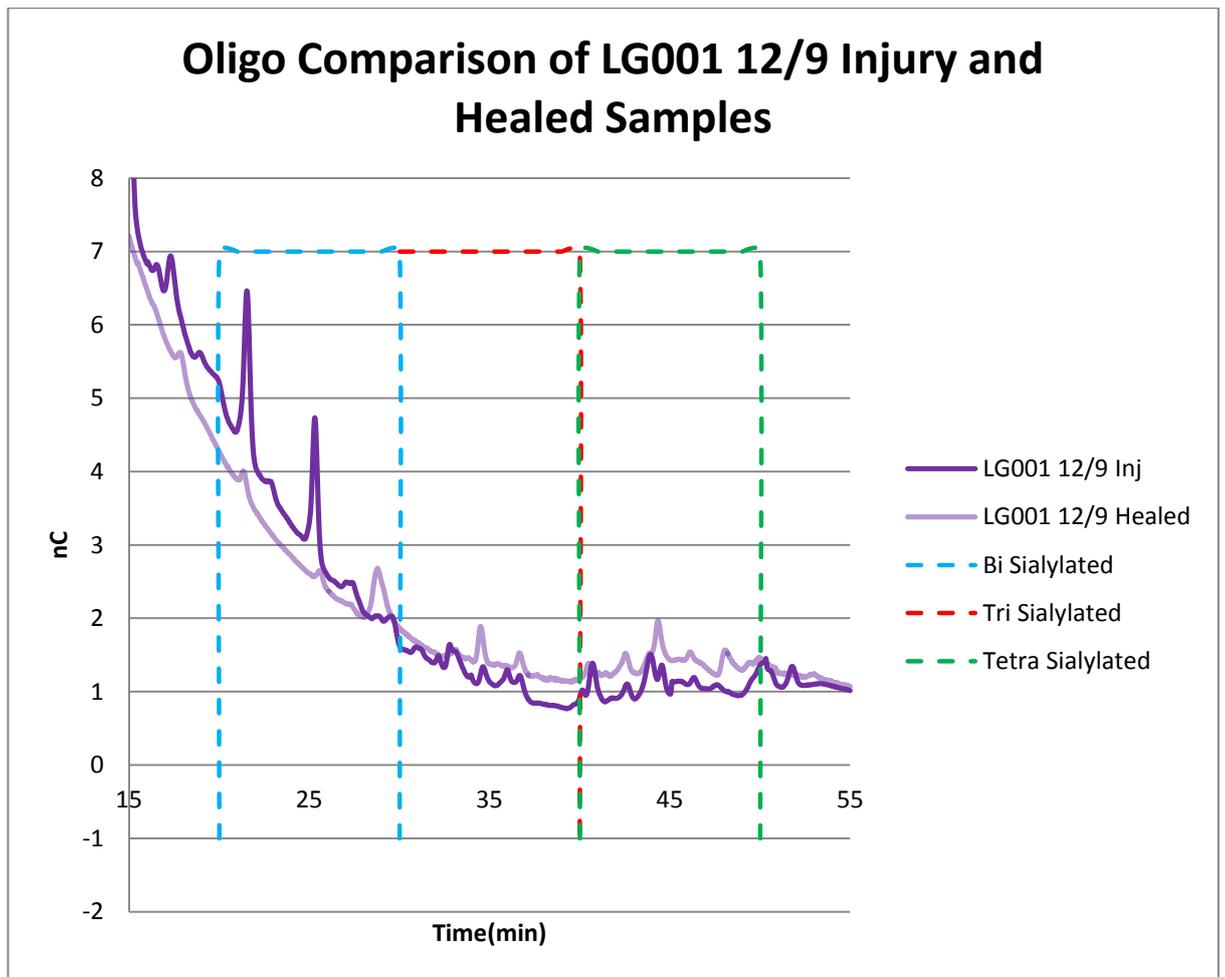
Graph 28. Comparison between GI001 7/6/10 Injury and Healed Sample Oligo Chromatograms.

The two samples are showing a very obvious difference from each other. As previously mentioned, peak activity and peak elution has increased over all fields of branching when the healed and injury sample are compared against one another. This certainly shows an obvious change has taken place within the AGP of the two samples. The four peaks within the healed sample chromatogram arguably share retention times with four of the corresponding peaks within the injury sample. This may possibly be an indication of the individual being in the process of recovering from their injury; this may show the possibility of AGP glycosylation patterns being used as a potential biomarker for the recovery from sports injury.



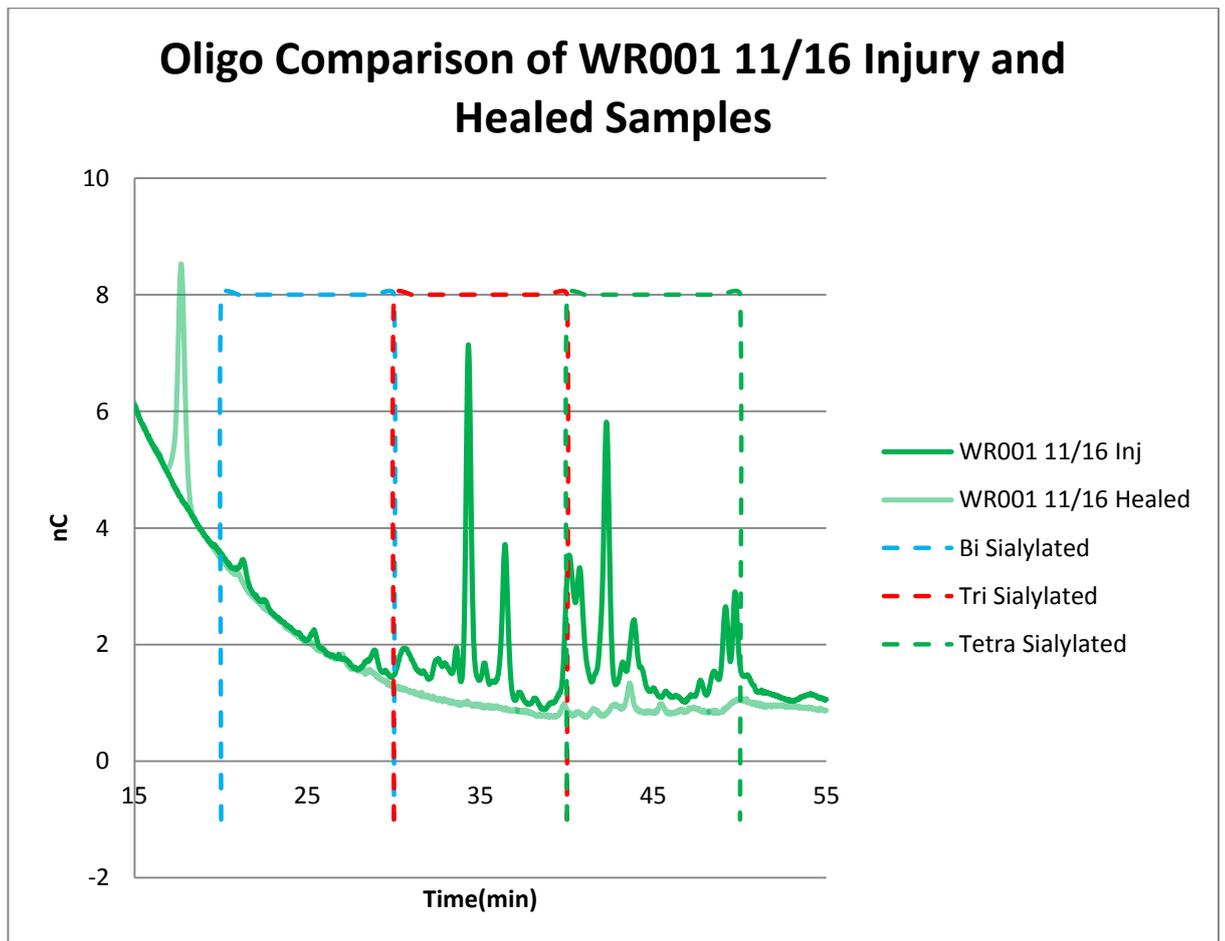
Graph 29. Comparison between EW001 20/3 Injury and Healed Sample Oligo Chromatograms.

