Sialic acids as biomarkers for cardiovascular disease

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Sialic acids are a family of over 50 derivatives of neuraminic acid that are widely available in Nature and that play many biological roles in health and disease. They are generally located at the terminus of glycans, which in turn form part of glycoproteins and glycolipids. *N*-Acetyl neuraminic acid (Neu5Ac), the most abundant sialic acid, has been hypothesised to be a biomarker for various diseases including cardiovascular disease (CVD). The presence of many diseases, including CVD can be linked to an increase in plasma and serum concentration of Neu5Ac in disease patients versus healthy controls. Increased Neu5Ac concentrations in plasma have also been linked to increased risk of CVD mortality. These studies have been limited in scope, studying only one derivative of sialic acid and only in plasma and serum. Multiple isomers of acetylated sialic acid exist, challenges have been encountered in synthesising these compounds however, which has hampered impact in this area. Given that Neu5Ac is associated with multiple diseases we need more precise biomarkers for diseases such as CVD. Therefore, the research described here aimed to investigate acetylated sialic acid derivatives as biomarkers for CVD and CVD mortality risk, not only in plasma and serum, but also in urine and saliva. Overall, the work carried out in this thesis details the utility of synthetic sialic acid derivatives as quantitative standards for the analysis of plasma, serum, urine, and saliva. The biomarker potential of Neu5Ac and Neu5,9Ac_2 for CVD risk and the presence of advanced CVD was also evaluated.

Chapter 1 A general introduction to sialic acid and sialoglycans is provided, demonstrating their importance within biological functions, and the role of sialic acid as a biomarker for cardiovascular disease, diabetes and cancer, as well as other inflammatory diseases. The synthesis of sialic acids and analysis of sialic acids in biological mixtures is also discussed.

Chapter 2 Since the discovery of sialic acid many assays have been developed for its qualitative and quantitative analysis. Many of these assays suffered from issues of sensitivity and specificity, more modern assays have been developed which overcome these challenges. Chapter 2 provides an in-depth review of a variety of assays for the analysis of sialic acid with a discussion of the advantages and disadvantages of different assay types (colorimetric, fluorometric, enzymatic, chromatographic). Many colorimetric and fluorometric assays are relatively quick, simple, and cheap to perform, they are inaccurate however, due to issues with sensitivity and specific. This has been overcome with more modern, mainly chromatographic, and enzymatic, assays that have high specificity for sialic acid and low limits of detection although they are more cumbersome to perform and require expensive equipment. This is followed by a future perspective on the analysis of sialic acids wherein the optimisation of

currently available assays to increase throughput is discussed, as well as development of plate-based assays to overcome challenges associated with chromatographic techniques.

Chapter 3 Sialic acid has been identified as a potential biomarker for many diseases with a large focus on cardiovascular disease, diabetes, and cancer. Chapter 3 provides insight into sialic acids as potential biomarkers for cardiovascular disease, diabetes and diabetic complications, and different types of cancer. The literature was reviewed for studies investigating sialic acid as a biomarker for these diseases after which the data from these studies was collated. The data included concentrations of Neu5Ac in disease cases and healthy controls as well as associated statistical significance. It was determined from the data reviewed that sialic acid shows potential as a biomarker for the presence of CVD, type-2 (but not type-1) diabetes and different types of cancer. However, the biomarker cannot discriminate between these different diseases and as such comorbid conditions may impact sialic acid concentration measurements and therefore reduce its biomarker potential. Utility was identified however in using sialic acid for the assessment of the severity of atherosclerotic diseases and the staging of malignant tumours, as well as the progression or success of treatments in a clinical setting.

Chapter 4 A distinct lack of quantitative standards of acetylated sialic acid derivatives has been identified, and these are key for the analysis of sialic acids in biological samples. Chapter 4 describes the synthesis of two acetylated derivatives of sialic acid: Neu4,5Ac_2 in four synthetic steps in 39% overall yield; Neu5,9Ac_2 in 1 synthetic step in 68% yield. Progress was also made towards the synthesis of Neu5,8Ac_2, Neu2,5Ac_2, Neu5,7Ac_2 and acetylated Neu5Gc derivatives. The two derivatives that were successfully synthesised were analysed using a QNMR technique to allow for their utilisation as quantitative standards. Concentrations of Neu5Ac, Neu5Gc, Neu5,9Ac_2 and Neu4,5Ac_2 in human plasma and guinea pig, porcine and ovine serum were determined. This research showcased the ability to label sialic acids with 1,2-diamino-4,5-methyleneoxybenzene (DMB) followed by analysis using liquid chromatography fluorescence detection. This could be utilised to effectively separate and analyse (both qualitatively and quantitatively) multiple sialic acid derivatives in one sample. The DMB method exhibited very low limits of detection and quantitation in line with the literature.

Chapter 5 Previous studies have investigated Neu5Ac as a potential biomarker for CVD risk, with elevated plasma Neu5Ac concentrations associated with increased CVD mortality risk. Information was collected from a cohort of volunteers who were at risk of cardiovascular disease or otherwise healthy. The volunteers were recruited *via* an ethically approved (UREC 18/39) study. The information was collected to determine the QRISK3 estimated relative risk

score for each volunteer. Plasma, serum, urine and saliva samples collected from the volunteers were subsequently analysed both qualitatively and quantitative for sialic acid derivatives whereby Neu5Ac and Neu5,9Ac_2 were identified. The concentrations of these derivatives in the biological samples were determined via the DMB labelling method. Following this, statistical analysis was performed to determine any potential associations between concentrations of sialic acids and QRISK3 estimated relative risk score, as well as CVD risk factors. Some associations were identified in women, but no associations were forthcoming in men. Evidence indicated that these sialic acid derivatives may be able to be utilised as markers for early CVD risk, but further studies are required to determine this.

Chapter 6 Neu5Ac has been widely researched and established as a potential biomarker for the presence of advanced CVD. Chapter 6 outlines the determination of the potential of Neu5Ac and Neu5,9Ac_2 as biomarkers for the presence of advanced CVD. Samples from thirty healthy controls and thirty patients with advanced CVD were purchased from BioIVT biobank. The samples were analysed for sialic acids whereby elevated concentrations of both Neu5Ac and Neu5,9Ac_2 were observed between CVD cases and healthy controls. The elevated concentrations were statistically significant (Neu5Ac: $P < 0.001$; Neu5,9Ac_2: $P < 0.04$) when comparing CVD cases and healthy controls although the P-value for Neu5,9Ac_2 was borderline significant. Further analysis *via* receiver operator curve analysis revealed the predictive power, sensitivity and specific of each marker. Neu5Ac was found to be a good predictor of advanced CVD with good specificity and sensitivity. Neu5,9Ac_2 performed similarly apart from very low specificity limiting its utility. A combination marker of Neu5Ac/Neu5,9Ac_2 however was found to offer similar predictive power as the individual markers but also much improved sensitivity and specificity.

Chapter 7 This chapter summarises the key findings of this project, provides critical evaluation of the work carried out and suggests potential avenues for future work. This thesis demonstrates the synthesis of two acetylated sialic acid derivatives using protecting group strategies. The utility of these derivatives as quantitative standards for the analysis of biological samples is assessed. Further to this, the quantitative standards prepared were used, along with sensitive chromatographic techniques to determine concentrations of sialic acids in plasma, serum, urine, and saliva. The concentrations determined were then utilised to establish to what extent whether Neu5Ac and Neu5,9Ac_2 can act as biomarkers for CVD and CVD risk.

List of publications

Assays for the identification and quantification of sialic acids: Challenges, opportunities and future perspectives. **J. Cheeseman**, G. Kuhnle, D. I. R. Spencer and H. M. I. Osborn, *Bioorganic Med. Chem.*, 2021, **30**, 115882. DOI: 10.1016/j.bmc.2020.115882

Sialic acid as a potential biomarker for cardiovascular disease, diabetes and cancer. **J. Cheeseman**, G. Kuhnle, G. Stafford, R. A. Gardner, D. I. Spencer and H. M. Osborn, *Biomark. Med.*, 2021, **15**, 911–928. DOI: 10.2217/bmm-2020-0776

Quantitative Standards of 4-*O* acetyl and 9-*O* acetyl *N*-acetyl Neuraminic Acid for the Analysis of Plasma and Serum. **J. Cheeseman**, C. Badia, R. I. Thomson, G. Kuhnle, R. A. Gardner, D. I. R. Spencer, H. M. I. Osborn *Accepted for publication in ChemBioChem, December 2021*. DOI: 10.1002/cbic.202100662

The evaluation of sialic acid and 9-*O*-acetyl sialic acid and their relationship to cardiovascular disease risk. **J. Cheeseman**, C. Badia, K. Jackson, R. A. Gardner, D. I. R. Spencer, H. M. I. Osborn, G. Kuhnle. *Submitted to PLOS One, December 2021*.

Elevated concentrations of Neu5Ac and Neu5,9Ac_2 in human plasma: Potential Biomarkers of Cardiovascular Disease. **J. Cheeseman**, C. Badia, G. Elgood-Hunt, R. A. Gardner, D. I. R. Spencer, G. Kuhnle, H. M. I. Osborn *Submitted to Scientific Reports, December 2021*.

List of conferences attended

Pharmacy PhD Showcase, 2021, University of Reading, UK, Oral Presentation: Sialic Acids as Biomarkers for Cardiovascular Disease. **J. Cheeseman**, D. I. R. Spencer, G. Kuhnle, H. M. I. Osborn.

RSC Carbohydrate ECR Webinar, Online Event, 2020

Eurocarb, Leiden, Netherlands, 2019, Poster Presentation: Sialic Acids as Biomarkers for Cardiovascular Disease. **J. Cheeseman**, D. I. R. Spencer, G. Kuhnle, H. M. I. Osborn.

M2M Science *Plus* Networking Event III, 2019, Thames Valley Science Park, UK.

Pharmacy PhD Showcase, 2019, University of Reading, UK, Oral Presentation: Sialic Acids as Biomarkers for Cardiovascular Disease. **J. Cheeseman**, D. I. R. Spencer, G. Kuhnle, H. M. I. Osborn. Awarded 'Best Second Year Presentation'.

RSC Carbohydrate Group Meeting, 2019, University of Reading, UK, Poster Presentation: Sialic Acids as Biomarkers for Cardiovascular Disease. **J. Cheeseman**, D. I. R. Spencer, G. Kuhnle, H. M. I. Osborn.

Pharmacy PhD Showcase, 2018, University of Reading, UK, Poster Presentation: Sialic Acid as a Biomarker for Cardiovascular Disease. **J. Cheeseman**, D. I. R. Spencer, G. Kuhnle, H. M. I. Osborn.

Table of Contents

Abbreviations

Asn	Asparagine
AUC	Area under the curve
BMI	Body mass index
BPH	Benign prostatic hypoplasia
BSA	Bound sialic acid
CAD	Coronary artery disease
Carotid IMT	Carotid intima media thickness
CEA	Carcinogenic embryonic antigen
CHD	Coronary heart disease
CHF	Coronary heart failure
CI	Confidence interval
COPD	Chronic obstructive pulmonary disorder
CRP	C-Reactive protein
CVD	Cardiovascular disease
d	Doublet
DMB	1,2-Diamino-4,5-methyleneoxybenzene
DMBA	4,5-Dimethylbenzene-1,2-diamine
Dol-P	Dolichol phosphate
ER	Endoplasmic reticulum
ERETIC	Electronic reference to access in vivo concentrations
ESI	Electro-spray ionisation
FSA	Free sialic acid
Gal-3	Galectin-3
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GlcNAc	*N*-Acetyl glucosamine
Golgi	Golgi apparatus
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HPLC	High performance liquid chromatography

HRMS	High resolution mass spectrometry
hs-CRP	High-sensitivity c-reactive protein
IR	Infra-red
J	Coupling constant
KDN	2-Keto-3-deoxy-D-glycero-D-galacto-nononic acid
LacNAc	N-*A*cetyl lactosamine
LC-FLD	Liquid chromatography-fluorescence detection
LC-MS	Liquid chromatography mass spectrometry
LDL	Low density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantitation
LSA	Lipid-bound sialic acid
m	Multiplet
m/z	Mass/charge ratio
MAG	Myelin-associated glycoprotein
Man	Mannose
ManNAc	*N*-Acetyl mannosamine
MI	Myocardial infarction
mp	Melting point
Neu5Ac	*N*-Acetyl neuraminic acid
Neu2,5Ac_2	5-Acetamido-3,5-dideoxy-2-*O*-acetyl-D-glycero-D-galactononulopyranosonate
Neu4,5Ac_2	5-Acetamido-3,5-dideoxy-4-*O*-acetyl-D-glycero-D-galactononulopyranosonate
Neu5,7Ac_2	5-Acetamido-3,5-dideoxy-7-*O*-acetyl-D-glycero-D-galactononulopyranosonate
Neu5,8Ac_2	5-Acetamido-3,5-dideoxy-8-*O*-acetyl-D-glycero-D-galactononulopyranosonate
Neu5,9Ac_2	5-Acetamido-3,5-dideoxy-9-*O*-acetyl-D-glycero-D-galactononulopyranosonate
Neu5Gc	*N*-Glycolyl neuraminic acid
NMR	Nuclear magnetic resonance
NT-proBNP	*N*-Terminal pro-brain natriuretic peptide

OSCC	Oral squamous cell carcinoma
PCa	Prostate cancer
ppm	Parts per million
PSA	Protein-bound sialic acid
p-TsOH	*para*-Toluene sulfonic acid
PULCON	Pulse length-based concentration determination
QNMR	Quantitative nuclear magnetic resonance
ROC	Receiver operator curve
s	Singlet
SBP	Systolic blood pressure
Ser/Thr	Serine/threonine
t	Triplet
T. cruzi	*Trypanosoma cruzi*
TACAs	Tumour associated carbohydrate antigens
TBDMS	*tert*-Butyldimethylsilyl
TBDMSCl	*tert*-Butyldimethylsilyl chloride
TFA	Trifluoroacetic acid
TSA	Total sialic acid
UHPLC	Ultra-high performance liquid chromatography
UREC	University of Reading ethics committee
VD	Vessel disease
WHO	World Health Organisation

Chapter 1:

General Introduction

1.1 Introduction

Sialic acids are a family of monosaccharides that are abundant in Nature and play many important biological roles in health and disease.[1] Overexpression of *N*-acetyl neuraminic acid (Neu5Ac, sialic acid) has been observed in biological fluids as a result of various diseases and as such is of interest as a potential biomarker.[2] One disease that has been widely studied is cardiovascular disease (CVD) with many studies evaluating associations between a diagnosis of CVD[3] or elevated CVD mortality risk[4,5] and Neu5Ac concentrations in plasma and serum. This thesis tested the hypothesis that acetylated Neu5Ac derivatives could act as biomarkers for CVD mortality risk and the presence of advanced CVD. Therefore, within this introductory chapter sialic acid, its bioavailability and its biological roles in health and disease are discussed. The qualitative and quantitative analysis of sialic acids is also covered herein as the ability to effectively measure concentrations of sialic acids in biological fluids is essential for their potential as biomarkers. Following this, the synthesis of acetylated Neu5Ac derivatives is discussed as access to these compounds is key to their utilisation as quantitative standards.

1.2 Glycans

Glycans are polysaccharide or oligosaccharide chains that form parts of glycoconjugates whereby a carbohydrate chain is covalently linked to a non-carbohydrate structure such as a protein or lipid. The synthesis of glycans is regulated by more than 200 enzymes, mainly glycosyltransferases and glycosidases.[6] Glycans are formed from a range of monosaccharide units which, when combined with different branching structures results in highly diverse structures.[7] Different linkages between monomer units, the addition of ‘capping’ units such as sialic acid, and fucosylation increases the diversity of these structures. Finally, functionalisation of the monomer units, such as through acetylation, increases this diversity further still.[8]

Glycans fit into two categories: *N*-linked glycans (*N*-glycans) and *O*-linked glycans (*O*-glycans). *N*-Glycans are covalently bound to asparagine (Asn) residues *via* an *N*-glycosidic bond. All eukaryotic *N*-glycans begin with an *N*-acetyl glucosamine unit and share a common core.[9] Three main types of *N*-glycan have been identified: oligomannose where only mannose residues are attached to the core; complex where ‘antennae’ are attached to the core; and hybrid which exhibits both features (Figure 1). *O*-Glycans generally begin with a *N*-acetyl galactosamine residue and are covalently linked to serine/threonine (Ser/Thr) residues via an

O-glycosidic bond. *O*-Glycans exhibit a wider range of common cores (Figure 2), these cores are much simpler in their structures, however.

N-Glycans and *O*-glycans can be modified by the addition of certain carbohydrate residues such as sialic acids which are generally located as the terminating unit of glycans. Sialic acids are added late in the stage of *N*-glycan synthesis,[8] with sialylated glycans containing one to four sialic acid residues, although not all *N*-glycans are sialylated.[10] *O*-Glycans once again differ from *N*-glycans in that sialic acids can be added at much earlier stages of synthesis which leads to simple sialylated glycans such as the sialyl Tn antigen.[11] If the biosynthesis is not blocked by sialylation, the core structures of *O*-glycans can undergo further manipulation to form more complex *O*-glycans. The addition of lactosamine repeat repeats which can be fucosylated and sialylated leads to a wide array of naturally occurring *O*-glycans.[9,12,13]

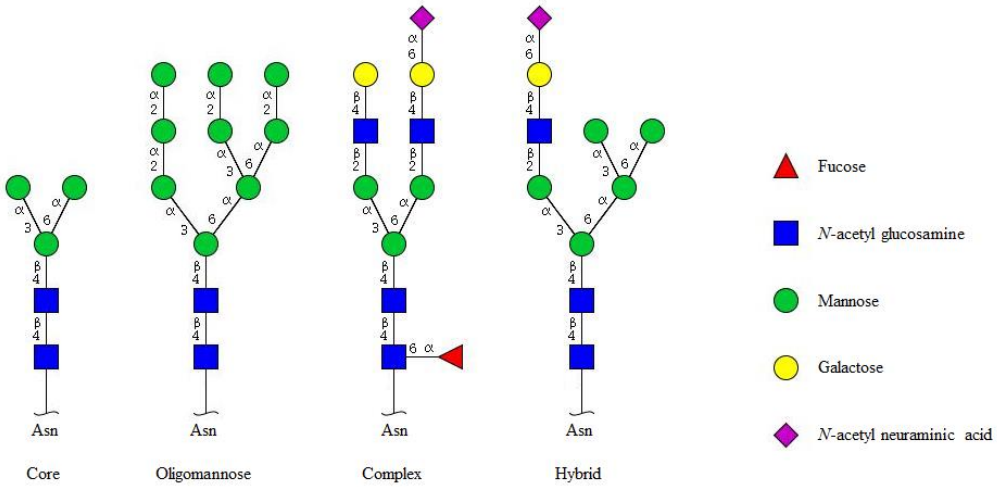


Figure 1: *N*-Glycan core structure as well as three glycan types: oliogimannoase, complex and hybrid.

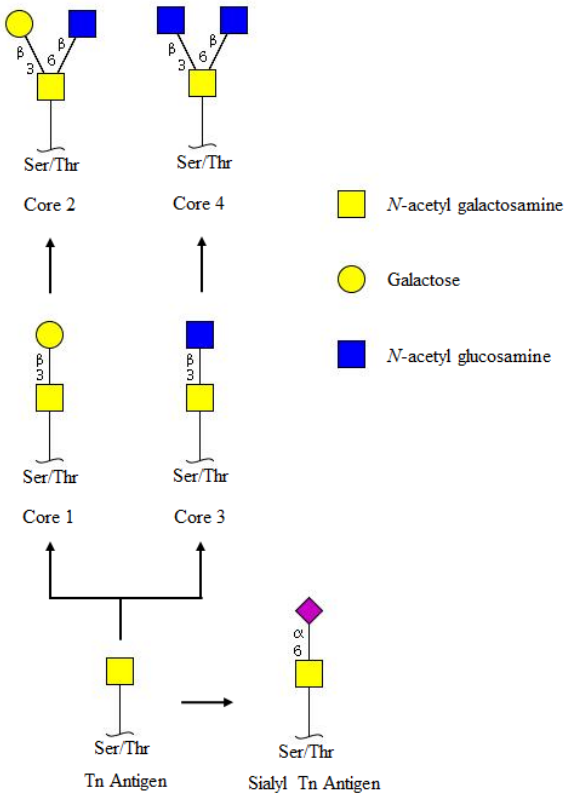


Figure 2: Core structures 1-4 of *O*-glycans formed from the Tn Antigen, also shows sialylated Tn antigen.