The biggest difference between the two samples here is the peak height; the chromatograms sit on top of each other. Peak elution has decreased within the injury sample from the healed sample. When considering peak activity there appears to be very little difference between the two samples throughout the tri- and tetra-sialylated branching areas, however, it could be argued, due to the presence of a larger peak within the bi-sialylated area of the injury sample, that there has been an increase in bi-sialylated activity within the injury sample.



Graph 30. Comparison between LG001 12/9 Injury and Healed Sample Oligo Chromatograms.

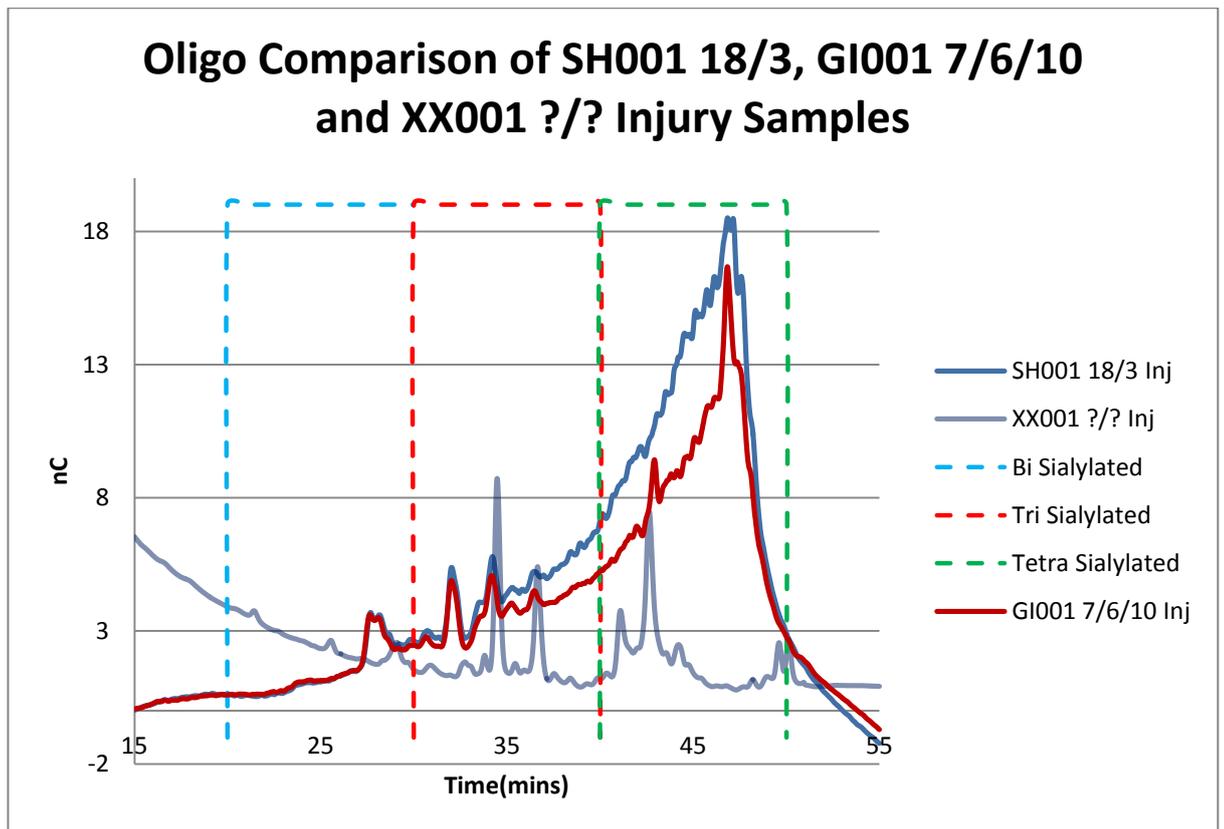
There has been an increase in bi-sialylated peak elution and peak activity within the injury sample, due to the presence of two large peaks within the bi-area of the graph that are not present within the healed sample. Peak elution within the injury sample then falls below the healed sample within the tri- and tetra-sialylated areas of the chromatograph. However, peak activities within these areas of the injury sample appear to have increased when compared to the healed sample.



Graph 31. Comparison between WR001 11/16 Injury and Healed Sample Oligo Chromatograms.

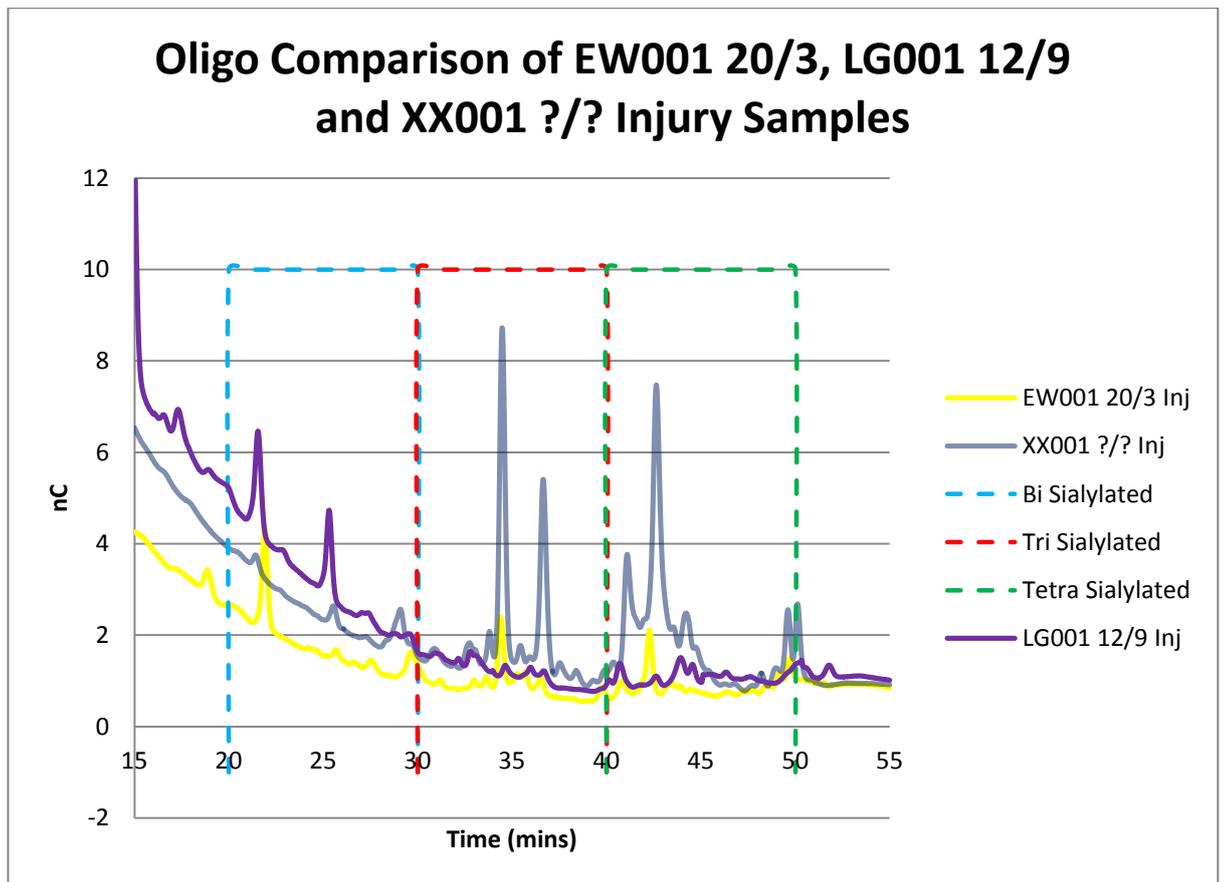
Peak elution has increased within the injury sample when compared to the healed sample, with bi-sialylated peaks seeing a slight increase and tri- and tetra-sialylated peaks showing a much more obvious increase. Peak activity has also increased between the two samples. Again, 10-20 minutes showing a slight increase in bi-sialylated peak activity, there being a dramatic increase in tri- and tetra-sialylated peaks from 20 minutes and onward. The evidence is suggesting that the injury was having an effect on the glycosylation patterns of the AGP within the volunteer at the time of the injury blood draw.