1.3 Sialic acid

N-Acetyl neuraminic acid (sialic acid) is a nine-carbon backbone acidic monosaccharide that was first isolated from bovine mucin in the 1930s.[9,14] The discovery of this compound sparked decades of research into its structure, bioavailability, and biological significance. Over the last 90 years, a family of over 50 neuraminic acids (sialic acids) have been identified.[15–17] Neuraminic acid (Neu) and 2-keto-3-deoxy-D-glycero-D-galatconononic acid (KDN), form the basis of most sialic acids (Figure 3).

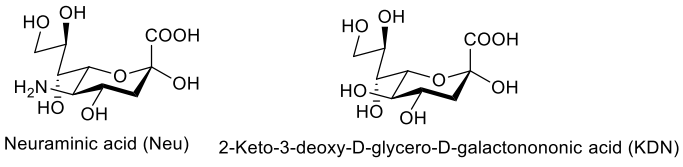


Figure 3: Neuraminic acid and KDN

Neuraminic acid is not found in its free form due to its instability. Rapid cyclisation of this monomer leads to the formation of a Schiff base.[18] However, KDN can be found in lower vertebrates such as fish.[19] Two of the most common and stable sialic acids in nature, both derived from neuraminic acid, are Neu5Ac and Neu5Gc (Figure 4). Although Neu5Ac is near ubiquitous in vertebrates it is only found in the higher invertebrates.[20] Neu5Ac is not found in plants and prokaryotes with one notable exception, some pathogenic bacteria exhibit sialic

acid on their cell surface.[21] Neu5Gc is particularly interesting in that humans lost the ability to synthesise it at a late stage of evolution, ~ 2 million years ago.[22] Neu5Gc is present in nearly all other vertebrates apart from a few exceptions such as new-world monkeys.

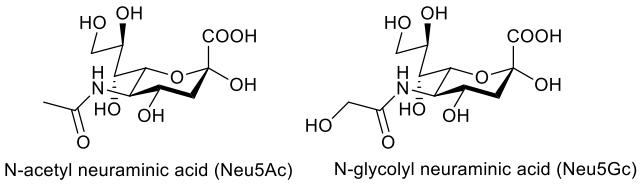


Figure 4: *N*-Acetyl neuraminic acid (Neu5Ac) and *N*-glycol neuraminic acid (Neu5Gc)

Sialic acid diversity comes from two main sources, the first of which is derivatisation of different sialic acids at the C-5 position (acetyl, glycolyl, hydroxyl) as well as at one or more hydroxyl positions (acetyl, methyl, sulfate, phosphate, lactyl) (Figure 5) with acetylated sialic acid derivatives the most abundantly found in nature.[15,16,23] Further to this, sialic acids are liable to form lactones and lactams, 2,3 unsaturated derivatives of sialic acids have also been identified.[17] The second level of diversity is derived from different linkages formed between the C-2 position of sialic acids and different carbohydrate frameworks (glycans).[9]

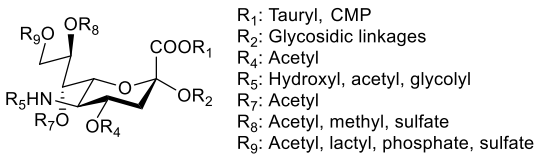


Figure 5: Naturally occurring substitutions of sialic acid

1.4 Biological functions of sialoglycans and sialic Acid

Sialoglycans, and by extension sialic acids play a large number of important biological functions in the structure and function of many cells and proteins.[24,25] These functions not only help with the regulation of normal cell function, but also aid in the protection of cells from infection by pathogens. [26,27] Sialic acids also appear to play roles in the presence and pathogenesis of various disease states, especially those associated with inflammatory diseases.[3,28,29] Given this information, it is not surprising that sialic acids are of great interest as a biomarker for various diseases. This topic is explored further in section 1.6. Some of the most vital biological functions of sialic acids, as well as how they are utilised by pathogens and other disease, are explored in subsequent sections.

1.4.1 Structural roles

The non-carbohydrate part of a glycoconjugate exhibits natural hydrophobicity and as such would usually suffer from issues of solubility in water.[25] Surface glycans and their terminal sialic acid units are responsible for the overall negative charge at the surface of glycoconjugates which aids with solubility in water.[1] The negative charge is also essential for the function of mucins, the hydrophilic capacity of the surface glycans leads to the binding of water which in turn leads to gelation.[30,31] The gelation observed results in the hydration and lubricating properties of mucins which help protect and ensure proper function of epithelial surfaces which are essential to key biological functions such as saliva production and swallowing.[32,33] Further to this, the negative charge is also important in other areas such as the prevention of the aggregation of red blood cells and erythrocytes by aiding in cell-cell repulsion.[34] Epithelial *O*-glycans are also important for maintaining the structure of mucins and preventing the mucin from folding into a globular structure.[35] The rigid *O*-glycan structure is formed by the binding of galectin-3 oligomers to epithelial cell membranes which forms a highly organised cell-surface barrier which can extend up to 300 nm from the cell membrane. This offers physical protection to epithelial cells by preventing potential infectious microorganisms from approaching target cells.[36,37] Sialoglycans also provide physical protection to proteins and cells from the action of proteases.[38]

1.4.2 Recognition of/masking by sialic acids

Sialic acid acts as a receptor determinant for numerous biological molecules such as lectins.[39] Sialic acid can also act as a receptor mask, by hiding the underlying glycan thus preventing binding.[34] One such example is found where terminal *O*-acetyl sialic acid can prevent the binding of influenza A and B viruses.[27] Sialic acid can also mask receptors on the cell surface such as siglecs in a cis-interaction to downregulate cell-cell trans-interactions.[40] This aids in the immune system recognising 'self' and thus not launching antibodies and causing autoimmune attack. The half-life and clearance of circulating cells and glycoproteins is also controlled by the presence of sialoglycans.[41] Sialic acid residues protect underlying galactose residues by preventing the binding of macrophages to these galactose residues. The binding of these macrophages would in turn lead to degradation and clearance such as in the case of desialylated platelets which have been exposed to the neuraminidase of pathogenic bacteria.[1,42]

1.4.2.1 Lectins

Sialic acid recognition is carried out by binding proteins belonging to a class known as lectins. Lectins are defined as proteins that preferentially bind to carbohydrate complexes found as part

of glycoconjugates.[43] Glycan binding by lectins is highly specific but the binding affinity is low, it is improved however by multivalent binding. Animal, plant, and microbial lectins have been identified and more specifically sub-types of lectins have been identified that specifically bind to sialylated structures.

1.4.2.2 Selectins

Selectins are a subgroup of the Ca^{2+} dependent C-type lectins.[44] Three selectins have been identified: L-selectin, E-selectin and P-selectin. L-selectin is expressed in all leukocytes; E-selectin is expressed in activated endothelial cells; and P-selectin is expressed in platelet granules and endothelial cells.[9] The selectins are released during an inflammatory response and play roles in responding to tissue injury and inflammation. Selectins modulate cell-cell interactions with L-selectin involved in leukocyte-leukocyte interactions and all three selectins involved in leukocyte-endothelial cell interactions.[45] The interactions of the selectins aid in recruiting leukocytes to injury sites by promoting leukocyte rolling on and adhesion to the endothelium.[45,46] Selectins bind to a number of sialylated carbohydrate structures, among these some notable examples are sialyl-Lewis^x, its mimic sialyl-Lewis^a epitopes (Figure 6) as well as sulfonated derivatives of these structures.[47,48]

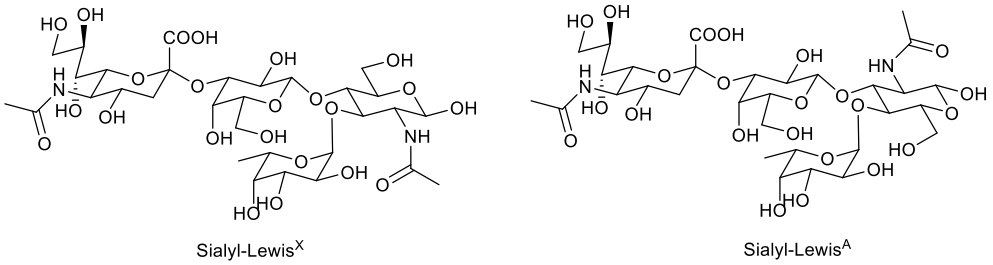


Figure 6: Structures of sialyl-Lewis^x and sialyl-Lewis^a epitopes

1.4.2.3 Siglecs

Sialic acid recognising immunoglobulin superfamily lectins, or Siglecs, are a family comprised of eleven lectins that recognise sialic acid as a ligand that are primarily expressed by immune cells.[40] They can be split into two groups: the first group contains sialoadhesin (Siglec-1), CD22 (Siglec-2), MAG (Siglec-4) and Siglec-15; the second group contains CD33 and related siglecs (Siglecs-5-11 and Siglec-14). Each siglec differs in its binding preferences, binding to sialyl-Lewis^x structures as well as other sialylated structures. Most siglecs can bind to both α-2,3 and α-2,6 structures with a preference for α-2,6 structures. Some siglecs cannot bind at all to α-2,3 linkages. Siglec activity is not only regulated by sialic acid linkage but also the sialic acid residue present on the sialoglycan. The presence of *O*-acetyl sialic acid residues interrupts binding of siglecs.[49,50] Siglec interactions are modulated by cis interactions with sialoglycan

ligands expressed on the same cell as the siglec. This downregulates but does not prevent trans interactions with other cells because it has been shown that high affinity ligands can outcompete cis ligands.[51] The siglec binding sites can also be exposed by the action of sialidase to remove terminal sialic acid residues from glycans, or by cellular activation downregulating cis interactions of siglecs. The biological roles of siglecs are summarised in Table 1:

Siglec	Functions	Occurrence	Ref
Sialoadhesin (Siglec-1)	1. Regulation of the function and survival of B-cells. 2. Removal of pathogens.	Macrophages	[40]
CD22 (Siglec-2)	1. Regulation of B-cell immune response, migration and signalling threshold.	B-cells	[52]
MAG (Siglec-4)	1. Promoting differentiation, maintenance and survival of oligodendrocytes. 2. Inhibits neurite growth.	Myelin sheaths	[53]
Siglec-15	1. Regulation of the immune system through activating processes	Macrophages and dendritic cells	[54]
CD33 related Siglecs	1. Regulation of the immune system through inhibitory processes	Haematopoietic cells	[55]

Table 1: Occurrence and biological functions of human siglecs.

1.5 Roles of sialic acid in infection/disease

Given the prevalence of sialic acid on the cell surface, it is not surprising that it is utilised by pathogenic bacteria, viruses, and parasites as a route to infection. Host systems require sialoglycans on cell surfaces for critical biological functions and pathogens use the prominent position of sialic acid as a binding site to mediate binding, cell entry and subsequent infection.

1.5.1 Bacterial infection

Pathogenic bacteria use sialic acid in two main ways. The first is for the key role of immune system evasion, by utilising sialic acid such that the immune system is not activated by the presence of the bacteria.[56] In this case sialic acid can be synthesised *de novo* by the bacteria such as in *E.coli*[57] or can be scavenged from the host system whereby bacteria secrete sialidases that release sialic acid from cell surface sialoglycans.[58] Some bacteria have been observed to not excrete sialidases but still exhibit cell surface sialic acid. This may be linked to the release of monomeric sialic acid by sialidases excreted by other pathogens or by the host itself in response to inflammation and infection, which may facilitate the uptake of sialic by these bacteria. Some bacteria have also been shown to utilise sialic acid as an energy source.[21]

1.5.2 Parasitic infection

Trypanosoma cruzi (*T. cruzi*) has been shown to exhibit *trans*-sialidase activity, meaning it has the ability to not only cleave host cell sialic acid but also add sialic acid to underlying carbohydrate frameworks through glycosidic linkages.[59] *T. cruzi* scavenges sialic acid similar to some bacteria and incorporates it into its surface to avoid host immune system detection and clearance. The expression of *trans*-sialidase also appears to affect host sialylation – *T. cruzi* infection is characterised by thrombocytopenia which may be a result of increased clearance of platelets because they have been desialylated by *trans*-sialidase expression.[60] Sialic acid is also of paramount importance for infection by *T. cruzi,* desialylated cells showed reduced levels of infection.[61] In comparison to this, desialylation of sialoglycans appears to play a key role in escape of the parasite from the cytoplasm of infected cells.[62]

1.5.3 Viral infection

Numerous viruses make use of sialic acid as a binding site for cell entry, the first stage in cell infection by a virus. The most well documented case of this is influenza, although his has been observed in noro- and rotaviruses.[63] Influenza A and B virus infect humans by attaching to and degrading α-2,6 linked Neu5Ac.[64] On the other hand, avian influenza viruses preferentially bind to α-2,3 linked Neu5Ac.[65] Other derivatives of sialic acid are also of interest here with 9-*O*-acetyl sialic acid (Neu5,9Ac_2) being the preferred binding site for influenza C as well as some human coronaviruses.[66,67] Interestingly, Neu5,9Ac_2 disrupts the binding of influenza A and B. Some viruses are also able to secrete neuraminidase which destroys surface sialic acids on glycoproteins promoting virus release from infected cells and neutralisation of sialic acid containing proteins that interfere with virus binding.[68] Of much more recent interest is the potential role that sialic acids play in infection by coronaviruses,

especially SARS-COV-2. Evidence points towards specificity for SARS-COV-2 to bind to Neu5Ac containing glycans,[69] with reduced binding to glycans containing Neu5Gc.[70] There is also evidence that shows, similarly to other human coronaviruses, that SARS-COV-2 binds to acetylated sialic acid derivatives, specifically Neu5,9Ac_2 and Neu4,5Ac_2.[71,72]

1.5.4 Cancer

Malignant tumours and cancer cells have been shown to decorate their surface with sialylated glycoproteins that express high numbers of tumour associated carbohydrate antigens (TACAs) and sialyl Lewis structures.[73] The terminal sialic acid residues hide the underlying galactose residues which prevents degradation and apoptosis of cancer cells.[74] Metastasis is also supported by these glycans. Loose cancer cells can escape into the blood stream to circulate throughout the body without the risk of clearance as they display a highly sialylated structure.[75] Overexpression of glycolipids such as GD2 and GD3 has also been observed in various types of cancer wherein it promotes cancer cell survival by inhibiting the immune system[76] and reduces cancer cell sensitivity to apoptosis and as such may play a role in cancer cell survival.[77] On the other hand, reduction of *O*-acetylation can also have undesirable outcomes for cancer cell progression. Reduction of *O*-acetylation appears to promote inflammation, especially in mucosal membranes, and may promote tumour growth such as the case of colorectal cancer.

1.5 Cardiovascular disease

While sialic acid has been studied in relation to numerous disease states, the focus of the researched detailed in this thesis focuses on CVD and associated risk factors. CVD is an umbrella term for a number of interlinked health conditions affecting the heart and blood vessels that include coronary heart disease (CHD), cerebrovascular diseases, rheumatic heart disease and venous thromboembolism.[78] CVD accounted for 34% of all deaths in 2020 with an estimated 500 million active cases.[79] CVD is the leading cause of death in most developed and developing countries and is the leading cause of disability-adjusted life years. The prevalence and mortality of CVD is expected to increase as the risk-factors for CVD (smoking, sedentary lifestyle, access to high salt and fat foods) increase globally especially in developing countries. CVD risk is also greatly increased if a person suffers from high-risk health conditions such as: high blood pressure, high cholesterol, atrial fibrillation and type-2 diabetes.[80]

The World Health Organisation (WHO) estimates that up to 80% of CVD is preventable and reduction in risk factors for CVD can reduce the global health burden and associated costs.[81] Early diagnosis of CVD is therefore of paramount importance for reducing mortality and

prevalence of CVD. Advances in biomarker and CVD research have led to improved clinical outcomes over the last 30 years.[82] Risk calculation algorithms have been developed such as QRISK3 which allows for the estimation of 10-year risk of developing heart attack or stroke.[83]

1.6 Biomarkers

A biomarker has been defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. An ideal biomarker must be able to: measure a specific pathology, add to clinical assessment, be acceptable to the patient, applicable to all sexes, ages and ethnicities, be easy to interpret, and have high sensitivity and specificity.[84,85] Many biomarkers are currently employed in diagnostic settings to aid in the detection and diagnosis of a wide range of diseases, for example: troponin for myocardial infarction, CEA for many types of cancer, and HbA1c for diabetes.

1.6.1 Sialic Acids as biomarkers

Sialic acid has been investigated as a biomarker in many studies over the last 30 years. Elevated concentrations of Neu5Ac in biological fluids such as plasma and serum have been associated with the presence and pathogenesis of a number of health conditions. These include: osteoarthritis,[86] sepsis[28] and COPD,[87] as well as diabetes and cancer which are discussed in greater detail in Chapter 3.[2,3,88–90] Many of these conditions are associated with, or lead to, inflammatory processes taking place, which may explain this overexpression of sialic acid and therefore its potential as a biomarker. This is discussed in greater detail in Chapters 5 and 6. Neu5Ac is not the only sialic acid derivative associated with these processes however, acetylated sialic acids are becoming of greater interest as biomarkers. This is due to their association with certain disease states, such as overexpression on the surface of cancer cells[91]. These sialic acid derivatives may also be of interest in relation to other health conditions, such as CVD.

1.6.2 Sialic acid as a biomarker for CVD

Numerous biomarkers have been studied and established for CVD; some are disease specific such as *N*-terminal pro-brain natriuretic peptide (NT-proBNP) or are related to a specific pathological process such as inflammation in the case of fibrinogen.[92] Sialic acid has been of interest in recent years as a marker for CVD and the associated inflammatory processes. It has been well established as a potential marker for the presence and pathogenesis of different cardiovascular diseases (coronary heart disease, coronary artery disease, atherosclerosis, hypertension) and associated inflammatory processes.[89,93–95] Elevated concentrations of

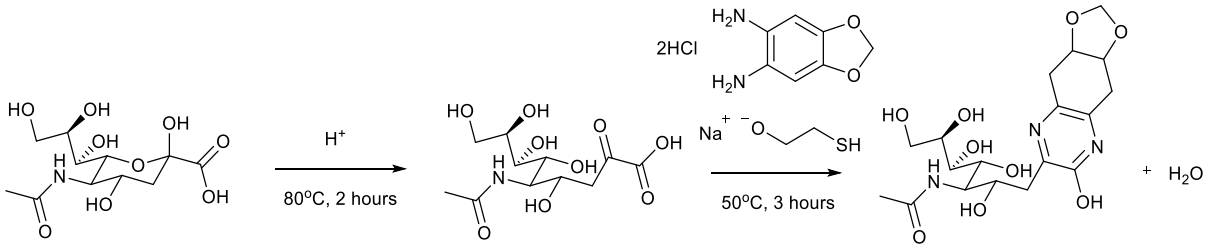
plasma sialic acid have been linked with an increase in cardiovascular mortality risk.[88] Chapter 3 provides a more detailed overview of sialic acid as a potential biomarker for cardiovascular disease.[2]

1.7 Analysis of biomarkers

Biomarker analysis requires robust methods that can effectively quantify specific compounds in complex biological mixtures. This means the method must be specific for the target compound so as not to produce inaccurate results by measuring interfering compounds. The methods must also be highly sensitive as some biomarkers are present in only small quantities.

1.7.1 1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) assay

In the context of this research, the DMB assay was utilised for the analysis of sialic acid. The DMB assay is a rapid, highly sensitive and highly selective method for the analysis of sialic acids (Scheme 1).[99,100] The method is performed by first releasing sialic acids from glycosidic linkages using mild acidic conditions, this facilitates glycosidic bond cleavage but is sufficiently mild to hamper acetyl group migration. Following acidic release, the sialic acids are labelled with the DMB fluorescent tag and subsequently analysed by liquid chromatography-fluorescence detection (LC-FLD). The method also allows for the differentiation and quantitation of multiple sialic acid derivatives simultaneously, such as *O*-acetylated sialic acid, thus reducing the need for multiple assays. More detail on this assay can be found in chapters 3, 4, 5 and 6.



Scheme 1: Acid release and DMB labelling of sialic acids

1.8 Synthesis of sialic acids

Quantitative analysis of sialic acids requires access to quantitative standards which must be obtained from nature or synthesised. Sialic acid can be isolated from edible birds nest.[101] Prior to this discovery, sialic acid was generally synthesised from compounds such as *N*-acetyl-D-glucosamine.[102] Enzymatic methods have also been employed for the synthesis of sialic acid.[103]. The ability to obtain large quantities of sialic acid opened doors for the chemical synthesis of natural and unnatural derivatives of sialic acid including acetylated sialic acid derivatives. This is of interest as while a variety of acetylated sialic acids can be isolated from

bovine submaxillary mucin[104], both the acidic release conditions and basic purification conditions can result in acetyl group migration and cleavage thus affecting the quantity of sialic acids in these mixtures.

Neu5Ac, Neu5Gc, Neu5,9Ac_2 and Neu4,5Ac_2 are the only sialic acids that are commercially available.[105–108] However, Neu5,9Ac_2 and Neu4,5Ac_2 are only available in small quantities at a high cost. As such, given the lack of availability of acetylated sialic acids, difficulty of isolating these derivatives from biological sources and the availability of Neu5Ac in large quantities from commercial sources, the synthesis of both natural and unnatural sialic acid derivatives has attracted much research in recent years.[16,109]

1.8.1 Synthesis of *O*-acetylated sialic acid derivatives

The work carried out in this thesis aimed to expand the scope of current research into sialic acids as biomarkers by accessing acetylated derivatives for use as standards. Synthesis of pure *O*-acetylated sialic acid derivatives requires the use of protecting group strategies to facilitate regioselective addition of acetyl groups. Care must be taken when adding and removing protecting groups especially when taking into account the lability of acetyl groups under basic conditions and the tendency for acetyl group migration under harsh acidic conditions.[110] More detail on the synthesis of acetylated Neu5Ac derivatives can be found in Chapter 4.[111]

1.9 Project overview

Cardiovascular disease is a highly prevalent disease responsible for the largest loss of disability-adjusted life years globally. Prediction of CVD is currently carried out using risk calculation algorithms such as QRISK3, although these can be unreliable in older populations.[112] A biomarker for CVD risk may supplement QRISK3 to allow for more accurate early prediction of CVD and allow for earlier intervention to reduce CVD prevalence. Most current analyses for sialic acid as a biomarker for CVD measure total sialic acid concentrations in plasma or serum, this research expanded this to include urine and saliva. This thesis also aimed to focus on different isomers of sialic acid by performing analysis using DMB labelling techniques which can separate different sialic acids in complex biological mixtures. It has been noted however that there is a lack of commercially available sialic acids for use as quantitative standards. Therefore, the overall aim of this project was to synthesise acetylated sialic acid derivatives for use as quantitative standards, and subsequently utilise these for the evaluation of the biomarker potential of these compounds. Analysis of these sialic acid derivatives in biological samples would be carried out to assess potential relationships between sialic acid concentrations and CVD, as well as CVD risk.

The general introduction is supplemented by two review articles in chapters 2 and 3. Chapter 2 provides a critical appraisal of numerous assays for sialic acid and its derivatives as well as advantages and disadvantages of each method with a comparison of different methods. This was carried out to give an overview of which methods for the analysis of sialic acids are best suited to different situations. Chapter 3 provides detail on the current state of research into sialic acid as a biomarker for CVD, diabetes and cancer. Data from the literature was collated and analysed to determine whether sialic acid concentrations were associated with different disease states. Chapter 3 also provides a critical appraisal of the utility of sialic acid as a biomarker for these diseases as well as potential future uses in areas such as diagnosis of disease severity and monitoring of treatments. Chapter 4 outlines the synthesis of two *O*-acetylated sialic acids (Neu4,5Ac_2 and Neu5,9Ac_2) employing protecting group strategies to perform regioselective acylation. Thereafter, the derivatives synthesised were analysed using quantitative NMR to allow for the accurate dispensing of standard samples for the preparation of standard curves. The utility of these compounds as quantitative standards was then demonstrated by the quantification of sialic acids in plasma and serum samples. Further synthetic work towards Neu5,8Ac_2, Neu2,5Ac_2, Neu5,7Ac_2 and acetylated derivatives of Neu5Gc is also outlined in Chapter 4. Chapters 5 and 6 used the standards developed for the analysis of sialic acid derivatives in biological samples. This was in order to assess the biomarker potential of sialic acid and its derivatives. Chapter 5 involves the recruitment of a cohort of volunteers who were either at risk of CVD or were otherwise healthy. This was then followed by the collection of biological samples (plasma, serum, urine, and saliva) and information for the measurement of QRISK3 estimated relative risk score. The samples collected were analysed to quantify the concentrations of Neu5Ac and Neu5,9Ac_2 in plasma, serum, urine, and saliva. These were compared with QRISK3 score and its constituent factors. Associations were observed in women between urinary Neu5Ac and QRISK3 estimated relative risk score, and urinary Neu5,9Ac_2 QRISK3 estimated relative risk score. Sensitivity analysis showed BMI as a potential driving factor behind this association. Inflammation linked physiological changes were posited as a possible explanation for these findings. Chapter 6 details the utilisation of the same sialic acid standards for the assessment of their potential as biomarkers for the presence of advanced cardiovascular disease. This was performed using a cohort of samples collected from healthy controls and patients diagnosed with advanced CVD. Elevated concentrations of plasma Neu5Ac, and Neu5,9Ac_2 were observed and the statistical significance of this was evaluated. Further to this, the predictive power of each marker was analysed using ROC analysis. Chapter 7 provides a summary of the key findings of this project followed by critical analysis of the work carried out. This is further supplemented by an

exploration of the potential future work for this project. The overview of this thesis is presented in Figure 7.

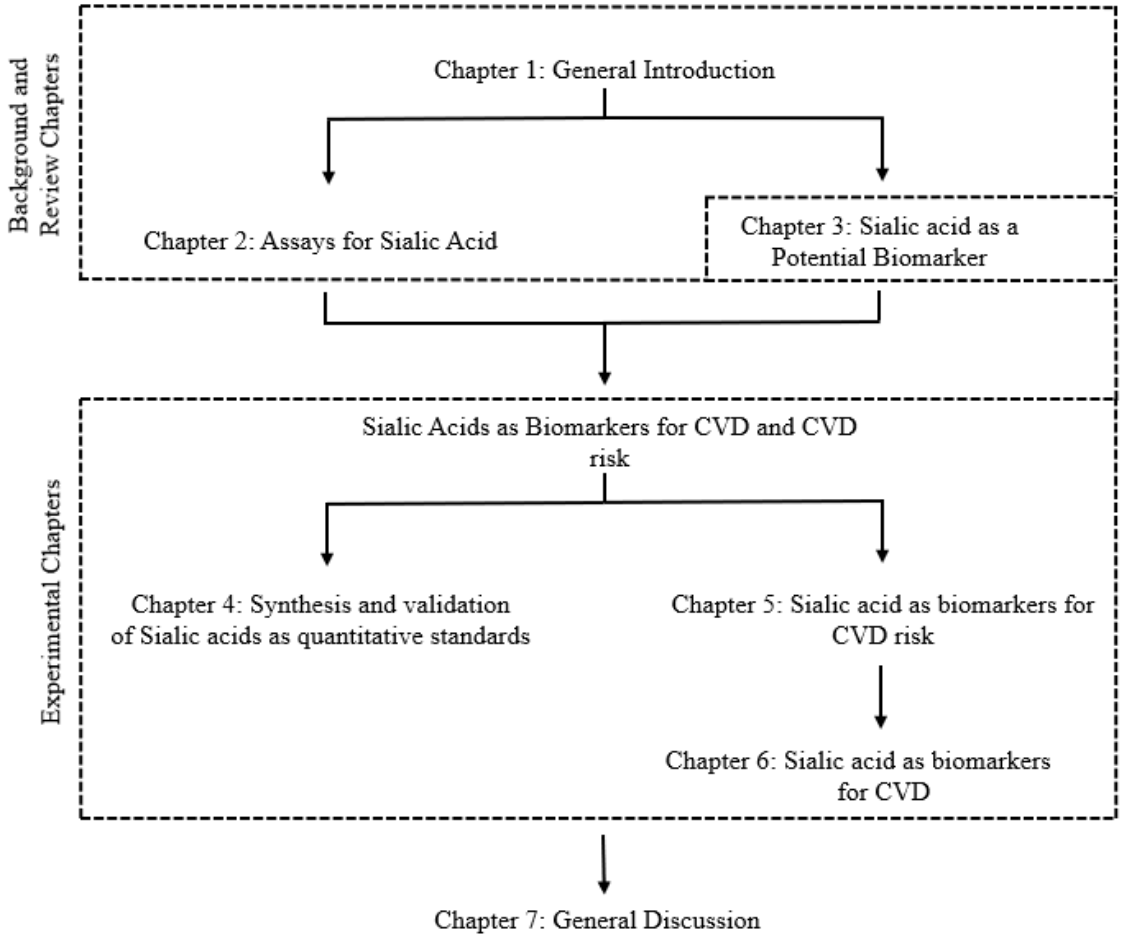


Figure 7: Thesis overview

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Chapter 2:

Assays for the identification and quantification of sialic acids: Challenges, opportunities and future perspectives.

Chapter Summary: This chapter explores the past and present literature with regards to assays for the qualitative and quantitative analysis of sialic acids. The assays are compared and critically evaluated to give an overview of the best assays currently available for the analysis of sialic acids. In addition, a future perspective on sialic acid analysis is provided.

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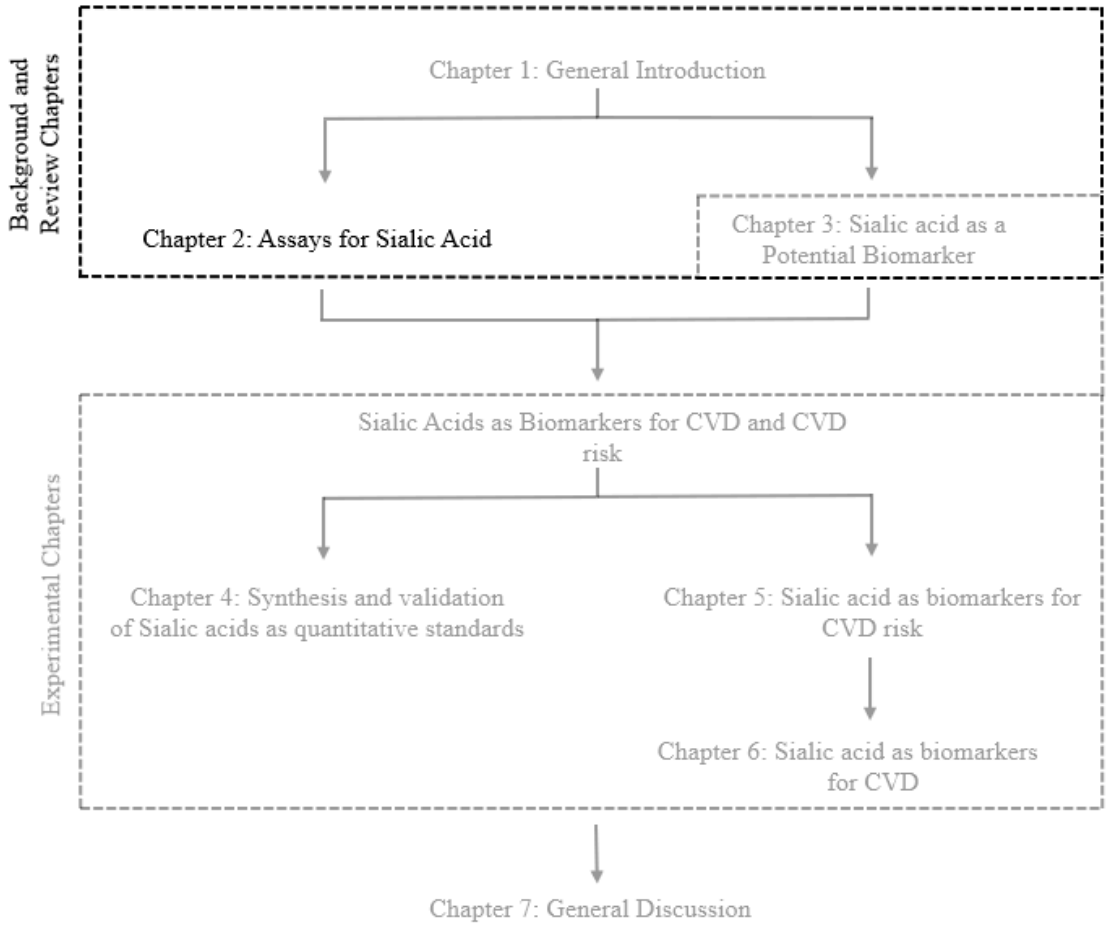

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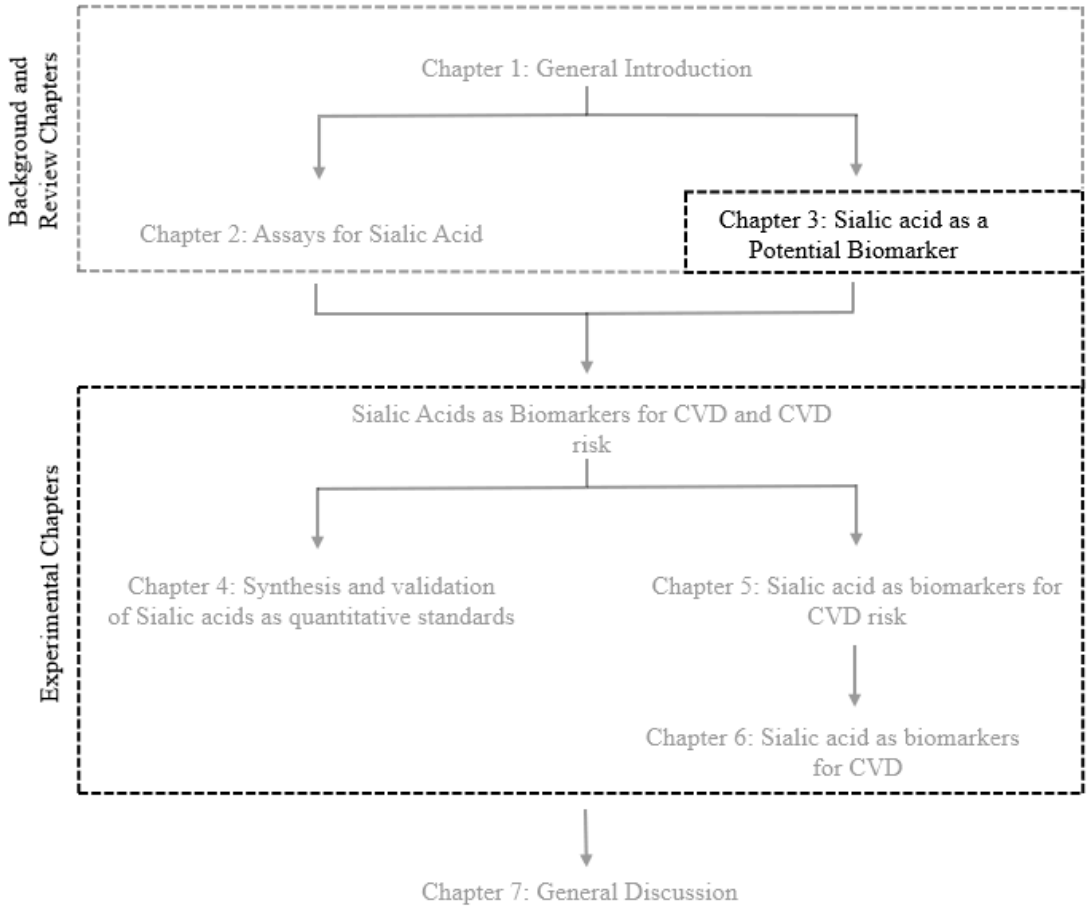
Chapter 3:

Sialic acid as a potential biomarker for cardiovascular disease, diabetes and cancer.

Chapter Summary: This chapter evaluates the data presented in the literature to provide an overview of sialic acid as a potential biomarker for cardiovascular disease, diabetes and cancer. The current and future utility of sialic acid as a biomarker not only for disease diagnosis, but also disease severity and treatment outcomes are discussed.

Bibliographic Details: Sialic acid as a potential biomarker for cardiovascular disease, diabetes, and cancer. **J. Cheeseman**, G. Kuhnle, G. Stafford, R. A. Gardner, D. I. Spencer and H. M. Osborn, *Biomark. Med.*, 2021, **15**, 911–928. DOI: 10.2217/bmm-2020-0776

Author Contributions: D.I.R.S, G.K and H.M.I.O designed the study, won funding for the programme and supervised the study. J.C performed the literature review, collated, and analysed all data. J.C. wrote the first draft of the main manuscript text apart from the section covering 'Oral Cancer' which was prepared by G.S. G.C prepared figures 4 and 5, J.C prepared all other figures. The manuscript was reviewed by all authors and J.C prepared the final draft for submission.



Appendix 1: References in the style of the Future Medicine journals

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Chapter 4:

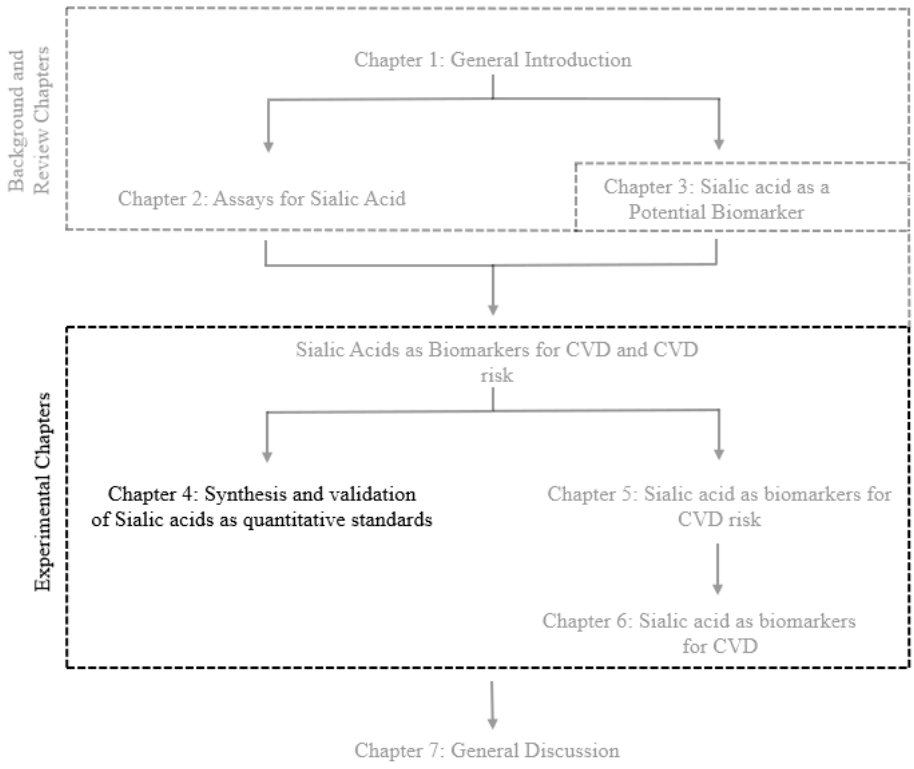
Quantitative Standards of 4-*O* acetyl and 9-*O* acetyl *N*-acetyl Neuraminic Acid for the Analysis of Plasma and Serum.