Finally, the following chromatograms show the differences in oligosaccharide composition between the injury samples, and the unknown sample (XX001 ??) in order to determine the diagnostic potential of AGP glycosylation patterns. Graphs 32-34 show the known injury samples when compared to the unknown injury sample.



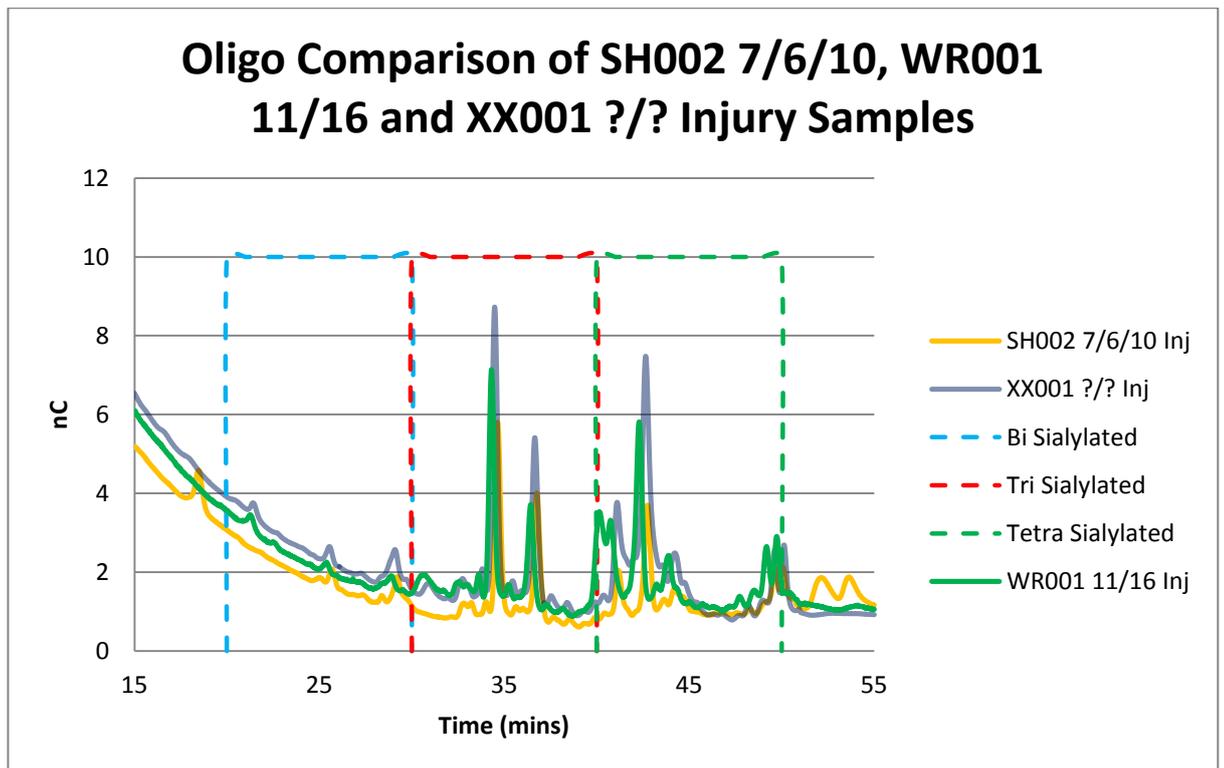
Graph 32. Comparison between SH001 18/3, GI001 7/6/10 and XX001 ?/? Oligo Chromatograms.

SH001 18/3 and GI001 7/6/10 show an increase in both peak activity and elution within the bi-sialylated branching area. The two injury samples then show a decreasing level of tri- and tetra-sialylated branching when compared to the unknown. The overall peak elution of SH001 18/3 is higher than that within XX001 ?/?, however, aside from bi-sialylated branching, there is a decrease in tri- and tetra-sialylated peak activity when comparing SH001 18/3 to the unknown. Based on a qualitative examination, it does not appear that SH001 18/3 and the unknown could be the same injury. When considering GI001 7/6/10 and the unknown, based on a qualitative examination, it does not appear that the two injuries could be the same.



Graph 33. Comparison between EW001 20/3, LG001 12/9 and XX001 ?/? Oligo Chromatograms.

When comparing EW001 20/3 and the unknown injury sample, it is interesting to note that both samples do look similar in terms of peak shape and retention time. However, EW001 20/3 does look to possess a slight decrease in peak activity for all branching types. EW001 20/3 does possess an increase in bi-sialylated peak elution than the unknown sample, while there is a decrease in the levels of tri- and tetra-sialylated peaks eluted within EW001 20/3. Based on this evidence, it is possible that the unknown injury may be that of an elbow injury. Peak elution and activity for the most part has decreased within LG001 12/9 compared to the unknown, with decreases being seen in tri- and tetra-sialylated peak elution within the known injury. However, there appears to be an increase in bi-sialylated peak elution and peak activity within LG001 12/9 when compared to the unknown. Based on this evidence, it is unlikely that the unknown injury sample is that of a leg injury.



Graph 34. Comparison between SH002 7/6/10, WR001 11/16 and XX001 ?/? Oligo Chromatograms.

SH002 7/6/10 and the unknown appear to share retention times, to the point of sitting on top of one another. There is a decrease in peak elution across all branching types within SH002 7/6/10 compared to the unknown sample. Based on this evidence, it is fully possible that SH002 7/6/10 and XX001 ?/? may be that of the same injury type (a shoulder injury). Again, when comparing WR001 11/16 to the unknown, there are minor differences. Peak elution within WR001 11/16 has decreased across all branching types compared to the unknown. Peak activity appears to show no major changes when comparing WR001 11/16 to the unknown, apart from a definite increase in tetra-sialylated peak activity within WR001 11/16. Again, there are very obvious similarities between the two samples here. Both samples are very close together in peak height, with the unknown edging out the known injury sample just slightly. However, the biggest difference here is that the peaks within WR001 11/16 appear to be coming off slightly earlier than those within the unknown sample. Aside from this, peak shape appears to be similar, including a small double peak within the tetra-sialylated area of the chromatogram of both samples. Based on this evidence, it is also possible that XX001 ?/? may be that of a wrist injury, with there being some strong evidence to suggest so.

3.3 Statistical Analysis of Results

Due to the relatively small sample size within this project and the possibility of missing/extra monosaccharides, statistical analysis of the sample group was limited. However, a small degree of statistical analysis was carried out using one sample t tests.