Chapter Summary: In this chapter, the synthesis of two acetylated sialic acid derivatives is described. This is followed by their utilisation as quantitative standards for their analysis in plasma and serum. The derivatives were successfully utilised as standards for the quantitative analysis of human plasma and animal serum samples. The method employed and developed upon allowed for the analysis of multiple sialic acid derivatives at once with very high sensitivity and specificity.

Bibliographic Details: Quantitative Standards of 4-*O* acetyl and 9-*O* acetyl *N*-acetyl Neuraminic Acid for the Analysis of Plasma and Serum. **J. Cheeseman**, C. Badia, R. I. Thomson, G. Kuhnle, R. A. Gardner, D. I. R. Spencer, H. M. I. Osborn *Accepted for publication in ChemBioChem, December 2021*. DOI: 10.1002/cbic.202100662

Author Contributions: D.I.R.S, G.K and H.M.I.O designed the study, won funding for the programme and supervised the study. J.C. designed the synthesis with the assistance of R.I.T and H.M.I.O. J.C performed synthesis and analysis of the samples with the assistance of C.B and R.G. Wider data analysis was performed by J.C. J.C prepared the first draft of the main manuscript text and prepared all figures. The manuscript was reviewed by all authors and J.C prepared the final draft for submission.

Appendices: Appendix 1 details further synthetic work outside the scope of the prepared journal article.



Quantitative Standards of 4-*O* acetyl and 9-*O* acetyl *N*-acetyl Neuraminic Acid for the Analysis of Plasma and Serum

Abstract

N-Acetyl neuraminic acid (sialic acid, Neu5Ac) is one of a large, diverse family of nine-carbon monosaccharides that play roles in many biological functions such as immune response. Neu5Ac has previously been identified as a potential biomarker for the presence and pathogenesis of cardiovascular disease (CVD), diabetes and cancer. More recent research has highlighted acetylated sialic acid derivatives, specifically Neu5,9Ac_2, as biomarkers for oral and breast cancers, but advances in analysis have been hampered due to a lack of commercially available quantitative standards. We report here the synthesis of 9-*O*-acetyl and 4-*O*-acetyl sialic acids (Neu5,9Ac_2 and Neu4,5Ac_2) with optimisation of previously reported synthetic routes. Neu5,9Ac_2 was synthesised in 1 step with a 68% yield. Neu4,5Ac_2 was synthesised in 4 steps with a 39% overall yield. Synthesis was followed by analysis of these standards via quantitative NMR (QNMR). Their utilisation for the identification and quantification of specific acetylated sialic acid derivatives in biological samples is also demonstrated.

Introduction

Neu5Ac is a nine-carbon backbone monosaccharide with a carboxylic acid functional group (Figure 1) and is one of a family of over fifty neuraminic acids (sialic acids).[1] Structural diversity arises from functionalisation at the 5-position (acetyl, glycolyl, lactyl, alcohol groups) as well as at one or more of the five hydroxyl positions (acetyl, methyl, phosphate, sulfate).[2] Further to this, glycosidic bonds between the 2-position of sialic acids and different carbohydrate frameworks allow for the creation of a range of different sialic acid-containing glycans. Lastly, different linkages of sialic acid (α-2,3, α-2,6, α-2,8, α-2,9) increase this diversity further still.[3]

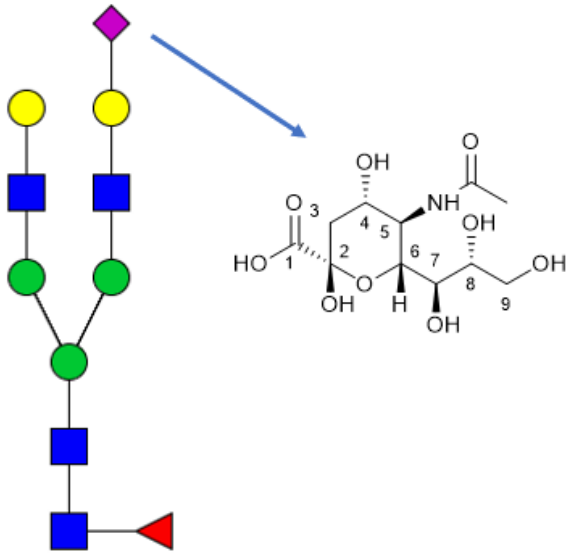


Figure 1: *N*-glycan and *N*-Acetyl neuraminic acid as the terminating unit

Sialic acids are generally located on *N*-glycans as the terminating unit (Figure 1),[4] forming parts of glycoconjugates such as glycoproteins and glycolipids. Sialic acids can also be found as internal residues of these glycans, particularly when an α-2,8 linkage is present, and as part of polysialic acid chains.[5] In far smaller quantities, free or unbound Neu5Ac is present in biological fluids. In primates and other mammals, the two major species found are Neu5Ac and Neu5Gc (Neu5Gc is not naturally produced in humans). Small quantities of other derivatives of Neu5Ac have been identified, for example Neu5,9Ac_2[6] in humans and a wider variety of sialic acids, including Neu4,5Ac_2, in other species.[6,7]

Neu5Ac has many important biological functions in humans including modulation of the immune system[8] and, due to the overall negative charge of the molecule, prevention of erythrocyte aggregation and thereby clotting.[9] Cancer cells also exhibit this characteristic by overexpressing cell surface Neu5Ac to avoid immune system detection and act as a biological mask, behaviour that aids cancer cell proliferation and promotes angiogenesis.[10] Neu5Ac is not the only neuraminic acid of biological importance. Neu5,9Ac_2 has also been indicated to play roles in modulating the immune system and stability of glycoproteins. Possible roles in cancer development, autoimmune conditions, and infection have also been articulated.[11] These roles are owed to different characteristics such as increased hydrophobicity, size and hydrogen bonding compared to Neu5Ac due to the presence of the additional acetyl functional group.[12] Expression of Neu5,9Ac_2 has also been observed in cancer cells.[13] Neu4,5Ac_2 is only expressed in certain vertebrates such as monotremes,[14] guinea pigs[15] and horses[16] where it plays roles in disrupting bacterial and viral activity. The disruption has been posited

to occur due to steric hindrance in binding sites posed by the protruding 4-position acetyl group present in Neu4,5Ac_2.[14,16]

Neu5Ac has previously been identified as a biomarker for cancer, diabetes and cardiovascular diseases.[17] Overexpression of sialic acid has been detected on the endothelium when CVD and therefore inflammation is present. There is a significant elevation in sialic acid in plasma and serum samples between healthy controls and disease patients. Sialic acid can be correlated with CVD risk, with volunteers that exhibit elevated sialic acid levels having higher mortality risk from CVD in a large scale long term follow-up study.[18] Neu5,9Ac_2 has also emerged as a potential marker for malignant tumours due to overexpression on the tumour surface.[13] The wide range of biologically available neuraminic acids and the array of biological roles they play, especially in immune response and infection, highlights the need for access to a broad range of sialic acid standards in order to probe their potential utility as biomarkers in a number of therapeutic areas.

Determining whether acetylated sialic acid derivatives are potential biomarkers for a therapeutic area of interest requires a high degree of method stability. Moreover, the reproducibility and reliability of such techniques would benefit from quantitative standards to measure the absolute quantitation of sialic acids in biological fluids. Neu5Ac and Neu5Gc are commercially available, but this is not the case for acetylated sialic acid derivatives. Currently the acetylated forms are derived from biological sources by performing acid release on readily available proteins such as bovine submaxillary mucin which has relatively high levels of a variety of sialic acid types.[19] The acid release of these sialic acids using 0.5 M formic acid and subsequent purification under basic conditions can cause challenges due to acetyl group migration with the former, and hydrolysis with the latter, conditions. This method also only results in a mixture of sialic acids that can be difficult to separate on a large scale due to the similarity in the polarities of the sialic acid derivatives. These mixtures can be useful for qualitative analysis and potential assignment of peaks. Individual standards are required, however, for more accurate assignment and quantitative analysis of sialic acids in biological samples.

To address the need for pure, well-characterised quantitative standards we report herein the further development and optimisation of previously reported synthetic routes towards two quantitative standards of acetylated sialic acid: Neu5,9Ac_2 and Neu4,5Ac_2 (Figure 2).[20] Then, to probe the utility of these synthetic derivatives as effective quantitative standards, quantitative nuclear magnetic resonance (QNMR) techniques were completed to determine the purity of the synthesised compounds. Finally, the synthetic compounds were used as qualitative

and quantitative standards for the analysis of sialic acids in biological samples derived from both humans and other species.

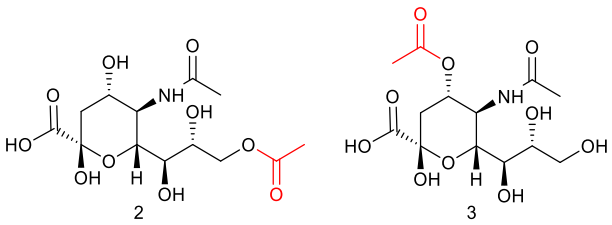
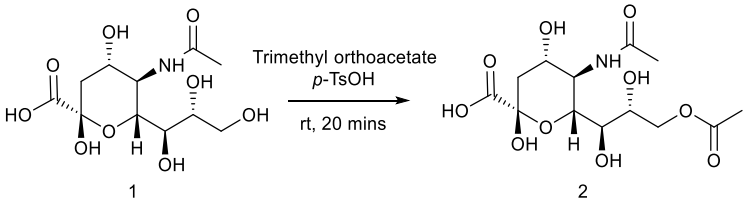


Figure 2: Neu5,9Ac$_2$ **(2)** and Neu4,5Ac$_2$ **(3)** with acetyl groups highlight in red.

Results and Discussion

Synthesis of Neu5,9Ac$_2$ and Neu4,5Ac$_2$

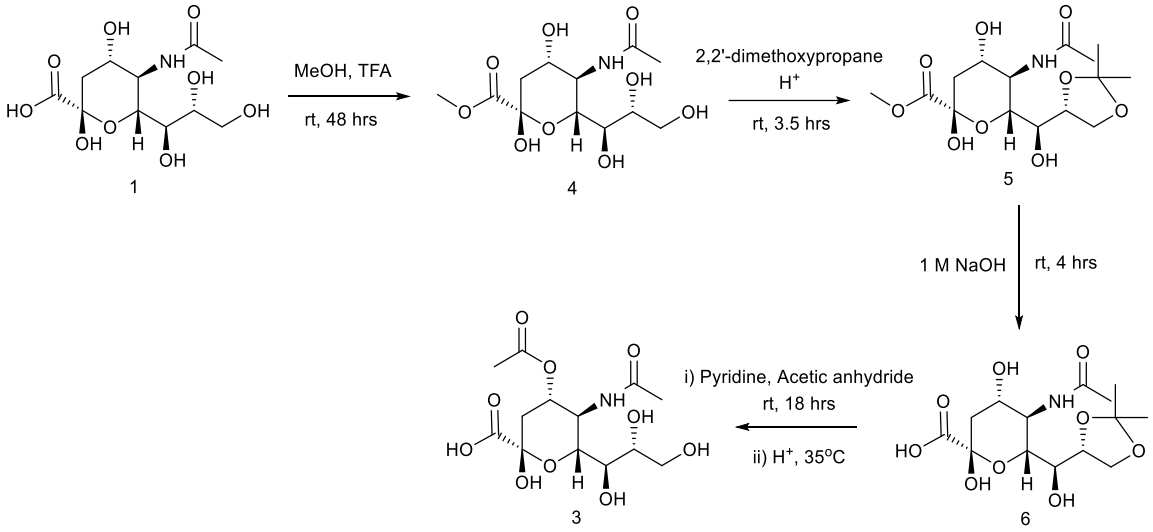
Neu5,9Ac$_2$ and Neu4,5Ac$_2$ have both been previously synthesised by Ogura *et al.*[20] and Clarke *et al.*[21] Neu5,9Ac$_2$ was synthesised in one step from Neu5Ac, and Neu4,5Ac$_2$ was synthesised in five steps from Neu5Ac by Ogura *et al.* and Clarke *et al.* albeit utilising different protecting group strategies. These approaches therefore lay the foundation for our approach. For access to Neu5,9Ac$_2$, Neu5Ac **(1)** was treated with trimethyl orthoacetate in the presence of catalytic *p*-toluene sulfonic acid for 20 minutes to afford Neu5,9Ac$_2$ **(2)** in 68% yield after purification using ion-exchange chromatography (Scheme 1). Acetylation at the 9-position could be confirmed by ^{1}H NMR spectroscopic analysis by the presence of a singlet at 2.00 ppm and the shift of the protons at the C-9-position hydrogens from 3.46 and 3.68 ppm in **(1)** to 3.95 and 4.26 ppm in **(2)**.



Scheme 1: Synthes of Neu5,9Ac$_2$

The route chosen to synthesise Neu4,5Ac$_2$ **(3)** was based on work by Ogura *et al.*[20] The synthesis of **(3)** therefore commenced with protection of the carboxylic acid group to give a methyl ester using conditions developed by Malapelle *et al.* employing methanol and trifluoroacetic acid to give **(4)** in quantitative yield.[22] This was then followed by the simultaneous protection of the 8 and 9-position hydroxyl groups with an acetonide protecting group using 2,2'-dimethoxypropane and Amberlyst 15 H^+ resin to give **(5)** in 65% yield. Deprotection of the methyl ester is required in the next step. The method put forth by Ogura *et al.* using 1 M NaOH was utilised to give **(6)** in quantitative yield. The acetyl group was installed

at the 4-position using excess pyridine and acetic anhydride and the crude mixture was then purified using ion exchange chromatography using 1 M formic acid as the eluant. Removal of the formic acid under reduced pressure at 35°C, as opposed to the use of lyophilisation by Ogura *et al.* then afforded **(3)** in 60% yield (Scheme 2). Pleasingly, it was therefore evident that removal of formic acid under these conditions also facilitated removal of the acetonide protecting group, as required, without the need for an additional synthetic step. As such, the approach detailed herein offers a higher overall yield compared with previous reports, specifically of a 39% overall yield versus 25% reported by Ogura *et al.* The successful acetylation at the 4-positon could be observed via ^{1}H NMR spectroscopic analysis by the appearance of a singlet at 1.95 ppm from the new acetyl group protons and the shift of the proton at C-4 from 3.95 in **(1)** to 5.17 ppm in the target compound **(3)**.



Scheme 2: Synthesis of Neu4,5Ac$_2$

Quantitative NMR of Standards

With Neu5,9Ac$_2$ and Neu4,5Ac$_2$ in hand, the next step was to determine the purity of the standards using QNMR techniques. The method chosen for this was based on the **pu**lse **l**ength based **con**centration determination (PULCON) method,[23–25] which utilises an external standard with a known concentration to calculate the concentration of an unknown sample. The PULCON method was chosen, as opposed to a method using an internal standard such as **e**lectronic **re**ference **t**o access **i**n vivo **c**oncentrations (ERETIC)[26], to avoid contamination of the sample. This was due to the limited quantities of material that were available and hence to facilitate further utilisation of the material after QNMR analysis. Analysis was performed by measuring the 360° pulse and comparing the integration of a known peak for both the reference and the unknown sample. The measurement of each spectrum was carried out with water suppression using presaturation to ensure that water in the sample would not obscure any peaks

and that the maximum peak intensity was obtained. Careful choice of the relaxation delay (d1) is also important; it is crucial for an accurate quantitation to ensure that all signals have fully relaxed between pulses. A d1 of at least five times the T1 of the slowest relaxing signal of interest in the spectrum should be used. T1 values for ^{1}H nuclei in medium-sized molecules typically range from 0.5 to 4. 0 seconds.

In our case, maleic acid was used as external standard at a concentration of 5.03 mmol. Maleic acid was chosen as its NMR resonance does not overlap with that of the synthesised standards and is not affected by water suppression. The 360^{o} pulse for both standards and samples was calculated using pulsecal; the pulse sequence used was noesypr1D with water suppression using presaturation, and the relaxation time chosen was 20 s.

Neu5Ac was first analysed to validate the method and hence five different concentrations were analysed via QNMR. The signals of interest (the 3-H, 12-H, 7-H and 9-H protons) in the NMR spectra obtained were integrated and normalised for the number of protons. These signals were chosen as they are distinct, and not overlapped by other resonances. They are also outside of the 3.8-5.8 ppm range and hence would not be inadvertently affected during water suppression. Signals were then compared with the signal of the maleic acid using the equation below (1) where S is the absolute area of the NMR signal, n the number of protons, U is the unknown sample, R is the reference standard and C is concentration.

$$c_U = c_R \frac{S_U n_R}{S_R n_U} \qquad (1)$$

The standard curve results were compared with the expected concentrations for each sample (Table 1) which showed that the determined values matched closely to the expected values, indicating the robustness of the PULCON method for concentration determination of a sample. A standard calibration curve is provided in the supporting information.

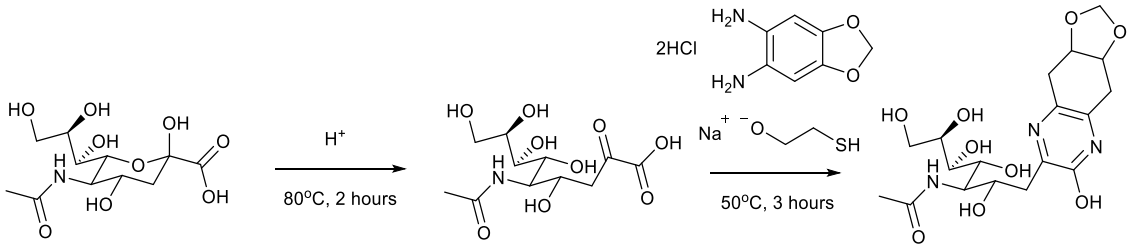
Standard Number	Prepared Concentration of Standard (mM)	Concentration Determined by QNMR (mM)
1	2.7	2.7
2	4.1	4.2
3	5.4	5.7
4	6.7	7.0
5	8.1	7.8

Table 1: Concentration of Neu5Ac standards determined by QNMR compared to the expected concentration of each standard

Having demonstrated the robustness of this QNMR PULCON method, the two synthesised sialic acid standards were next each analysed in triplicate. For Neu5,9Ac_2 the 3-H, 7-H, 12-H and 15-H protons were selected for study. For Neu4,5Ac_2 the 3-H, 7-H, 9-H and 12-H protons were selected for study. This procedure allowed for the concentrations of the Neu5,9Ac_2 and Neu4,5Ac_2 in the standard solutions to be determined, again using equation (1), as 4.04 mM and 3.71 mM respectively.