First of all, known injury samples were compared against the Normal blood sample. It was necessary to determine if the collected injury samples were significantly different from the Normal blood sample. As such, the collected monosaccharide means were compared against the null “hypothesis” or the mean of the Normal blood sample. A comparison of the monosaccharide means of SH001 18/3, GI001 7/6/10, EW001 20/3, and LG001 12/9 was carried out against a four monosaccharide averaged Normal sample; the resulting P value (two-tailed)= 0.1082, as such there were no significant differences of injury samples against the Normal at a statistical level. A comparison of the monosaccharide means of SH002 7/6/10, WR001 11/16 and XX001 ??/? was carried out against a five monosaccharide averaged Normal sample; the resulting P value (two tailed)= 0.0418, as such these injury samples were found to be significantly different from the Normal.

A comparison of injury samples against their respective healed samples could not be carried out within this study, as such a comparison of the healed samples against the Normal sample was carried out statistically instead. For the first comparison, the monosaccharide means of GI001 7/6/10 H, and LG001 12/9 were compared out against a four monosaccharide averaged Normal sample; the resulting P value (two tailed)= 0.1257, as such, these two healed samples were not significantly different from the Normal. Furthermore, the monosaccharide means of EW001 20/3 H, and WR001 11/16 H were compared against a five monosaccharide averaged Normal sample; the P value (two tailed)= 0.3556, these two samples were not significantly different from the Normal also.

Finally, it was necessary to determine the statistical significance of the monosaccharide means of the known injury samples against the unknown injury sample. A comparison of the monosaccharide means of SH001 18/3, GI001 7/6/10, EW001 20/3, LG001 12/9, SH002 7/6/10, and WR001 11/16 (all

averaged for five monosaccharides) against the five monosaccharide mean of the unknown sample to determine if the known injury samples as a whole were significantly different from the unknown; the P value (two tailed)= 0.0301, which means the known injury samples are significantly different from the unknown injury sample. However, a further comparison was carried out to determine if there were any significant differences between the SH002 7/6/10 and WR001 11/16 against the unknown injury sample, due to the similarities between these three samples at the monosaccharide and oligosaccharide level; the P value (two tailed)= 0.1772, meaning that the known injury samples were not significantly different from the unknown injury sample.

4. Discussion

The main aims within this project were three fold. First, it was necessary to determine whether or not an Acute Phase Response initiated by a physical injury would in fact alter the glycosylation patterns of the AGP molecule; should this aim have proved successful, then this would form the basis of the other aims within the project. These were to determine whether or not different types of injury produce different glycosylation patterns within AGP, and whether or not AGP glycosylation patterns can be used to diagnose a physical injury.

Within this project, injury samples from mountain biking accidents were selected for use within the project due to the high speed and high risk of injury associated from the sport, as well as the profile that Edinburgh Napier University possesses within the mountain biking community. While the APR is activated by bacterial infection (Gupta *et al.*, 2010), disease (Poland *et al.*, 2001), viral infection, strenuous exercise, and physical injury (Kushner and Rzewnicki, 1994), and with AGP glycosylation pattern having been affected by various different disease types (Gallacher, 2009, Anderson, 2002), up till now no studies have been carried out into the effects of physical injury on the glycosylation patterns of AGP; although, it has been suggested by Goldberger *et al.*, (1987) and Kageyama *et al.*, (1985) that there is a limited linear relationship between the severity of the injury suffered and the degree of plasma protein expression. The fundamental hypothesis of this project is to determine whether or not a physical injury induced APR will affect AGP glycosylation patterns in the same way.

When considering monosaccharide analysis, the level of difference between the tested samples and the normal sample was minute in the microgram level. The normal blood sample was found to contain 0.66 μ g of fucose, 0.46 μ g of glucosamine, 1.6 μ g of galactose and 1.32 μ g of mannose; this would be considered the “normal” glycosylation composition for the general population within this project. At the monosaccharide level, there is no injury sample that matches the normal blood sample exactly. The biggest difference between an injury sample and the normal sample is that of SH001 18/3, in which only two of the expected monosaccharides, glucosamine and mannose, were detected within SH001 18/3, while all four monosaccharides were found within the normal blood sample. It should also be noted that of the two detected

monosaccharides, there was a 0.3 fold decrease of glucosamine within SH001 18/3 compared to the normal, while there was a 0.98 fold decrease in mannose within the SH001 18/3. A similar scenario had appeared when GI001 7/6/10 was tested, in which only three of the expected monosaccharides (Glucosamine at 0.17 μ g, Galactose at 1.21 μ g, and Mannose at 1.18 μ g) were found within the sample when compared to the normal blood sample; these sugars when compared to their respective levels within the normal blood sample were found to be 0.37 fold, a 0.76 fold and 0.89 fold decreases respectively. What is interesting to note, is that these two samples were found to contain the highest absorbance under 280nm when tested within a spectrophotometer, and as such, were calculated to contain the highest concentration of AGP within them. When considering this evidence, the levels of glucosamine and mannose within SH001 18/3 were found to be no higher than some other samples which possessed lower absorbencies and were calculated to contain less AGP than either SH001 18/3 and GI001 7/6/10. As such, it may be possible that there was a contamination issue within those two samples which affected the resulting chromatographs.

With regards to monosaccharide analysis, most samples showed some differences when compared to the normal blood sample; in fact, most samples, apart from SH001 18/3, GI001 7/6/10 and GI001 7/6/10 Healed, showed the expected four monosaccharides present within the monomix solution. As previously stated, the differences between the tested samples and the normal blood sample were minute; with fucose showing the most consistency of all the monosaccharides with a 0.04 μ g range of values within all of the samples it was detected within. This is not consistent with the evidence seen within Gallacher's study (2009), in which hyperfucosylation was seen as the severity of the cancer observed was increased. It was also seen within the statistical analysis section of the thesis, that there are no statistical differences when comparing the monosaccharide means of SH001 18/3, GI001 7/6/10, EW001 20/3, and LG001 12/9 against the Normal blood sample, however, differences have been seen at the monosaccharide and oligosaccharide level. Furthermore, when comparing SH002 7/6/10, WR001 11/16 and the unknown against the Normal blood sample, statistically significant differences were observed. The monosaccharides glucosamine, galactose and mannose showed a greater

variation in the samples in which they were detected; with a range of 0.12 μ g (LG001 12/9 Healed) to 0.46 μ g (normal blood) for glucosamine, 1.19 μ g (EW001 20/3) to 1.95 μ g (LG001 12/9) for galactose, and 1.18 μ g (three samples) to 1.77 μ g of mannose (GI001 7/6/10). Perhaps the biggest difference that some samples possessed was that of the presence of the unexpected monosaccharide glucose. This sugar was detected in three injury samples and two healed samples, out of 12 samples tested (42%) within the HPAEC. It's presence within monosaccharide testing was unexpected, as glucose is normally removed in the AGP glycosylation pattern building process, thus, it was not found within the normal blood sample, and the majority of the collected samples for the project. Therefore, the presence of glucose may then show an obvious effect that the physical injury has had on the volunteer's glycosylation patterns. The presence of unexpected monosaccharides is not uncommon, in both Gallacher's (2009) and Anderson's (2002) studies, the monosaccharide GalNAc was detected within the more aggressive forms of their respective diseases, suggesting that the presence of GalNAc was as a result of the breast cancer and the liver diseases experienced by the individuals within each study. It is then possible that the presence of glucose within the samples within this study is as a result of the injuries suffered by the volunteers. However, it should be noted that the level in which glucose was found in each of the tested samples proved to be fairly consistent, with one sample (SH002 7/6/10) containing 0.55 μ g of the carbohydrate, and the other four glucose containing samples showing 0.53 μ g of the carbohydrate.

When considering a comparison between the tested injury samples and their respective healed counterparts, subtle differences emerged. For example, the biggest difference between the two samples relating to GI001 7/6/10 (seen within Graph 10), was that of the level of mannose detected; with 1.18 μ g detected within the injury, and 1.77 μ g detected when healed, resulting in a 0.66 fold decrease when injured. No fucose was detected in either sample, however, each detected monosaccharide was found to decrease within the volunteer when they were injured. When the results of EW001 20/3 are considered (Graph 11), no change was detected in fucose amongst EW002 20/3 injury, healed and the normal blood sample. While the levels of galactose and mannose decreased (by 0.78 fold and 0.83 fold respectively) when the

volunteer was injured, their level of glucosamine increased by 2.86 fold. There was also no change in the levels of fucose when LG001 12/9 injury and healed (Graph 12), when compared to the normal blood sample. While the volunteers levels of mannose decreased by 0.79 fold once injured, their levels of glucosamine and galactose increased, by 1.17 fold and 1.64 fold respectively. And finally, when considering WR001 11/16 injury and healed samples (Graph 13), and comparing them to the normal blood sample, it was seen that the level of mannose had once again, albeit subtly this time, decreased by 0.98 fold when the volunteer became injured, from 1.63 μ g within the healed sample 1.6 μ g within the injury sample; their level of fucose (0.68 μ g) had also been minutely higher than that of the normal at 0.66 μ g; this level did not alter when the injury had occurred. The volunteer's sample also showed the presence of glucose within their injury and healed samples, however, this level did not change over the course of testing, staying at 0.53 μ g. The volunteer's levels of glucosamine would also decrease by 0.1 μ g from 0.24 μ g to 0.14 μ g (a fold difference of 0.58) once the injury had taken place (while still being lower than the level within the normal sample at 0.46 μ g), and their level of galactose would increase by 0.08 μ g from 1.64 μ g to 1.72 μ g (a fold difference of 1.05) as the injury had healed; while still being higher than the normal blood sample. Perhaps the biggest link between these four samples, is that after the injuries had taken place, their levels of mannose would decrease to levels that were lower than those seen within the healed samples themselves. This perhaps shows that mannose levels decrease when an individual is injured; the level of this decrease would also be dependant on the individual as well. The stability of fucose levels throughout the testing period suggest that fucose levels may not be affected by injury induced APR, as previous studies have shown fucose levels to change dramatically when the APR is induced, particularly when the APR is induced by disease. Fujimura *et al.*, (2008) showed fucose levels to increase when an individual was suffering from prostate cancer, while invasive breast cancer samples (Gallacher, 2009) and hepatitis samples (Anderson, 2002) have also shown fucose levels to increase within those suffering from these conditions. Progression from hepatitis to cirrhosis infections have also shown fucose levels to decrease over time (Anderson, 2002). The evidence of the stability of fucose levels in this study is in contradiction to evidence

presented by Higai *et al.*, (2005), who found that levels of fucose increased within those suffering from acute inflammation. However, Higai's study used AGP collected from those experiencing acute inflammatory states brought on by disease, not physical injury. The evidence presented in the present study suggests that fucose may not be affected by physical injury induced immune responses.

Moving onto oligosaccharide analysis, it is easier to see that a difference has taken place within the tested AGP samples. As previously noted, the first aim of this project was to determine whether or not a physical injury induced APR would affect AGP glycosylation pattern by analyzing the change in the complexity of the profile produced by an injury sample when compared against the normal, a healed sample, or the unknown. Change in complexity is defined as being either the increase or decrease of a samples peak elution (peak height) and/or peak activity (number of peaks produced). While it may not be obvious at first glance to determine whether or not a change as taken place when looking at the monosaccharide composition of the AGP samples, it is much easier to determine if a change has taken place when looking at the oligosaccharide composition of the molecule. In fact, when compared to the N-linked library (Graph 14), there is no tested sample that matches the line produced when the N-Lib was tested. Every sample is showing an obvious difference from that of the N-Lib. For example, when compared to the N-Lib, EW001 20/3 and LG001 12/9 (Graph 22) show far smaller peak heights, possessing no shared peak shapes or retention times with the N-lib.

However, what is also interesting to note is the difference at the oligosaccharide level between those injury samples from which there is a healed sample available for testing. When compared against each other, the two samples do not share peak elution or complexity and only share peak retention times for a small number of peaks. This evidence is seen again when comparing the two samples relating to WR001 11/16 (Graph 28); again, peak elution and complexity are not shared between the two samples and few peak retention times are shared. This evidence is supported by Anderson's study (2002), Gallacher's study (2009) and Behan's study (2010), where the tested samples have the potential to have little in common with the collected normal

samples. This shows that there is potential for injury and healed samples to be very different at the oligosaccharide level.

However, it should also be noted, that the evidence provided by these differences may be limited, as seen with EW001 20/3 (Graph 26) and LG001 12/9 (Graph 27). EW001 20/3 paints a different picture here; peak elution is again the difference that separates the injury and healed samples, in addition to a solitary peak within the injury sample that is higher than others detected within the healed sample; these samples do share a large degree of peak retention times and shape, showing that the two samples did not possess many differences. These similarities somewhat continue when looking at LG001 12/9. Again, the two samples can be separated by peak height, however, as opposed to what was seen in EW001 20/3, the differences in peak height are a bit more evenly distributed, with both samples showing peak heights that are higher than the other sample at varying points of the chromatograph i.e. the injury sample possesses higher peak heights within the bi-sialylated area of the chromatograph, and the healed sample possessing higher peak heights within the tetra-sialylated area. However, there appears to be an increase in the levels of peak activity within the injury sample when compared to its respective healed sample showing that differences between the two samples are present, if not obvious at first. The evidence provided by these two volunteers suggests that oligosaccharide level differences between injury and healed samples may be limited. It should be noted that patterns possessing similarities to this degree were not seen in Anderson's (2002), Gallacher's (2009) or Behan's studies (2010). However, this study has also had an opportunity to analyze AGP collected from those who had recovered from their injuries, allowing a perspective within this project that other studies have not had; understandably due to the severity of the conditions that were being analyzed, the ability to acquire a healed blood sample from volunteers once they had healed may not have been possible. Any similarities within injury and their respective healed samples could then be as a result of the two samples having come from the same person.

Previous studies, such as that carried out by Gallacher (2009), show a correlation between the levels of galactose, a main component of the branches

of AGP, and the complexity (changes in peak elution and peak activity) of the branches. Within this project, this correlation was seen to a large degree. When comparing the injury samples to the normal blood sample, a correlation was seen between the levels of galactose and the complexity of the branching patterns at the oligosaccharide levels within six of the seven injury samples. A decrease in the level of galactose was found to correlate with a decrease in the complexity of the branching patterns, while an increase in the level of galactose was found to correlate with an increase in the complexity of the branching patterns. Previous studies, such as that by Anderson (2002), and Fournier *et al.*, (2000), have found the level of fucose to be related to the degree of branching at the oligosaccharide level, with an increasing presence of fucose suggesting a reduction in the number of bi-sialylated glycans, however, the evidence seen within this study does not support these conclusions. Higai *et al.*, (2005), found the level of fucose and degree of branching to be independent of each other, which was seen within this project. Only LG001 12/9 (seen in Graph 4 and Graph 16), in which it's levels of galactose would suggest an increasingly complex branching pattern, differed from this evidence; it's oligosaccharide branching pattern would show a less complex branching chain. This correlation between the levels of galactose and the complexity of the branching patterns was seen again when comparing the monosaccharide composition and oligosaccharide branching patterns of the injury samples and those with healed samples. In this case, three out of four of the sample sets would support this theory, with the levels of galactose in these sample sets suggesting a change in complexity of the branching patterns.