Utilisation of Standards for Quantitation

Standards of 1 nmol of Neu5,9Ac_2 and Neu4,5Ac_2 were next dispensed using a liquid handling robot to minimise the introduction of errors. Commercially available 1 nmol Neu5Ac and Neu5Gc provided by Ludger Ltd were also utilised. Neu5Ac and Neu5Gc have previously been quantified using DMB assays[27,28] and provide a good point of comparison for our standards. The standards were labelled with 1,2-diamino-4,5-methyleneoxybenzene (DMB) in the presence of β-mercaptoethanol and sodium dithionite in 1.4 M acetic acid (Scheme 3, step 2) and analysed via ultra-high performance liquid chromatography (UHPLC).[27,29] This method was chosen as it is highly specific for sialic acids and allows for the detection of low levels of these carbohydrates. Furthermore, analysis of sialic acids via the DMB method does not suffer from issues such as interference that hinder many other assays utilised for the quantitative analysis of sialic acids. Compounds other than sialic acid present in the biological sample do not react with the labelling reagent and as such cannot interfere with the assay, removing any issues with identification or quantitation of sialic acids.[30]



Scheme 3: Sialic Acid release and DMB labelling reaction

Once the standards were qualitatively assessed, the next stage was to utilise the standards for the quantitation of sialic acids in biological samples. Samples that contained acetylated sialic acid derivatives were chosen to validate Neu5,9Ac_2 and Neu4,5Ac_2 as quantitative standards. A literature search of biologically available Neu5Ac derivatives led to the purchase of recombinant human plasma[31] and guinea pig serum[15] from Sigma-Aldrich, and dried porcine and ovine serum[1,3] from First Link (UK). The sialic acids were released from the biological samples using 2 M acetic acid at 80°C for 2 hours, these conditions are kept intentionally mild to ensure that no acetyl group migration takes place (Scheme 3, step 1). Following this, both standards and samples were labelled with DMB at 50°C for 3 hours (Scheme 3, step 2). The standards were used to qualitatively assess which sialic acids were present in each sample and to create standard curves for quantitative analysis. Standard curves with the range 0.01-1 nmol were used. A variety of sialic acids were detected and quantified in each sample. Neu5Ac and Neu5,9Ac_2 were detected in human plasma (Figure 3). Neu5Gc, Neu5Ac and Neu4,5Ac_2 were detected in guinea pig serum (Figure 4), and this was the only sample to contain Neu4,5Ac_2. Porcine and ovine serum showed Neu5Ac and Neu5Gc (Figures 5 and 6). The information obtained matched the expectations derived from previous literature; Neu5Ac and Neu5,9Ac_2 are the main neuraminic acid derivatives identified in humans.[32,33] The animal serum samples also match previous literature, as all three samples contained Neu5Gc, which is not present in human samples.[34] Neu4,5Ac_2 was detected in guinea pig serum.[15] This method therefore allowed for the identification of a variety of different sialic acid derivatives in complex biological mixtures with the need for only one assay per sample, greatly reducing the time required to perform analysis for these compounds. Other unknown peaks were also observed in the traces obtained for the animal samples, but their identification was outside of the scope of this analysis, which was to determine the validity of the synthesised compounds as quantitative standards.

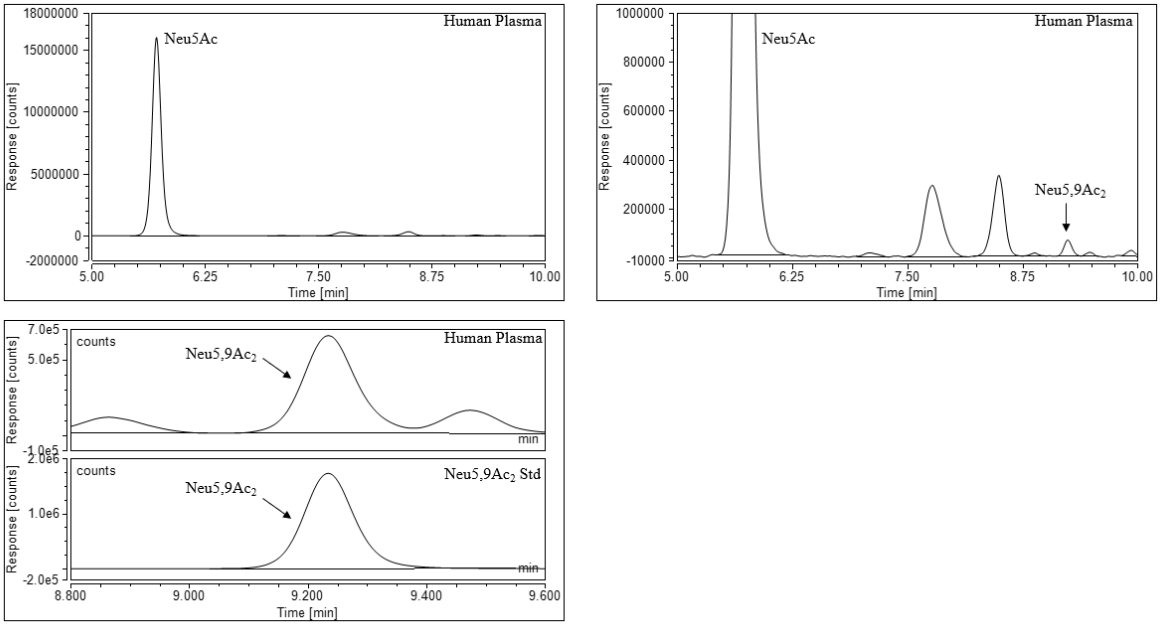


Figure 3: Human plasma after DMB labelling and UHPLC analysis (full trace and zoomed in)

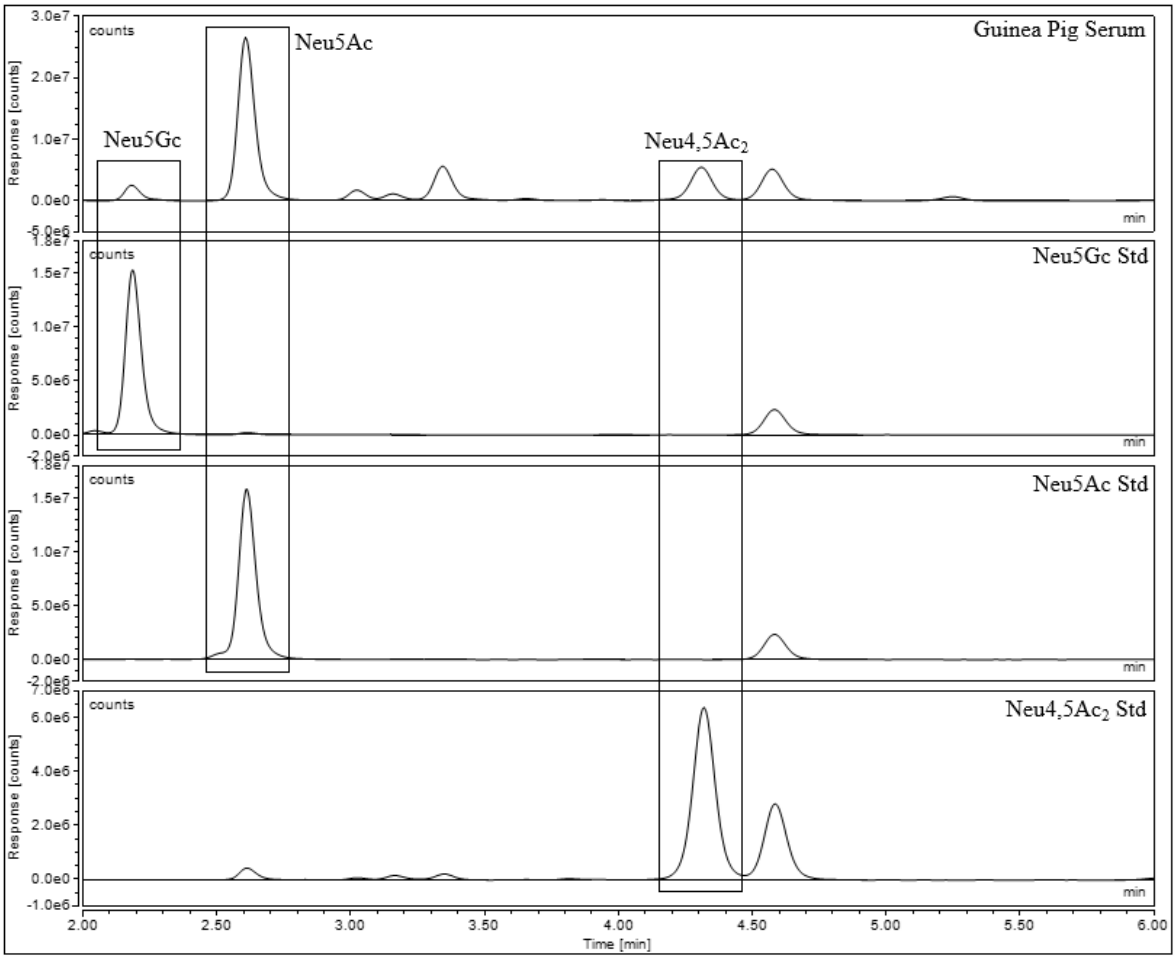


Figure 4: Guinea pig serum after DMB labelling and UHPLC analysis

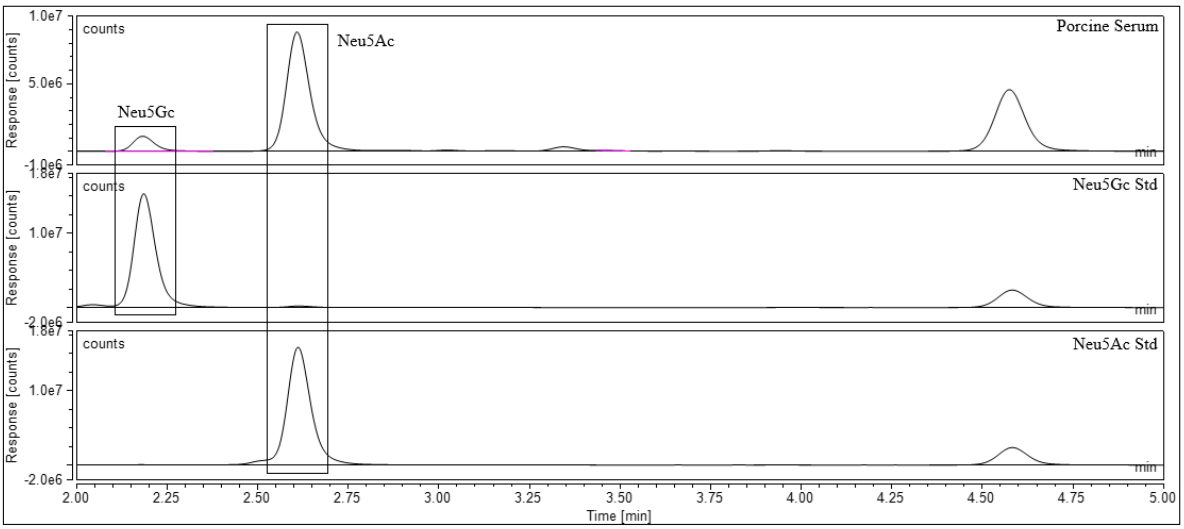


Figure 5: Porcine serum after DMB labelling and UHPLC analysis

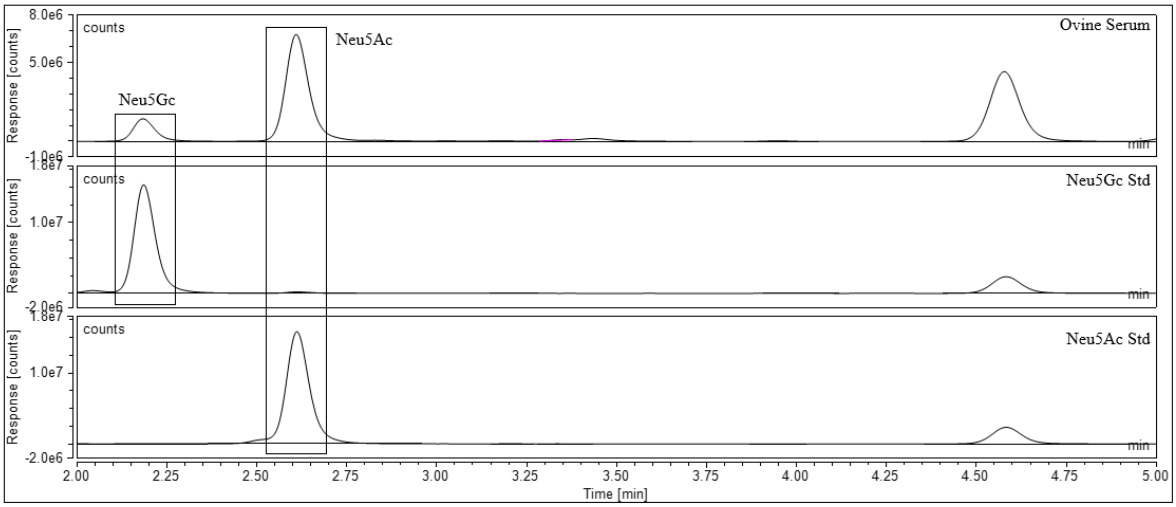


Figure 6: Ovine serum after DMB labelling and UHPLC analysis

Neu5Ac, Neu5Gc and Neu4,5Ac_2 could be effectively quantified in small quantities of the relevant biological samples with ease: the peaks met the thresholds for limit of detection (LOD) and limit of quantitation (LOQ). LOD and LOQ were calculated for standard curves prepared in water as a solvent. The signal-to-noise ration for LOD was ten and for LOQ was three. Neu5,9Ac_2 posed an issue in the plasma sample in that it was present in quantities 50-100 times smaller than Neu5Ac and as such the samples were concentrated before analysis to ensure that they met the thresholds of the LOD and LOQ (Table 2). This sample also suffered from impurities overlapping the Neu5,9Ac_2 peak. The lack of sufficient resolution was overcome by changing the solvent system to effectively separate Neu5,9Ac_2 from any impurities - using a gradient solvent system starting with low acetonitrile content and slowly increasing the volume to give a greater retention time for sialic acid derivatives allowed for any impurities to

elute first. The values obtained for quantitative analysis of the samples is shown in tables 3 and 4. Each sample was analysed in triplicate. The values were as expected: Neu5,9Ac_2 is generally detected in quantities far smaller than that of Neu5Ac.[32,33] Neu4,5Ac_2 was found to comprise 34% of total sialic acid in guinea pig serum, which matched the previously reported data for this material.[15]

Standard	LOD (mol)	LOQ (nmol)	R^2 std curve
Neu5Ac	10.0	30.5	1.0
Neu5Gc	8.5	25.7	1.0
Neu5,9Ac_2	5.9	18.0	1.0
Neu4,5Ac_2	10.8	32.9	1.0

Table 2: Limit of detection and limit of quantitation of Neu5Ac, Neu5Gc, Neu5,9Ac_2 and Neu4,5Ac_2

Sample	Average Neu5Ac (mg/100 mL)	Average Neu5Gc (mg/100 mL)	Average Neu5,9Ac_2 (mg/100 mL)	Average Neu4,5Ac_2 (mg/100 mL)
Human Plasma	45.5 ± 1.0	N/A	0.29 ± 0.0	N/A
Guinea Pig Serum	37.80 ± 1.2	4.21 ± 0.1	0.33 ± 0.0	21.25 ± 0.4

Table 3: Quantity different sialic acids in biological samples. The data are presented as mean ± standard deviation.

Sample	Average Neu5Ac (mg/100 mg serum)	Average Neu5Gc (mg/100 mg serum)	Average Neu5,9Ac_2 (mg/100 mg serum)	Average Neu4,5Ac_2 (mg/100 mg serum)
Porcine Serum	0.7 ± 0.0	0.1 ± 0.0	N/A	N/A
Ovine Serum	0.4 ± 0.0	0.1 ± 0.0	N/A	N/A

Table 4: Quantity different sialic acids in biological samples. The data are presented as mean ± standard deviation.

The LOD and LOQ found for Neu5Ac were in line with previous literature[27] with the method outlined here boasting a similar LOD and LOQ for Neu5Gc, Neu5,9Ac$_2$ and Neu4,5Ac$_2$. The intra-assay variation for the samples in triplicate was <10% indicating that the assay has good precision and allows for repeatable results while offering the ability to identify and quantify multiple sialic acid derivatives in the same assay.

Conclusions

The synthesis of 4-*O*-acetyl and 9-*O*-acetyl sialic acids (Neu5,9Ac$_2$ and Neu4,5Ac$_2$) was achieved through optimisation of previously reported synthetic routes. Neu5,9Ac$_2$ was synthesised in 1 step in 68% yield. Neu4,5Ac$_2$ was synthesised in 4 steps in 39% overall yield. The analysis of these standards by QNMR revealed the concentration of each standard present in the prepared samples and gave an accurate determination of the quantity of sample to dispense that could then be utilised as qualitative and quantitative standards for the analysis of biological samples. Methods were developed for the utilisation of these standards for the quantitation of Neu5,9Ac$_2$ and Neu4,5Ac$_2$. The method was able to separate, detect and quantify multiple derivatives of sialic acid simultaneously, forgoing the need for multiple assays to detect different derivatives in the same sample. The method was also highly sensitive, exhibiting a limit of detection and limit of quantitation comparable with previously reported DMB assays for Neu5Ac, thereby indicating that these standards combined with the DMB labelling method employed here can be used to analyse acetylated sialic acid derivatives even when present in small (<0.1 nmol) quantities without interference from impurities. The ability to detect and quantify different derivatives of sialic acid in biological samples opens doors for the investigation of these derivatives as biomarkers for different diseases such as CVD or malignant tumours.

Acknowledgements

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Experimental

Instrumentation

Melting points were determined using a Stuart SMP10 melting point apparatus. Optical rotations were carried out using a Perkin Elmer Polarimeter 341 with reference to the sodium D line (λ = 589 nm, sodium lamp) and values are given in 10^{-1}deg.cm^2.g^{-1}. Infra-red spectra were recorded using a Thermo Scientific Nicolet IS5 FT-IR spectrophotometer with an ID5 ATR accessory onto which a small quantity of sample (10 mg) was placed as a solid and values are given as the wavenumber (cm^{-1}). Mass Spectrometry was performed by the University of Reading Chemical Analysis Facility using a Thermo Scientific LTQ OrbiTrap XL with an attached ACCELA LC Autosampler. NMR spectra were recorded using either a Nanobay or Bruker DPX 400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C in either DMSO or D_2O. Quantitative NMR was carried out using a Bruker Advance III 500 MHz instrument in D_2O. Chemicals shifts are quoted in parts per million. Coupling constants are quoted in Hertz (Hz) and rounded to the nearest 0.5 Hz.