The slight decrease in the level of galactose between GI001 7/6/10 Healed and GI001 7/6/10 Injured would suggest a slight decrease in the complexity of the branching patterns, however, when the volunteer was injured, their oligosaccharide branching pattern became more complex, i.e. the pattern produced by the injury sample possessed a higher peak activity and peak elution than the sample taken once the volunteer had healed.

In summary, it was seen throughout the project testing, that the majority of injuries produced an effect on the AGP contained within their samples. By testing at the monosaccharide levels, the carbohydrates that make up each samples respective AGP have been determined and quantified. These

carbohydrates were then compared to the gathered normal blood sample for this project, and the healed samples that have been provided. While any detected differences have been minute, an effect on the AGP, presumably as a result of the APR from the injury, has been established. Taking this evidence in conjunction with the oligosaccharide evidence of all the samples gathered also, shows even further than an effect on the glycosylation patterns of AGP has taken place. Kushner and Mackiewicz (1987) suggest that the qualitative patterns of APPs (of which AGP is included) are not influenced to a significant degree by the type of injury sustained, this contradicts evidence found within this study. This change can be further established and quantified when observing the levels of galactose within the injured and healed samples. Based on the findings within the scope of this project, the findings at this point may be limited, however, this work can then be used as the basis for further research into the effect that physical injury has on the glycosylation pattern production of AGP.

It could also be argued that the small scope of the study (seven injury samples, four healed samples, and a normal blood sample), could have been a limiting factor within this study, and arguably, more samples could have added more information to this study. However, it should also be noted that it could take one month and over to purify a blood sample from venepuncture to analysis of the results within the HPAEC. As a result, the number of collected samples gathered within the project was ideal for the time frame of this project. Should further research within this area be carried out in the future, a longer timeframe would allow a higher sample count to be analyzed. The single sport of choice may have also been a limiting factor; a wider approach to sport choice would also presumably increase the chances for injury. However, by focusing on one sport, this study has managed to gather important evidence on the relationship between AGP and sports injury that can act as a foundation for further project research where a wider sports range can be used within the study. Finally, the Beer Lambert Law was used to determine the concentration of AGP within the collected injury sample. While in the majority of cases the Beer Lambert Law can be used to successfully determine the concentration of AGP and other solutions, the Beer Lambert Law also has its limitations; high concentrations can cause deviations in absorption coefficients due to the

interactions of the molecules at close proximity. This could potentially explain the two anomalous results (SH001 18/3 and GI001 7/6/10), where these results produced very high absorbance values for (apparently) a low concentration of AGP, and as such, the limitations of the Beer Lambert Law should be known when using the technique.

Following on from the first aim of the project, it was then necessary to determine whether or not different injury types would then produce an individual effect on the glycosylation patterns of AGP. As previously stated, the ability to determine a difference in a sample's AGP glycosylation using monosaccharide composition alone may be limited. There was very little variation in the levels of fucose within the scope of the seven injury samples gathered, suggesting that fucose may not be affected by injury induced APR as opposed to hyperfucosylation seen in Gallacher's study (2009), Anderson's study (2002) and Fujimura (2008), and the levels of glucose within the three samples it was found in showed very little variance. While more variance was found within the levels of glucosamine, galactose and mannose, there is perhaps not enough evidence to identify specific injury types on their own. The evidence gathered here provides important information on the carbohydrates that make up the glycosylation patterns of the AGP molecule. However, it is much easier to determine individual injury types using the oligosaccharide analysis chromatographs gathered. Out of the seven injury types, the chromatographs produced by SH001 18/3 and GI001 7/6/10 were markedly different from the other five injury types, with these samples showing a rising curve as the analysis goes on within the HPAEC, these samples are the only samples which pass over the 15nC range. However, these samples do not show as many discrete, definite peaks when compared to the other five samples, which while they do not possess any peaks higher than 15nC, they do possess definite, distinguishable peaks. In addition to the obvious differences seen in SH001 18/3 and GI001 7/6/10, LG001 12/9 produced the smallest chromatogram seen throughout the study, with very low peak elution and decreasing peak activity compared to the other samples, suggesting a dramatic decrease in the complexity of the glycoform and supporting the theory that the injury suffered by the volunteer has produced an individual effect on the glycosylation of their AGP.

The other four samples, EW001 20/3, SH002 7/6/10, WR001 11/16 and XX001 ?/?, produced chromatographs that were similar in shape overall but do possess subtle differences. Removing the unknown from consideration at this point, as it is the focus of Aim 3, it has been noted that EW001 20/3, SH002 7/6/10, and WR001 11/16 (Graphs 16 and 17), all possess a similar shaped curve, with minor differences between the three. What is interesting to note, is that these samples also share a degree of homology in peak shape and retention time. It should be noted that EW001 20/3 possesses a much smaller peak height than the other two samples, and possesses a peak within the bisialylated area that is not present within the other two samples. It should also be reported, that WR001 11/16 possesses the highest peak heights of the three samples, and these peaks were shown to have slightly earlier retention times than those within the other two samples. What is interesting to note is the homology that WR001 11/16 and SH002 7/6/10 share in particular; WR001 11/16 is a wrist fracture injury, and it has been found since the project had finished that SH002 7/6/10 was a misdiagnosed shoulder fracture injury, this shows the potential of injury types to produce similar glycosylation patterns when considering one type of injury, i.e. fractures. Conversely, these samples may lead to the suggestion that perhaps that AGP glycosylation pattern alteration may be dependant not on the type of injury, but the area the injury has occurred in, due to the obvious similarities between the three samples regardless of the injury type, while all three injuries had taken place within the arm of these volunteers. However, the evidence in support of this theory is limited as there are two shoulder injury samples within this project, and neither of these samples bear any resemblance to each other, most probably due to these injuries being two different types of shoulder injury.

It has also been seen in Gallacher's study (2009), that tested samples of the same degree of breast cancer from different volunteers have also produced slightly different oligosaccharide patterns, this was also found within Anderson's study (2002) when considering multiple samples from the same types of liver diseases. This variability within sample groups was also found within Behan's study (2010) when studying the glycosylation patterns within volunteers on different types of methadone recovery therapy. This evidence suggests that the oligosaccharide patterns may not be entirely reproducible, however, it has been

seen within this study that different injury types have the ability to produce different types of oligosaccharide patterns and the ability to produce similar (not exact) glycosylation patterns from similar injury types, with SH002 7/6/10 and WR001 11/16 representing this best. Due to the limited sample group for this project, the evidence of the reproducibility of glycosylation patterns between similar injury types from different volunteers may be limited. In conclusion, the evidence presented in this section of the project suggests that different injury types have the potential to produce different effects on the glycosylation patterns of AGP, however, further research must be carried out in order to definitively confirm this theory.

Finally, the last aim of this project was to determine the diagnostic potential of glycosylation patterns of AGP, having presented evidence of an effect on the glycosylation patterns of AGP as a result of an APR induced by a physical injury, and that different injury types can produce different effects on AGP glycosylation. Thus, the final stage of the project was to determine whether or not there is any diagnostic potential in AGP glycosylation patterns when these glycosylation changes have been induced by an injury APR. The diagnostic potential of AGP glycosylation patterns has been seen before, with Gallacher's study (2009) showing that glycosylation patterns of AGP can distinguish between different severities of breast cancer, Anderson (2002) showing the potential of AGP to diagnose different types of liver diseases; in addition to these advancements, altered fucosylation of AGP has also been shown potential to act as a biomarker during pregnancy development (Orczyk-Pawilowicz *et al.*, 2009).