5-Acetamido-3,5-dideoxy-9-*O*-acetyl-D-glycero-D-galactononulopyranosonate (2)[20]

To a solution of *N*-acetyl neuraminic acid **1** (0.25g, 0.81 mmol, 1 eq.) in DMSO containing *p*-TsOH (10 mg) was added trimethyl orthoacetate (0.205 μL, 1.62 mmol, 2 eq.). The reaction was stirred at room temperature for 30 minutes. The resultant reaction mixture was poured directly onto a column (2 x 10 cm) of DOWEX-1X8 formate anion exchange resin (100-200 mesh). The column was washed with water (3 x 10 mL) and eluted with formic acid (50 mL). The formic acid was removed under reduced pressure to give a clear residue. The residue was dissolved in water (5 mL) and lyophilised to give 9-*O* acetyl *N*-acetyl neuraminic acid **2** as a white solid (175 mg, 68% yield). $[\alpha]_D^{20}$ -13^o (c 1.0, H_2O); mp. 153-155^{o}C; ^{1}H NMR (400 MHz, D_2O): δ 4.26 (1H, dd, *J*=11.5, 2.5 Hz, H-9), 4.07 (1H, dd, *J* = 6.5 Hz, H-4), 4.00-3.90 (2H, m, H-6 + H-9), 3.87-3.77 (2H, m, H-5 + H-8), 3.49 (1H, d, *J* = 8.0 Hz, H-7), 2.19 (1H, dd, *J* = 13.0, 5.0 Hz, H-3eq), 2.00 (s, 3H, H-15), 1.94 (s, 3H, H-12), 1.81-1.72 (1H, m, H-3ax ^{13}C NMR (100 MHz, D_2O): δ 174.73 (C13), 174.41 (C11), 171.22 (C14), 95.48 (C2), 70.15 (C7), 69.2 (C8), 67.59 (C6), 66.80 (C4), 66.26 (C9), 52.03 (C5), 38.89 (C3), 22.6 (C12), 20.19 (C15) ppm; IR ν_{max} [cm^{-1}] (powder) 3295 (O-H, br), 1719 (C=O), 1034 (C-O); HRMS (ESI): m/z calc for $C_{13}H_{22}O_{10}N$: 352.1238 $[M+H]^+$; found: 351.1239

Methyl 5-Acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranosonate (4)[22]

To a solution of *N*-acetyl neuraminic acid **1** (2.0 g, 6.46 mmol, 1 eq.) in methanol (40 mL) was added TFA (0.98 mL, 12.92 mmol, 2 eq.). This was stirred at room temperature for 24

hrs. The solvent was removed under reduced pressure to give the methyl ester **4** as a white solid (2.1 g, quant.) $[\alpha]_D^{20}$ -26° (c 1.0, MeOH); mp. 177-178°C; ^{1}H NMR (400 MHz, D_2O): δ 3.94 (2H, m, H-4 + H-6), 3.80 (1H, m, H-9), 3.72 (4H, m, H-5 + H-14), 3.60 (1H, m, H-8), 3.50 (1H, m, H-9), 3.42 (1H, m, H-7), 2.19 (1H, dd, H-3eq *J* = 13.0, 5.0 Hz), 1.92 (3H, s, H-12), 1.79 (1H, m, H-3ax) ppm ^{13}C NMR (100 MHz, D_2O): δ 174.79 (C13), 171.36 (C11), 95.29 (C2), 70.29 (C6), 70.05 (C8), 68.14 (C7), 66.62 (C4), 63.10 (C9), 53.43 (C14), 52.00 (C5), 38.60 (C3), 22.00 (C12) ppm; IR ν_{max} [cm^{-1}] (powder) 3256 (O-H, br), 2944 (C-H), 1751 (C=O), 1026 (C-O); HRMS (ESI): m/z calc for $C_{12}H_{21}O_9NNa$: 352.1109 $[M+H]^+$; found: 346.1102

Methyl 5-acetamido, 3,5-dideoxy-8,9-*O*-isopropylidene-D-glycero-β-D-galactononulopyranosonate (5)[20]

To a solution of methyl ester **4** (1.0 g, 3.09 mmol, 1 eq.) in acetone (50 mL) was added Amberlyst 15 H^+ resin (1.5 g) and 2,2-dimethoxypropane (0.48 mL, 3.71 mmol, 1.2 eq.). This was stirred for 3.5 hrs at room temperature before being filtered to remove the resin. The resin was washed with acetone and the filtrate collected. After removing the solvent under reduced pressure, the resulting crude material was passed over a silica plug and eluted with ethyl acetate (4 x 200 mL) to give acetonide **5** as a beige foam (0.73 g, 65% yield) $[\alpha]_D^{20}$ -24° (c 1.0, MeOH); mp. 164-166°C; ^{1}H NMR (400 MHz, DMSO-d6): δ 8.07 (1H, d, *J* = 8.0 Hz, H-10), 6.71 (1H, d, *J*= 2.0 Hz, O-H2), 4.82 (1H, d, J = 6.0 Hz, O-H4), 4.75 (1H, d, *J* = 5.0 Hz, O-H7), 4.02-3.94 (1H, m, H-8), 3.90-3.86 (1H, m, H-6), 3.85-3.78 (2H, m, H-4 +H-9), 3.68 (3H, s, H-14), 3.56 (1H, t, *J* = 9.5 Hz, H5), 3.52-3.49 (2H, m, H-7 + H-9), 2.03 (1H, dd, *J* = 13.0, 5.0 Hz, H-3eq), 1.88 (3H, s, H-12), 1.61 (1H, m, H-3ax), 1.26 (3H, s, H16), 1.23 (3H, s, H-16) ppm ^{13}C NMR (100 MHz, DMSO-d6): δ 179.94 (C13), 171.68 (C11), 107.22 (C15), 94.57 (C2), 75.92 (C8), 71.70 (C7), 68.38 (C6), 65.20 (C9), 64.94 (C4), 52.89 (C14), 52.12 (C5), 40.23 (C3), 26.55 (C16), 25.72 (C16), 22.55 (C12) ppm; IR ν_{max} [cm^{-1}] (powder) 3291 (O-H, br), 2926 (C-H), 1740 (C=O), 1032 (C-O); HRMS (ESI): m/z calc for $C_{15}H_{25}O_9NNa$: 386.1422 $[M+Na]^+$; found: 386.1416

5-Acetamido-3,5-dideoxy-8,9-*O*-isopropylidene-D-glycero-D-galactononulopyranosonate (6)[20]

The acetonide **5** (0.25 g, 0.68 mmol) was dissolved in 1 M NaOH (5 mL) and stirred for 4 hrs at room temperature. This was then diluted with water (10 mL) and deionised with Amberlyst H^+ resin. The mixture was filtered, the filtrate was collected and lyophilised to give a white solid **6** (0.24 g, quant.). $[\alpha]_D^{20}$ -25° (c 1.0, H_2O); mp. 157-158°C; ^{1}H NMR (400 MHz, D_2O):

δ 4.25 (1H, dd, *J*=12.5 Hz, 6.0 Hz, H-8), 4.20-4.11 (1H, m, H-6), 4.07-3.97 (2H, m, H-4 + H-9), 3.95-3.85 (2H, m, H-5 + H-9), 3.62 (1H, m, *J* = 7.5 Hz, H-7), 2.09 (3H, s, H-12), 1.46 (3H, s, H-15), 1.36 (3H, s, H-15) ppm; ^{13}C NMR (100 MHz, D_2O): δ 176.75 (C13), 174.43 (C11), 168.30 (C2), 109.58 (C14), 75.10 (C8), 70.59 (C7), 69.07 (C6), 66.99 (C9), 66.14 (C4), 52.16 (C5), 25.77 (C15), 24.27 (C15), 21.99 (C12) ppm; IR ν_{max} [cm^{-1}] (powder) 3282 (O-H, br), 1735 (C=O), 1427 (COOH) 1057 (C-O); HRMS (ESI): m/z calc for $C_{14}H_{23}O_9NNa$: 372.1265 $[M+H]^+$; found: 372.1263

5-Acetamido-3,5-dideoxy-4-*O*-acetyl-D-glycero-D-galactononulopyranosonate (3)[20]

To a solution of **6** (100 mg, 0.29 mmol, 1 eq.) in dry pyridine (0.5 mL) under nitrogen was added acetic anhydride (30 μL, 0.32 mmol, 1.1 eq.). This was stirred at room temperature for 18 hours after which ethanol was added to remove excess acetic anhydride and solvent removed under reduced pressure by co-evaporation with toluene (3 x 100 mL). The resultant residue was dissolved in water (10 mL) and passed through a column (2 x 10 cm) of DOWEX-1X8 formate anion exchange resin (100-200 mesh). The column was washed with water (3 x 10 mL) and eluted with formic acid (50 mL). The formic acid was removed under reduced pressure to give a clear residue. The residue was dissolved in water (5 mL) and lyophilised to give 4-*O* *N*-acetyl neuraminic acid **3** as a white solid (60 mg, 60% yield). $[\alpha]_D^{20}$ -34° (c 1.0, H_2O); mp. 170-172°C; ^{1}H NMR (400 MHz, D_2O): 5.22-5.12 (1H, m, H-4), 4.18-3.99 (2H, m, H-5 + H-6), 3.76- 3.72 (1H, m, H-9), 3.63 (1H, m, H-8), 3.50 (1H, m, H-9 + H-7), 2.24 (1H, m, H-3eq), 1.95 (3H, s, H-15), 1.87 (4H, m, H-12+H-3ax); ^{13}C NMR (100 MHz, D_2O): δ 174.7 (C14), 173.38 (C13), 173.31 (C11), 95.24 (C2), 70.07 (C8), 70.00 (C7), 68.01 (C6), 63.09 (C4), 53.50 (C9), 49.38 (C5), 36.02 (C3), 21.85 (C12), 20.30 (C15) ppm; IR ν_{max} [cm^{-1}] (powder) 3340 (O-H, br), 2933 (C-H), 1727 (C=O), 1019 (C-O); HRMS (ESI): m/z calc for $C_{13}H_{22}O_{10}N$: 352.1238 $[M+H]^+$; found: 351.1239

Quantitative NMR analysis

Into an NMR tube was placed 600 μL of a 5.03 mMol maleic acid solution in D_2O. A 1.8 mg quantity of synthesised standard was dissolved in 1.8 mL of D_2O. This was split into three 600 μL samples in order to analyse the standards in triplicate. After matching and tuning, each NMR sample was analysed on a 500 MHz NMR spectrometer where a 1D ^{1}H spectrum with water suppression was obtained with a relaxation delay of 20 seconds. The 360° pulse was obtained for each spectrum using pulsecal. The pulse sequence used was noesypr1D. Equation 1 was used to determine the concentration and purity of each synthesised standard.

DMB labelling of the Neu5Ac, Neu5Gc, Neu5,9Ac$_2$ and Neu4,5Ac$_2$ standards

Neu5Ac, Neu5Gc, Neu5,9Ac_2 and Neu4,5Ac_2 standards were labelled using LudgerTagTM DMB sialic acid (LT-KDMB-96). DMB labelling solution (20 µL) was added to the standards. The samples were then vortexed and centrifuged followed by incubation at 50°C for 3 hours. The labelling reaction was quenched by the addition of water to give a final volume of 500 µL (480 µL). Standard curves were prepared by performing serial dilution to create a standard curve with points: 0.01-1 nmol. Each standard was made up to a volume of 200 µL. The procedure was carried out using a Hamilton MICROLAB STARlet Liquid Handling Robot.

Sialic acid release and DMB labelling of human plasma and guinea pig serum

Release of sialic acid and DMB labelling of the samples was achieved using LudgerTagTM DMB sialic acid (LT-KDMB-96). A 5 µL aliquot of each sample was dispensed into a 96-well plate in triplicate. To this was added 25 µL of 2 M acetic acid. The sample was vortexed and centrifuged followed by incubation at 80°C for 2 hours. The sample was allowed to cool to room temperature and a 5 µL aliquot of each sample was transferred to a new 96-well plate. To this was added 20 µL of DMB labelling solution. The sample was then vortexed and centrifuged followed by incubation at 50°C for 3 hours. The labelling reaction was quenched by the addition of water (475 µL) to give a final volume of 500 µL. The samples were then subjected to a 1 in 10 dilution (50 µL sample, 450 µL water). All procedures, except the dispensing of the samples on the plate, were conducted using a Hamilton MICROLAB STARlet Liquid Handling Robot.

Sialic acid release and DMB labelling of porcine and ovine serum

Release of sialic acid and DMB labelling of the samples was achieved using LudgerTagTM DMB sialic acid (LT-KDMB-96). A stock solution was first prepared by dissolving 10 mg of porcine or ovine serum in 1 mL ultra-pure water. A 10 µL aliquot taken from the solution of each sample was dispensed into a 96-well plate in triplicate. To this was added 25 µL of 2 M acetic acid. The sample was vortexed and centrifuged followed by incubation at 80°C for 2 hours. The sample was allowed to cool to room temperature and a 5 µL aliquot of each sample was transferred to a new 96-well plate. To this was added 20 µL of DMB labelling solution. The sample was then vortexed and centrifuged followed by incubation at 50°C for 3 hours. The labelling reaction was quenched by the addition of water (475 µL) to give a final volume of 500 µL. The samples were then subjected to a 1 in 10 dilution (50 µL sample, 450 µL water). All procedures, except the dispensing of the samples on the plate, were carried out using a Hamilton MICROLAB STARlet Liquid Handling Robot.

Sialic Acid release and DMB labelling of plasma for Neu5,9Ac$_2$

Release of Sialic Acid and DMB labelling of the samples was achieved using LudgerTagTM DMB Sialic Acid (LT-KDMB-96). A 25 µL aliquot of each sample was dispensed into a 96-well plate in triplicate. To this was added 75 µL of 2.7 M acetic acid. The sample was vortexed and centrifuged followed by incubation at 80ºC for 2 hours. The sample was allowed to cool to room temperature and a 20 µL aliquot of each sample was transferred to a new 96-well plate. To this was added 20 µL of DMB labelling solution. The sample was then vortexed and centrifuged followed by incubation at 50ºC for 3 hours. The labelling reaction was quenched by the addition of water to give a final volume of 500 µL (475 µL). The sample was subjected to filtration through a Ludger Clean Protein Binding Membrane filtration plate (LC-PBM-plate) to remove excess protein. All procedures, except the dispensing of the samples on the plate and LC-PBM-plate filtration, were carried out using a Hamilton MICROLAB STARlet Liquid Handling Robot.

Fluorescence analysis of DMB labelled sialic acid derivatives by LC-FLD

DMB labelled sialic acid derivatives were analysed by LC-FLD. A 5 µl aliquot of each sample was injected to a LudgerSep™ uR2 UHPLC column (2.1 × 150 mm, 1.9 µm, C18,175 Å pore size) at 30 °C on a Dionex UltiMate™3000 RSLCnano system with a fluorescent detector (λex = 373 nm, λem = 448 nm). For Neu5Ac analysis, an isocratic solvent system was used (7:9:84 %v/v MeOH:ACN:H_2O) for 15 minutes including an ACN wash. For Neu5,9Ac$_2$ analysis a variable solvent system was used: 7:6:87 %v/v MeOH:ACN:H2O for 6.5 minutes followed by 6:9:85 MeOH:ACN:H2O for 11.5 minutes. Integration of resultant peaks was performed using Chromeleon 7. LOD and LOQ regression analysis and calculation was carried out using Microsoft Excel 2019.

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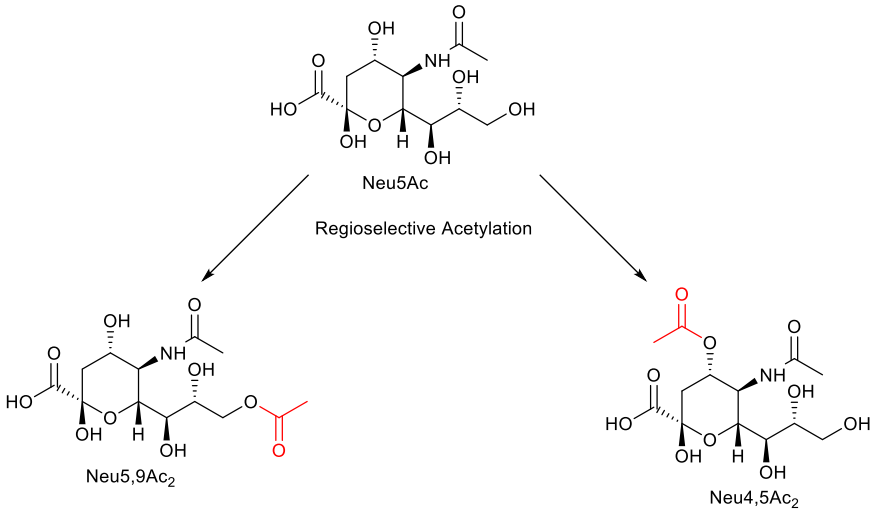
Keywords: Acetylated sialic acid, carbohydrates, DMB assay, QNMR spectroscopy, UHPLC

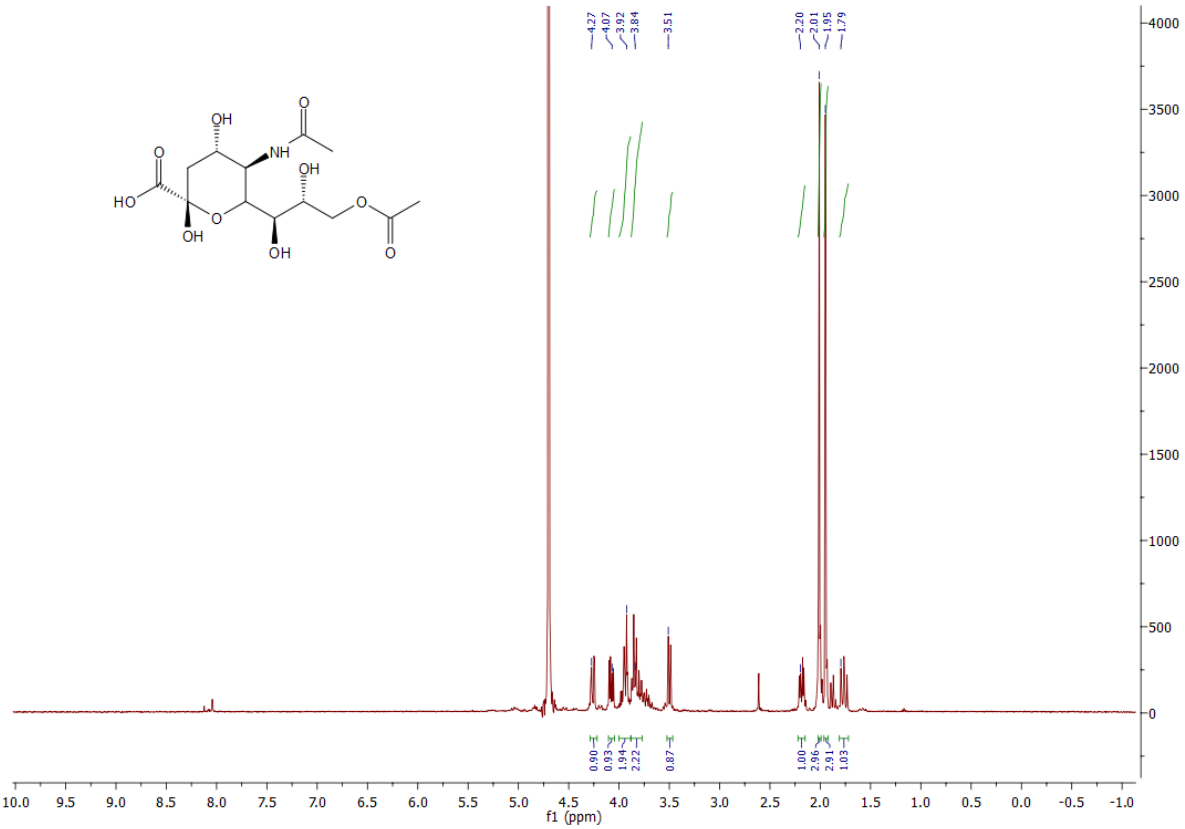
Table of contents graphic:



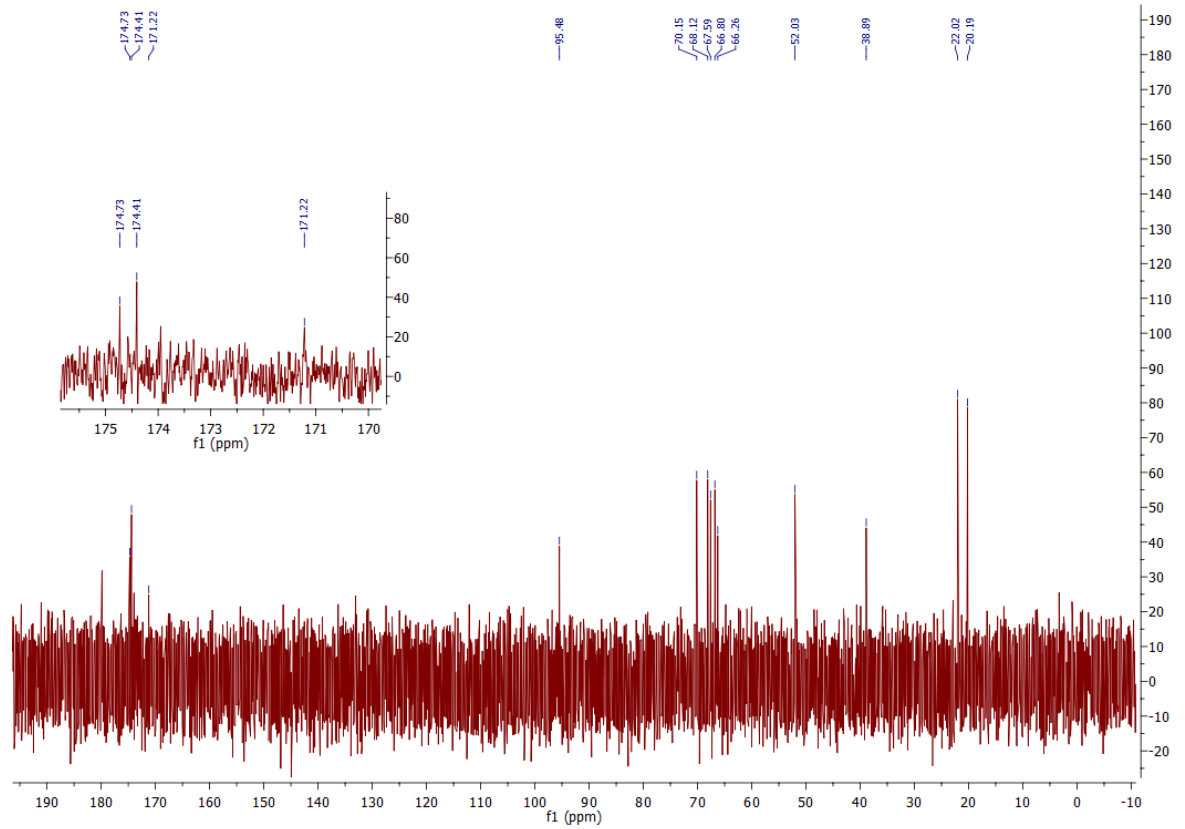
Accompanying text:

Synthesised acetylated derivatives of *N*-acetyl neuraminic acid, Neu5,9Ac_2 and Neu4,5Ac_2, alongside commercially available Neu5Ac and Neu5Gc were utilised as standards for the quantitative analysis of these derivatives in plasma and serum samples. Multiple derivatives could be detected in one assay, which exhibited high specificity and low limits of detection and quantitation.