The approach of this author to answering this question was to follow the same two fold analysis that was used for Aims 1 and 2 within this project. An unknown sample was collected from a project volunteer, the identity of the injury remained anonymous throughout the project so that this aim could possibly be determined without bias being a factor. This sample, XX001 ??/ (Graphs 6 and 18), was collected and processed along with the other samples in the study, while the paperwork relating to the volunteer was kept in a sealed folder until analysis had been completed, after which, the sample's injury type was revealed once.

The following speculative discussion and conclusion as to the injury under question was made without any knowledge of the injury, which remained sealed until the author had drawn these conclusions. To determine the identity of the unknown sample, identification would be made on the basis of three comparisons; first, the monosaccharides present within the unknown were compared to those within the known injury samples, followed by a comparison in the amounts of monosaccharides detected. This was achieved by looking at the percentage change of monosaccharides detected within a known injury sample against the monosaccharides detected within the normal blood sample, and comparing it to the percentage change of monosaccharides detected within the unknown sample against the normal blood sample. The two main monosaccharides of interest within this comparison were that of fucose and galactose, due to their status as potential markers for the extent of glycosylation (Fournier *et al.*, 2000, Anderson, 2002, Gallacher, 2009). The level of percentage change allowed to be considered for a match was +/- 5% in order to be significant. Finally, a visual comparison of the oligosaccharide trace gathered from the unknown was then carried out against the oligosaccharide traces from the known injury samples.

When comparing the monosaccharide composition's of the tested samples, on a visual level the samples that were found to be the closest match to that of the unknown were SH002 7/6/10 and WR001 11/16 (see Table 9). In fact, when statistical analysis was carried out, it was seen that differences between SH001 18/3, GI001 7/6/10, EW001 20/3, and LG001 12/9 against the unknown injury sample were statistically significant. Furthermore, this was supported by a statistical comparison of SH002 7/6/10 and WR001 11/16 against the unknown sample, where it was seen that these differences were not statistically significant, suggesting that it could be possible to match one of these two samples to the unknown sample. The unknown could match that of SH002 7/6/10, with there being no change in the levels of fucose, and 0.6% increase in the levels of galactose within the unknown sample when compared to the known injury sample. They both contain all the expected monosaccharides and the unexpected monosaccharide in glucose, however, the biggest difference between the two samples is that of their levels of mannose, with a 0.4µg difference in the level of mannose between the samples.

As a result of this difference, it could then be argued that the unknown sample is that of WR001 11/16 also; the levels of fucose and galactose within WR001 11/16 also fall within the 5% range, with there being a 2.9% increase in fucose and 3.6% decrease in galactose within the unknown injury sample when compared against the respective levels within the known injury sample. Again, the two samples share the presence of all of the expected monosaccharides and glucose, however, there is also a 185.7% increase in the level of glucosamine within the unknown compared to the known injury sample. As a result of these similarities, theories as to the identity of the unknown sample were able to form, however, it was not possible to make a conclusive diagnosis based on this information; as such the oligosaccharide structure of the unknown was then compared to the oligosaccharide results gathered from the other tested samples within the project.

When considering the oligosaccharide structures of the tested samples of the project, it was quick to remove SH001 18/3 and GI001 7/6/10 from consideration due to the large differences from the unknown sample within them. It was found in a study by Brinkmann-van der Linden *et al.*, (1996) that acute inflammation states would produce an increase in bi-sialylated glycans, however, four out of the seven samples tested, would show an increase in more complicated branching types. Gallacher's work in (2009) would also support this theory due to the increased presence of bi-sialylated glycans in invasive breast cancer samples, however, Anderson's (2002) when studying liver diseases found that as the liver disease severity increased, the branching complexity would see a shift from bi-sialylated branches to tri- and tetra-sialylated branches (particularly, when hepatitis C infections progressed from acute to chronic). LG001 12/9 was removed from consideration due to the vast differences it possessed against the unknown. EW001 20/3 does share some similarities with the unknown, with a degree of homology between the unknown and EW001 20/3 in terms of peak complexity and retention time, which while not exact, there are definite similarities between the two samples. However, EW001 20/3 possesses a prominent peak within the bi-sialylated area of the chromatograph, along with a marginally different peak elution from the unknown. When considered along with the monosaccharide results (EW001 20/3's levels of

fucose and glucose fall outside the allowed 5% range), it may be unlikely that the unknown is that of EW001 20/3, an elbow injury.

This then leads to the consideration of SH002 7/6/10 and WR001 11/16 (see Graphs 13 and 31). When comparing SH002 7/6/10 and the unknown sample, there is very little that can separate the two samples. The two samples possess a large degree of homology in terms of peak complexity and peak retention time, with the biggest difference between the two samples being that of peak height; the unknown possessing larger peak heights than that of SH002 7/6/10, however, the two chromatograms could be placed upon one another. When considered with monosaccharide results, where the biggest difference is still the difference in the levels of mannose, there is strong evidence that SH002 7/6/10 could be the same injury type as the unknown sample.

When considering WR001 11/16 and the unknown sample, again there is very little that can separate the two samples. The two samples share a large degree of peak complexity, and WR001 11/16 possesses a closer peak elution to the unknown than that of SH002 7/6/10. However, the peaks within WR001 11/16 have come off slightly earlier than those within the unknown sample. Again, the biggest difference when considering monosaccharide analysis is the levels of glucosamine between WR001 11/16 and the unknown sample.

As such there are two samples of which could be related to the unknown sample, SH002 7/6/10 and WR001 11/16. There is very little that can separate these two samples from being homologous to that of the unknown sample. When the sealed folder containing the volunteer's unknown paperwork was opened, it was found that the injury suffered by the volunteer was that of an elbow fracture. As a result of this injury, the evidence provided by the unknown sample supports two theories proposed by this study. Firstly, the revelation of the unknown being that of an elbow fracture, while producing similar oligosaccharide chromatographs and being of similar monosaccharide compositions to the wrist fracture seen in WR001 11/16, and seen in SH002 7/6/10, when it was subsequently seen to be a shoulder fracture, supports the theory that injury induced alterations of AGP glycosylation patterns can produce changes within the carbohydrate of the AGP molecule that are injury specific. Secondly, as a result of this information, the revelation of the unknown injury

type through the comparison of monosaccharide composition and oligosaccharide structure of other injury samples shows the diagnostic potential of changes to AGP glycosylation patterns when induced by injury. It should also be noted the similarities that the unknown shares with EW001 20/3 could perhaps suggest that EW001 20/3 (an abrasion injury to the elbow) could perhaps have been a more serious injury than first thought due to the similarities that EW001 20/3 shares with the known fracture injuries within this study. However, the medical potential of this evidence is limited due to the small scope of this project.

5. Conclusions

In conclusion, there were three aims in this project. These were to determine whether or not AGP glycosylation patterns could be altered as a result of an injury induced APR process, and if so, could different injury types produce different changes to the glycosylation patterns of AGP? Finally, is there any diagnostic potential within the glycosylation patterns of AGP when altered by physical injury?

As it formed the basis of the entire project and any potential future work within this area, the biggest aim was to determine whether or not physical injuries can in fact alter the glycosylation patterns of AGP. Through the monosaccharide composition and oligosaccharide structural analysis of the gathered seven injury samples and the four normal samples, a carbohydrate fingerprint was constructed for each sample, which would then allow comparisons and similarities between each sample to be drawn. When the injury samples had been analysed and compared to the normal blood sample there was evidence within the injury samples that a change had taken place.

As seen throughout the project, the level of differences between the injured samples and the normal blood sample was minute in the μg level, however, changes were observed and quantified. The research within this project adds to what is already known about AGP, by showing that injury induced APR does have an effect on the glycosylation patterns of AGP. Within the scope of this project, each injury was shown to affect the glycosylation pattern of the volunteers AGP through the comparison of injury AGP samples against a normal blood sample, and subsequently in some cases, a healed blood sample from the same volunteer. While obvious differences were observed between the injury AGP samples and the normal blood sample, there were more subtle differences observed within comparisons of injury AGP samples and healed injury samples from the same volunteer. However, while more subtle differences were observed within these comparisons, the information gathered was invaluable as it allowed the observation of a before and after state of an individual's injury, while at the same time showing the differences that can occur between individuals with different injuries.

Furthermore, when it was confirmed that different injuries can produce different effects on the glycosylation patterns on an individual's AGP, it was the aim of this project to determine whether or not there was any diagnostic potential in AGP glycosylation patterns. When the unknown sample was compared against the evidence collected from the known injury samples, it was found that the unknown sample shared a great deal of homology at the monosaccharide and oligosaccharide levels with the known wrist fracture and shoulder break within the project, leading to the theory that changes to the glycosylation patterns of AGP were dependant on the injury received with no regards to where the injury had taken place. This theory was then strengthened when the unknown injury sample was revealed to be an elbow fracture, thus showing that there is diagnostic potential within the glycosylation patterns of AGP that have been affected by injury induced APR.

In conclusion, the main aim of the project was to determine whether or not physical injury induced APR can produce changes in the glycosylation patterns of AGP, akin to those changes seen to have taken place within different severities of breast cancer and different types of liver diseases. Through the monosaccharide and oligosaccharide analysis of the gathered injury samples and their comparisons against the normal samples, it has been determined that injury induced APR does produce an effect on the glycosylation patterns on AGP within those injured individuals. It is possible to categorize this change both qualitatively and quantitatively. Furthermore, it has been determined that there is diagnostic potential within the glycosylation patterns of AGP with regards to injury induced APR. This information can then be used as the basis for further research into the effects of injury induced APR changes on AGP glycosylation patterns.

6. Further Research

As a result of the conclusions reached within this project, should further research into the effects of injuries on glycosylation patterns, then further research recommendations are;

- Increase the sample size for further project research; within this project, seven injury samples from volunteers were gathered, four healed reference samples were gathered from previous volunteers, along with a normal blood sample, monomix solutions and an N-Lib. Should time allow, a larger sample group would be ideal, as would an increased sample type range (with nearly half of these project samples containing fracture/break injuries) would also be beneficial.
- If the acquirement for more and different sample types is possible, then the development of a “glycosylation pattern database for injury types” would allow for the storage of reference information relating to the glycosylation patterns of specific injuries. This would then allow further research into the diagnostic potential of the glycosylation patterns of AGP, with the comparisons of newer unknown injury types against the known injury types within the database.
- Should more research be carried out within this area, a further recommendation would be to analyze a persons glycosylation patterns over the course of their recovery, to determine how the glycosylation patterns of AGP behave throughout the recovery process, so that it may be possible to develop a biomarker to show a persons recovery from a physical injury. Could glycosylation patterns of AGP be useful to track a person’s recovery from overuse/over training injuries?
- Finally, should it be possible to develop a biomarker for the recovery from physical injury, then would there be potential to chart the effectiveness of different therapies for more serious injuries, i.e. which is a more effective treatment for a torn bicep, surgery or physiotherapy?

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Appendix

Appendix 1 Volunteer Information Sheet

Alpha-1 acid glycoprotein (AGP) as a serum biomarker for sport injury

We would like volunteers to take part in a Research Study funded by Edinburgh Napier University. The study is designed to examine the structural changes that occur to Alpha-1-acid Glycoprotein (AGP) under the stresses of a physical sporting injury and to determine if AGP can be used to diagnose and chart the recovery of the individual from their injury.

Why have I been chosen?

Inclusion in this project will be based on the severity of the injury to the individual. All ages and gender of rider will be considered, however, those under the age of 18 will have to obtain written consent from their parents before inclusion in the project.

What will happen to me if I take part?

We would like to collect one 5ml sample (about three teaspoonfuls) of blood from you. The blood sample which is collected from you will have your name and address removed so that you cannot be recognised during the course of the research. There will also be no transfer of any identical information about you nor will your medical record be accessed.

You are free to withdraw from participation in the study at any point without explanation. If, at all possible, your blood sample, and all associated data, will be removed from the study and destroyed.

Should you have any enquiries into the project or would like to take part, please take one of the tags below and email me regarding your interest.

Thank you for taking the time to read this leaflet. Your help is very much appreciated.

William Surradge, Edinburgh Napier University, Sighthill Campus

Email: 06017014@live.napier.ac.uk

This study has been reviewed, and approved, by Faculty of Health, Life and Social Sciences Ethics and Governance Committee.

Appendix 2 Venepuncture Consent Form



Subject Declaration for Vene Puncture Blood Donation

You have consented to donate blood in the School of Life Sciences. The School phlebotomists have all undergone an approved training course and have Hepatitis B immunity. The blood you are donating will be used for

.....but will not be screened for pathogenic organisms that could adversely affect the health of any exposed person. It is therefore important that you do not donate blood if any of the risk factors listed below apply to you. At the end of the experiment the cells will be disposed of and not stored for future experiments.

Please read the list below and think very carefully if any apply to you. If any factors do apply please do not sign the declaration and do not offer your services as a donor. You do not have to say which risk factors apply.

Risk Factors

Recent –

- Ill-Health
- Contact with infectious diseases
- Vaccinations or immunisations

In the last year-

- Tattoo or body piercing
- Childbirth
- Blood transfusion
- Tissue or skin graft
- Hormone treatment
- Major surgery
- Travel to a malarial area or in sub Saharan Africa, Asia or South America

At any time –

If you have lifestyle factors which would pose a risk please do not donate blood.

Declaration

I have read the risk factors and have considered my lifestyle factors and to the best of my knowledge none of them apply to me and I am in good health. I understand that my blood will be used for research purposes.

Name of Donor: Name of Phlebotomist:

Signature of Donor:..... Signature of Phlebotomist:.....

Date:..... Date:.....

Patient Information Questionnaire

Adapted from Stuart Aitken (details below)

Glentress Mountain Biking Injury Study

July 2007 – Jun 2008

Stuart Aitken

Orthopaedic Research Fellow, Royal Infirmary Edinburgh

PLEASE CIRCLE THE MOST APPROPRIATE ANSWERS, OR SIMPLY WRITE IN THE SPACES PROVIDED.

Name

Age & Gender

Postcode & Town

Do you wear a helmet when you ride? Always Usually Sometimes Never
Is it XC, Skater-style or Full-face?

Do you wear gloves or mitts? Always Usually Sometimes Never

Do you wear any other protective gear? Always Usually Sometimes Never
If so, what kind of gear?

What kind of pedal system do you use? SPDs or Cleats Flats Toe cages

Which runs do you use most often? (Please circle).

GREEN Every time Usually Seldom Never

BLUE Every time Usually Seldom Never

RED Every time Usually Seldom Never

BLACK Every time Usually Seldom Never

FREERIDE Every time Usually Seldom Never

What kind of bike do you use?

BMX Rigid frame Hardtail Full Suspension Other

How many months / years experience of trail riding do you have?

What was the injury that you suffered while you were mountain biking?

Did the injury require an extended hospital stay? (This is anything longer than an overnight stay.)

How long has it been since the injury occurred (till the time of the provision of the blood sample)?

What is the projected recovery time for this injury?

How many times in the last 12 months have you had to go to Hospital with a Mountain biking injury?