Supporting Information – NMR Spectra



^{1}H NMR (400 MHz, D_2O): 5-Acetamido-3,5-dideoxy-9-*O*-acetyl-D-glycero-D-galactononulopyranosonate **(2)**



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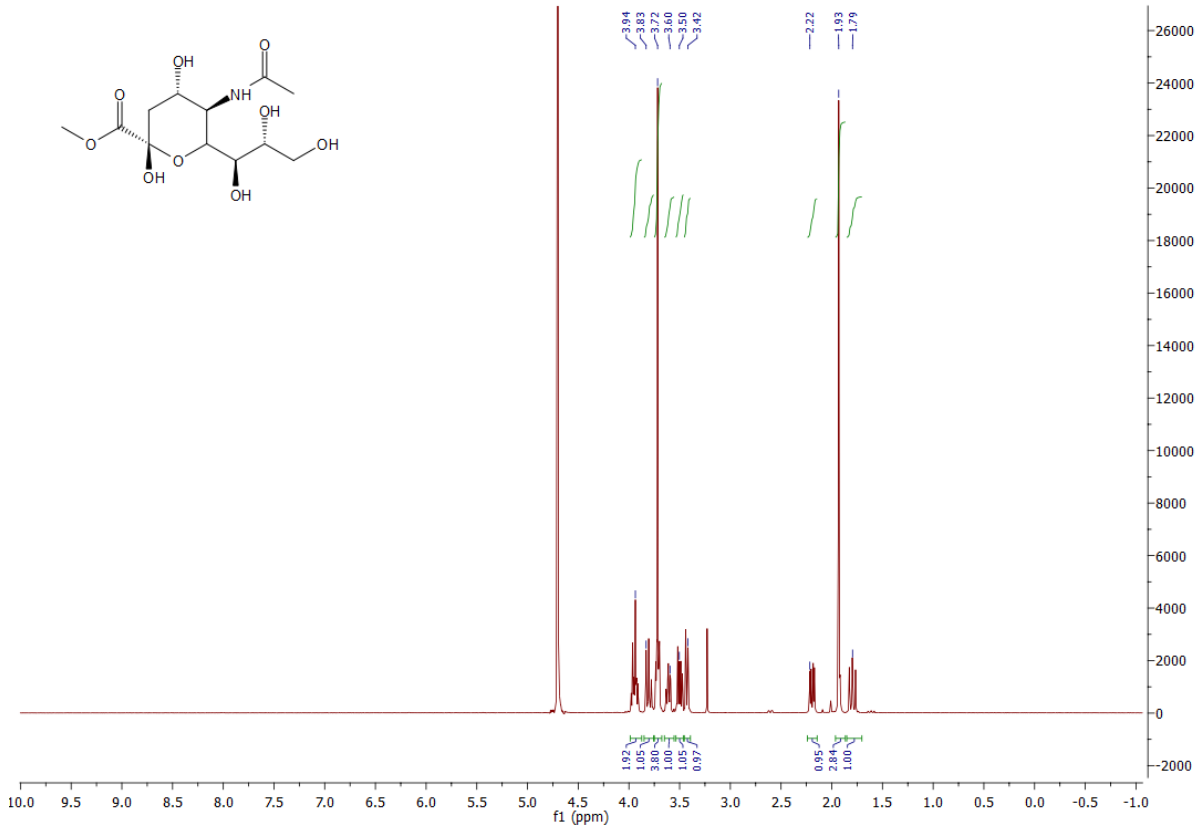
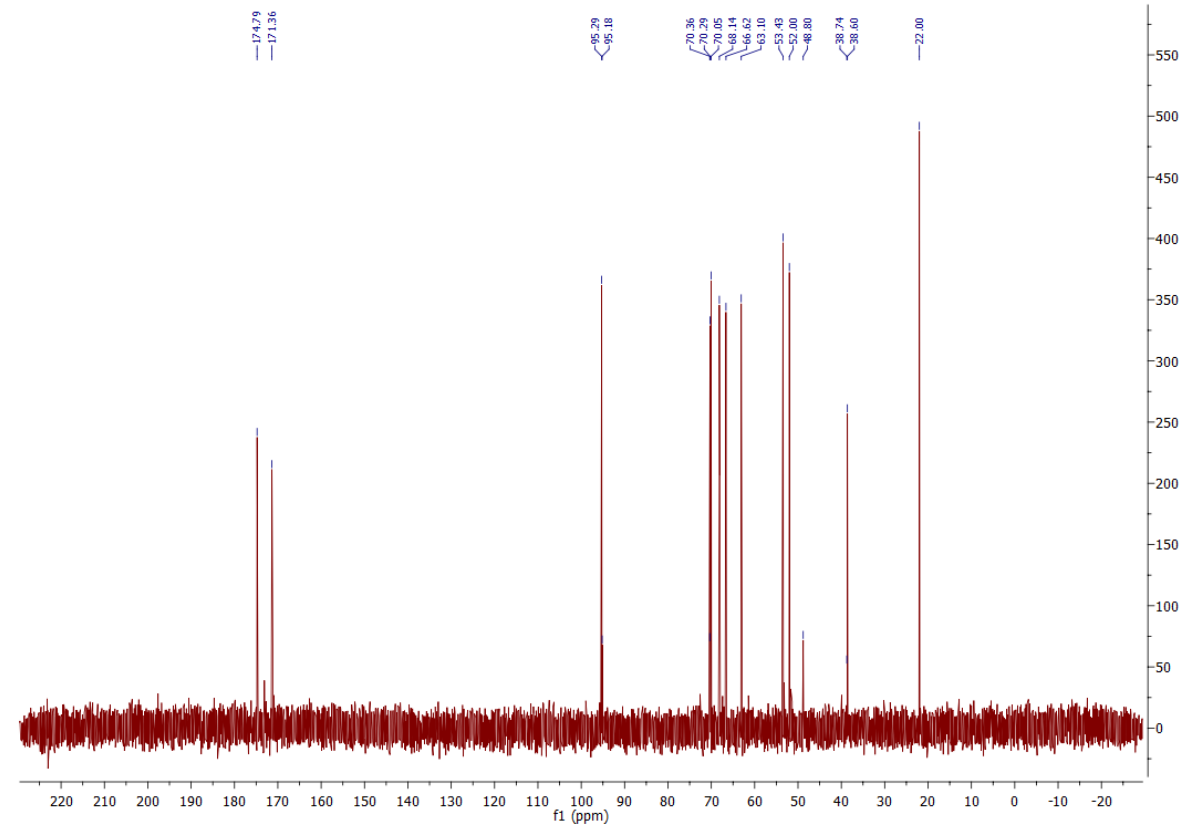
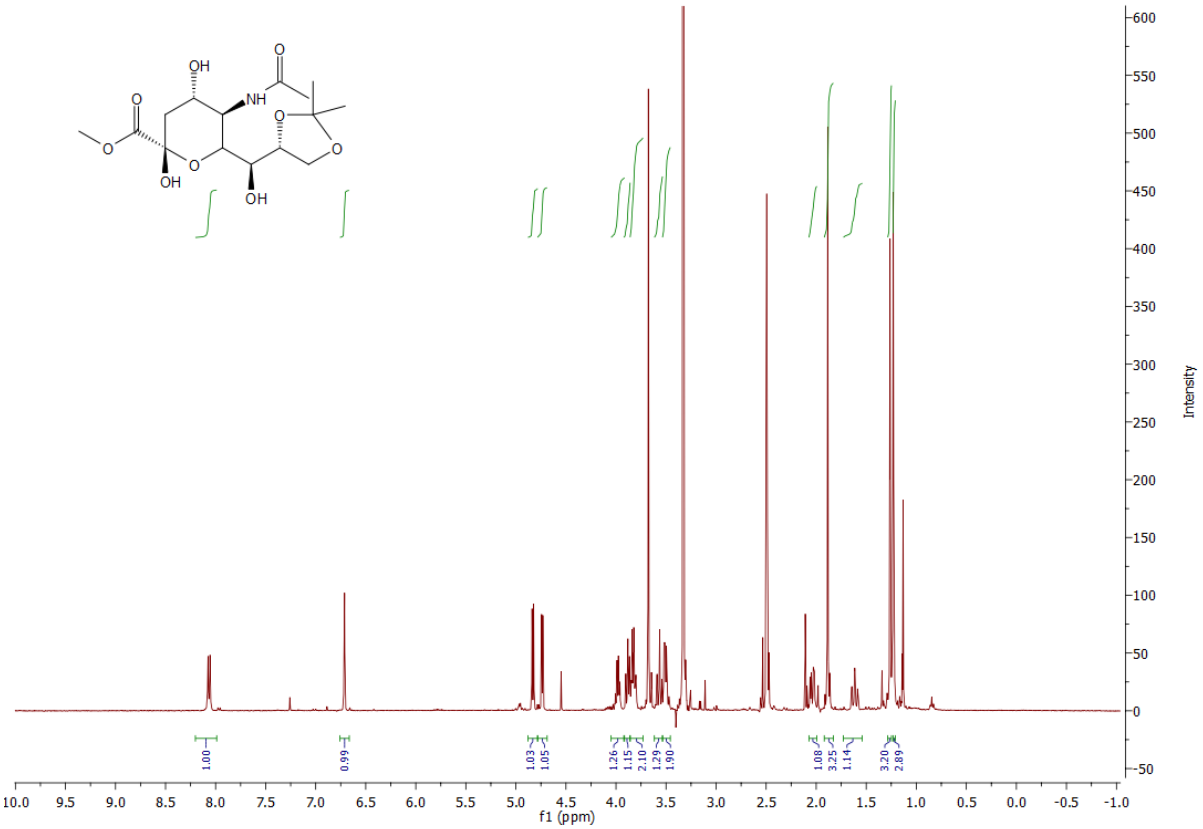


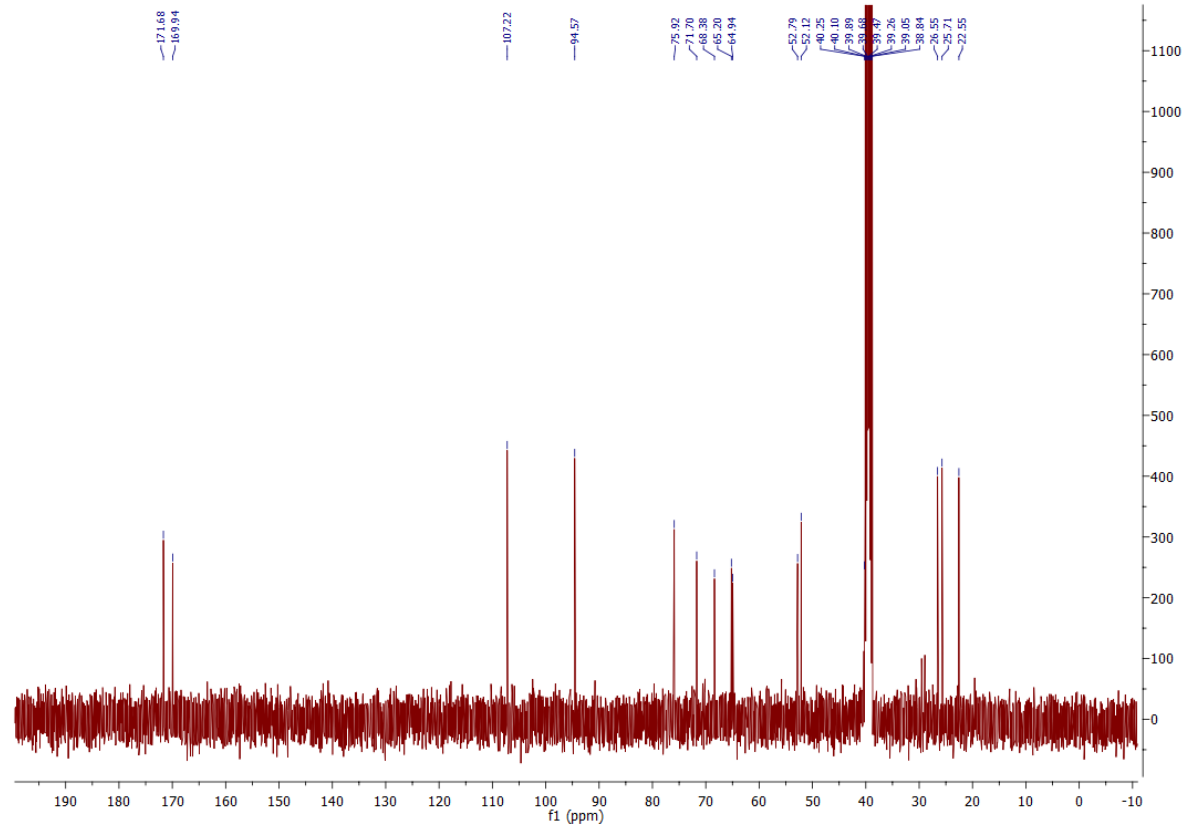
^{1}H NMR (400 MHz, D_2O): Methyl 5-Acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranosonate **(4)**



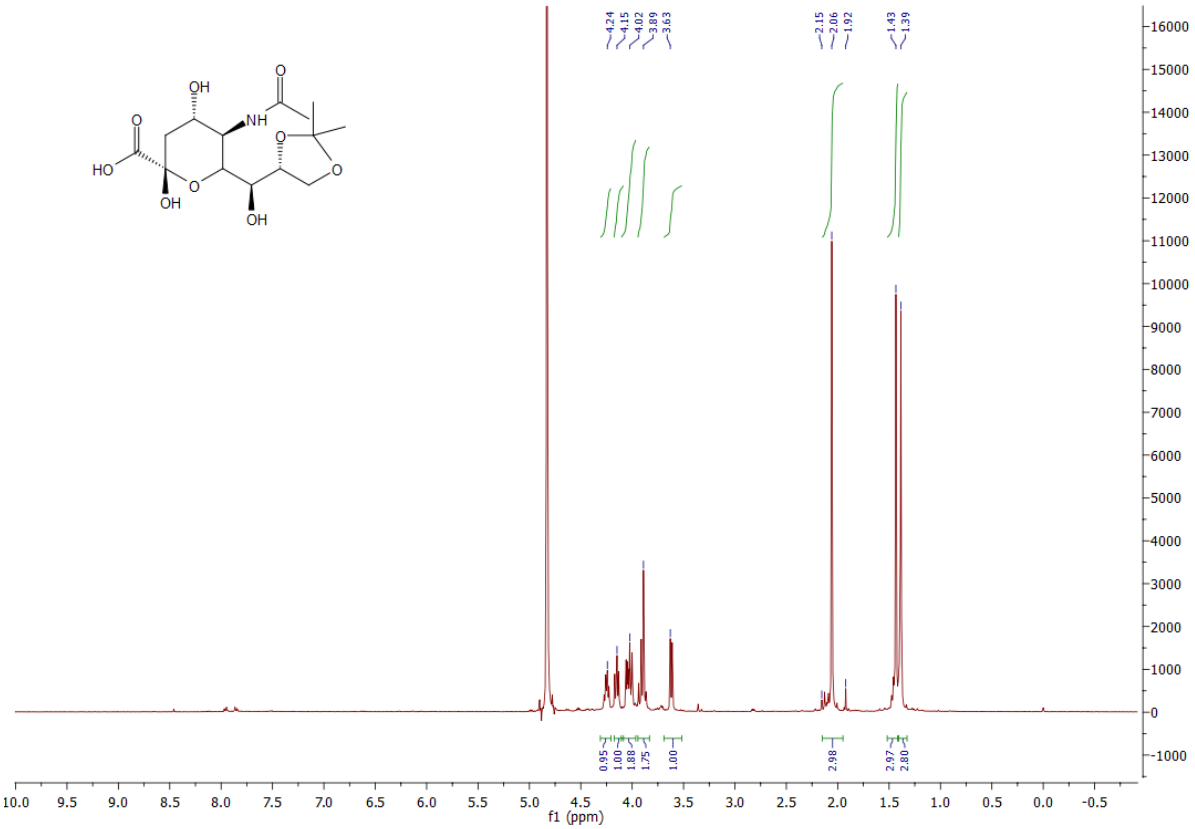
^{13}C NMR (100 MHz, D_2O): Methyl 5-Acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranosonate **(4)**



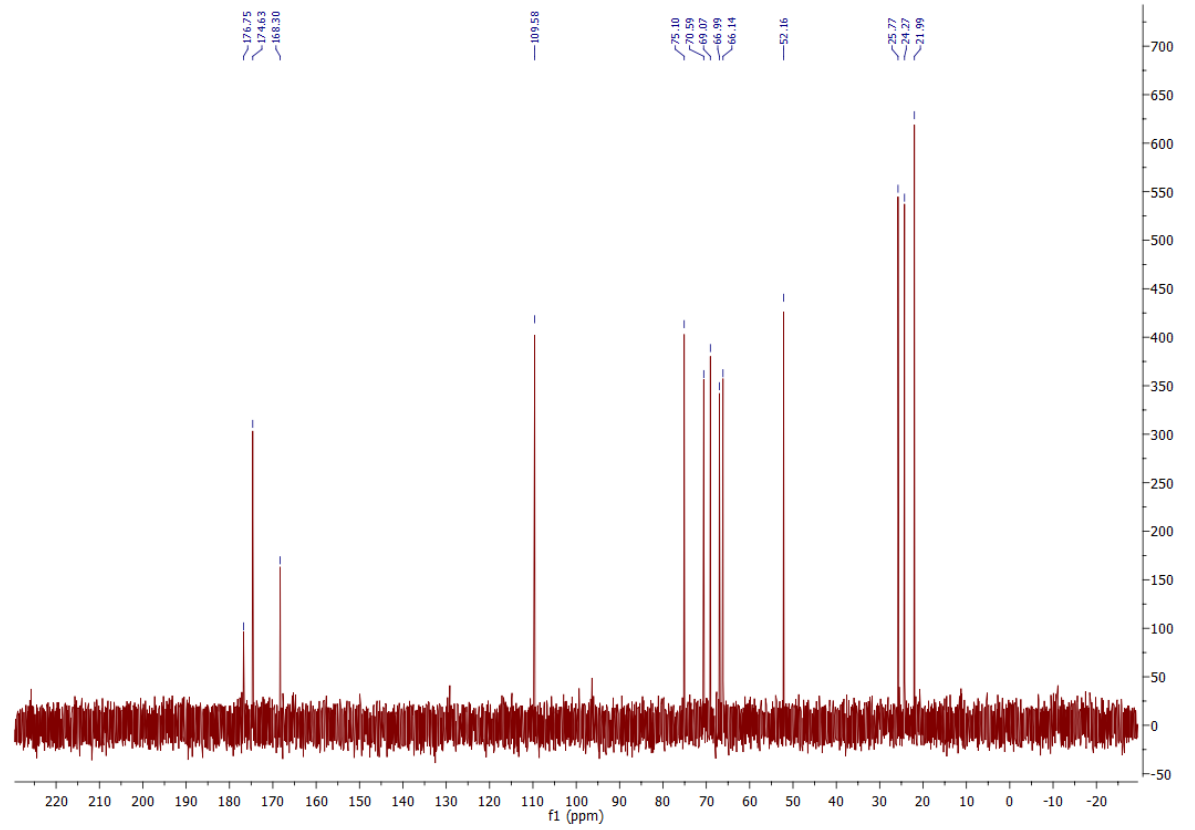
[1]H NMR (400 MHz, DMSO-d6): Methyl 5-acetamido, 3,5-dideoxy-8,9-*O*-isopropylidene-D-glycero-β-D-galactononulopyranosonate **(5)**



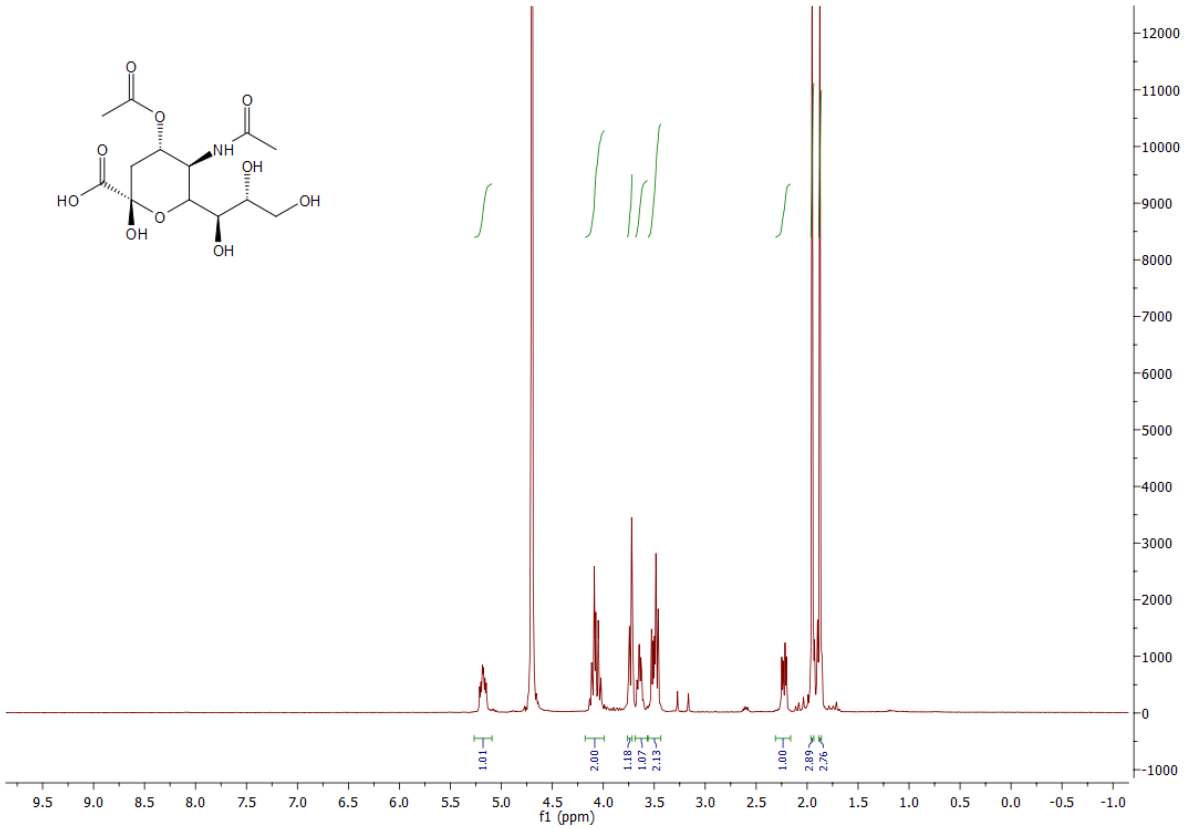
[13]C NMR (100 MHz, DMSO-d6): Methyl 5-acetamido, 3,5-dideoxy-8,9-*O*-isopropylidene-D-glycero-β-D-galactononulopyranosonate **(5)**



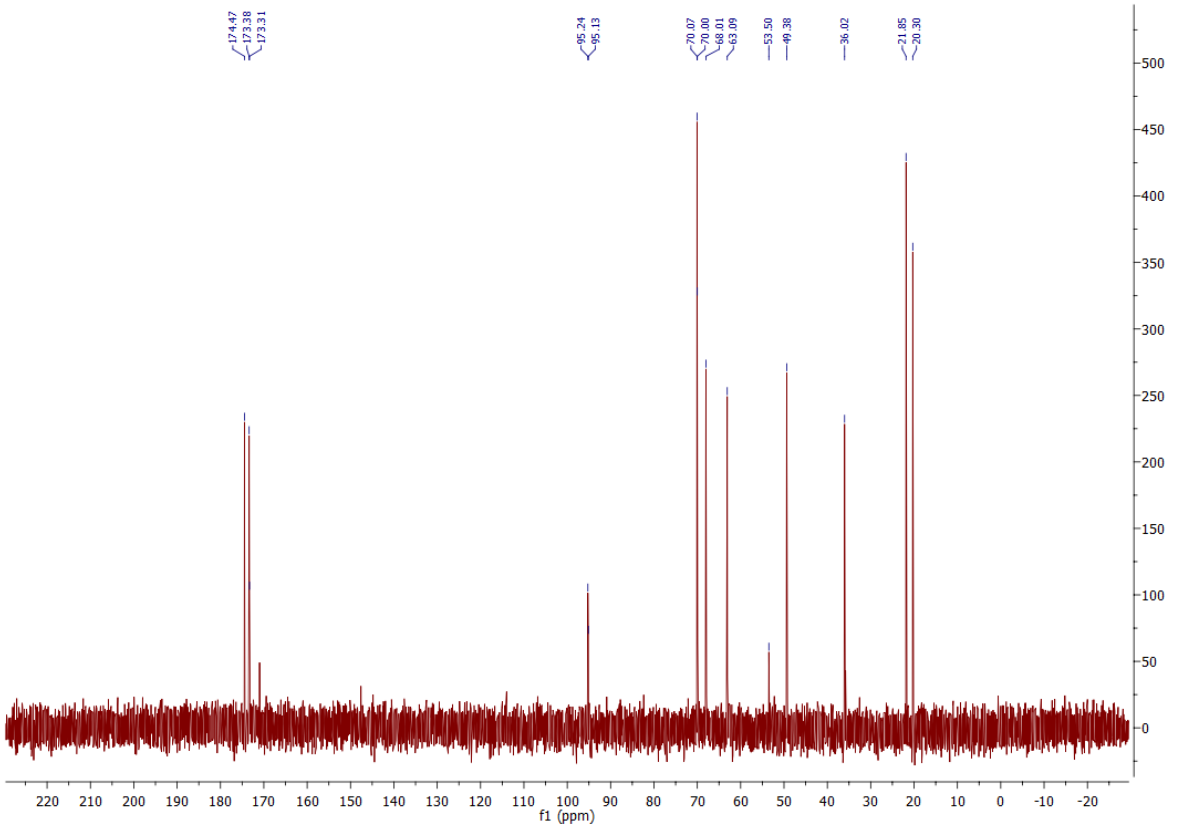
[1]H NMR (400 MHz, D_2O): 5-Acetamido-3,5-dideoxy-8,9-*O*-isopropylidene-D-glycero-D-galactononulopyranosonate **(6)**



[13]C NMR (100 MHz, D_2O): 5-Acetamido-3,5-dideoxy-8,9-*O*-isopropylidene-D-glycero-D-galactononulopyranosonate **(6)**

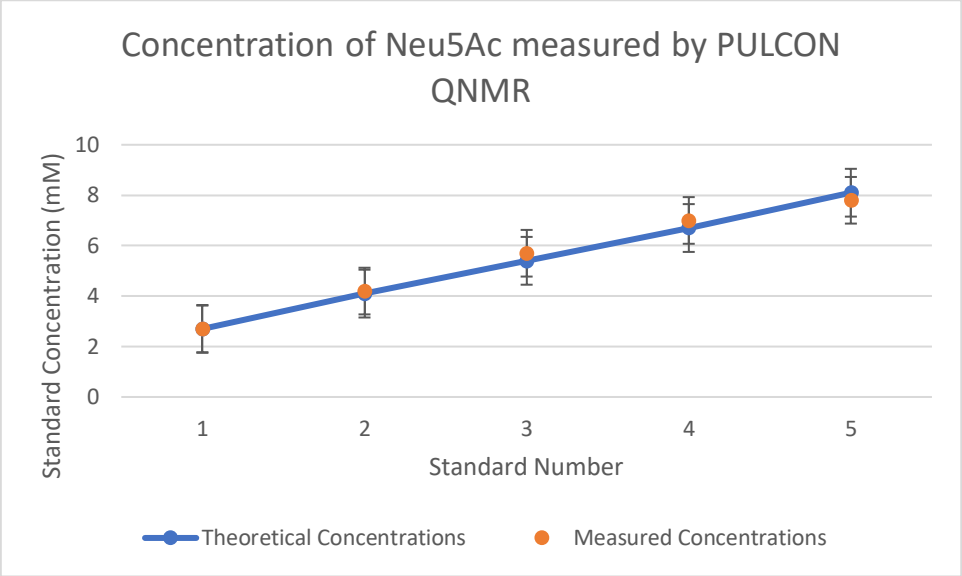


^{1}H NMR (400 MHz, D_2O): 5-Acetamido-3,5-dideoxy-4-*O*-acetyl-D-glycero-D-galactononulopyranosonate **(3)**



^{13}C NMR (100 MHz, D_2O): 5-Acetamido-3,5-dideoxy-4-*O*-acetyl-D-glycero-D-galactononulopyranosonate **(3)**

Quantitative NMR calibration curve



Concentration of Neu5Ac standards determined by QNMR (orange points) compared to the expected concentration of each standard (blue line)

Appendix 1: Work Towards the Synthesis of Neu5,8Ac$_2$, Neu2,5Ac$_2$, Neu5,7Ac$_2$ and acetylated Neu5Gc derivatives

Introduction

Further to the synthesis of Neu5,9Ac$_2$ and Neu4,5Ac$_2$ work was undertaken to synthesise Neu5,8Ac$_2$, Neu2,5Ac$_2$, Neu5,7Ac$_2$ and acetylated derivatives of Neu5Gc. Synthetic routes were devised from the literature (Figures 1-5).[1–6]

Synthesis of Neu5,8Ac$_2$ (8)

The synthesis of Neu5,8Ac$_2$ required selective protection of the C-4 and C-9 hydroxyl groups to leave the C-8 position open to acylation. The synthesis of Neu5,8Ac$_2$ **(8)** was started by protecting the carboxylic acid group to give a methyl ester **(2)** in quantitative yield. This was then followed by simultaneous protection of the C-7 and C-9 hydroxyl groups with a benzylidene protecting group by the action of benzaldehyde dimethyl acetal and *p*-TsOH using Dean-Stark apparatus to give **(3)** in 5% yield. The low yield unfortunately prevented further work using this route, as such a second route was devised. This once again started the synthesis of **(2)** by the protection of the carboxylic acid functional group to give a methyl ester in quantitative yield. This was followed by the simultaneous protection of the C-4 and C-9 positions with TBDMS protecting groups by the action of TBDMSCl and imidazole to give the disilyl compound **(9)** in 78% yield. The methyl ester was then converted back to the carboxylic acid using potassium trimethyl silanolate which yielded **(10)** in 83% yield while leaving other protecting groups intact. Attempts at acetylation were performed using acetic anhydride and pyridine, these were unsuccessful however and this synthesis was not pursued further.

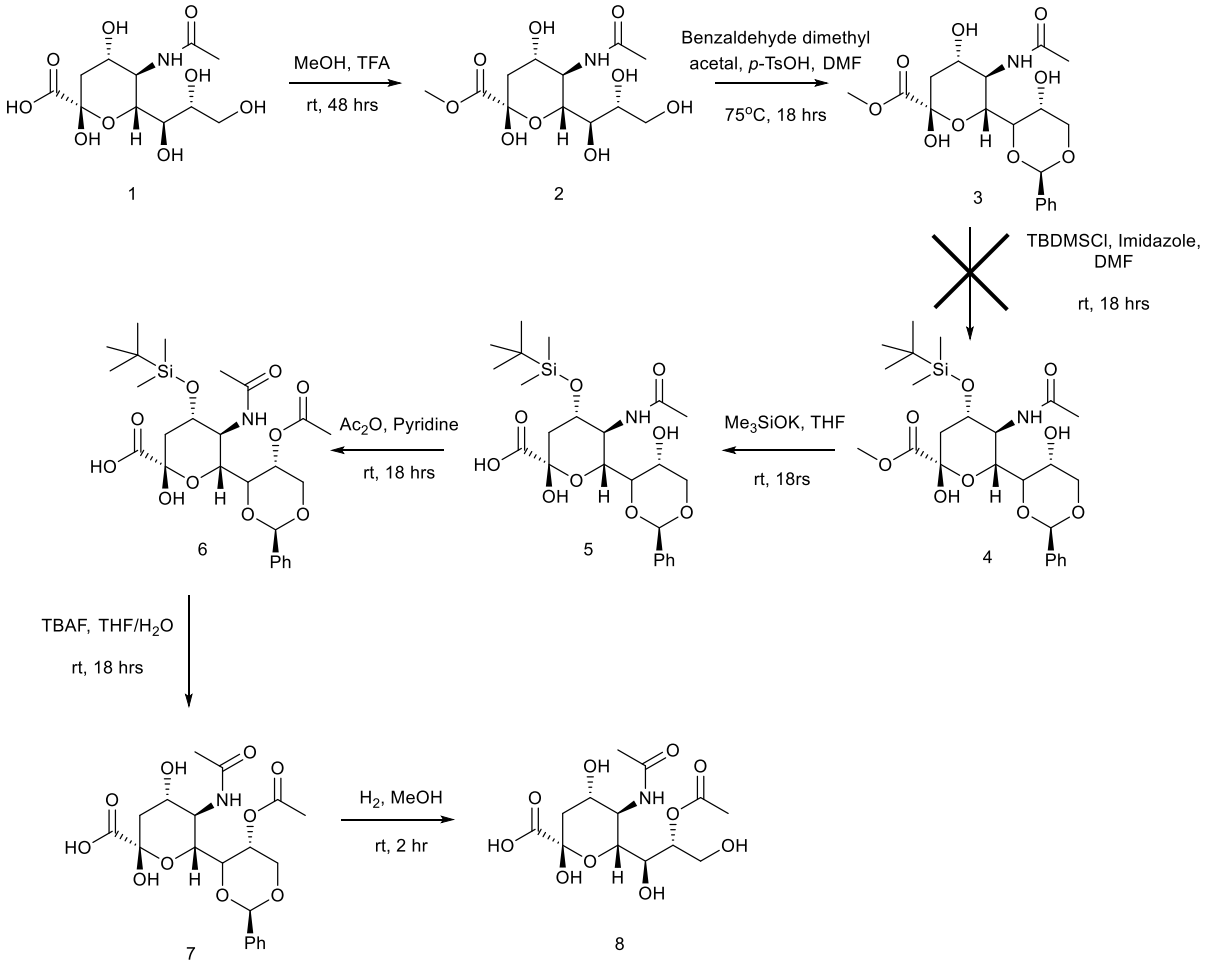


Figure 1: Synthesis of Neu5,8Ac$_2$ **(8)** employing benzylidene and silyl protecting groups

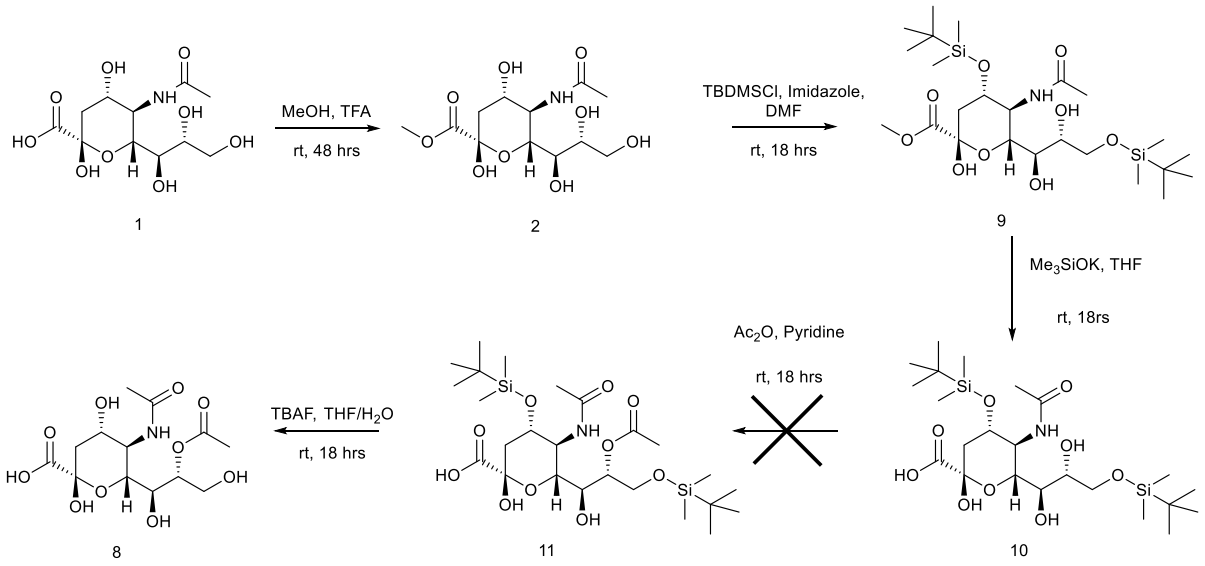


Figure 2: Synthesis of Neu5,8Ac$_2$ **(8)** employing two silyl protecting groups

Synthesis of Neu2,5Ac$_2$ (17)

The synthesis of Neu2,5Ac$_2$ commenced with protecting the carboxylic acid group to give a methyl ester **(2)** in quantitative yield. This was then followed by the simultaneous protection of the C-8 and C-9 hydroxyl groups with an acetonide protecting group using 2,2'-dimethoxypropane and Amberlyst 15 H^+ resin to give **(12)** in 65% yield. Subsequent protection of the C-4 hydroxyl by the action of TBDMSCl and imidazole yielded the silyl protected sialic acid **(13)** in 88% yield. Deprotection of the methyl ester was then carried out using potassium trimethyl silanolate to give the unprotected carboxylic acid **(14)** in 76% yield. Acetylation of C-2 position was attempted but was unsuccessful, possibly due to poor reactivity of tertiary alcohols.

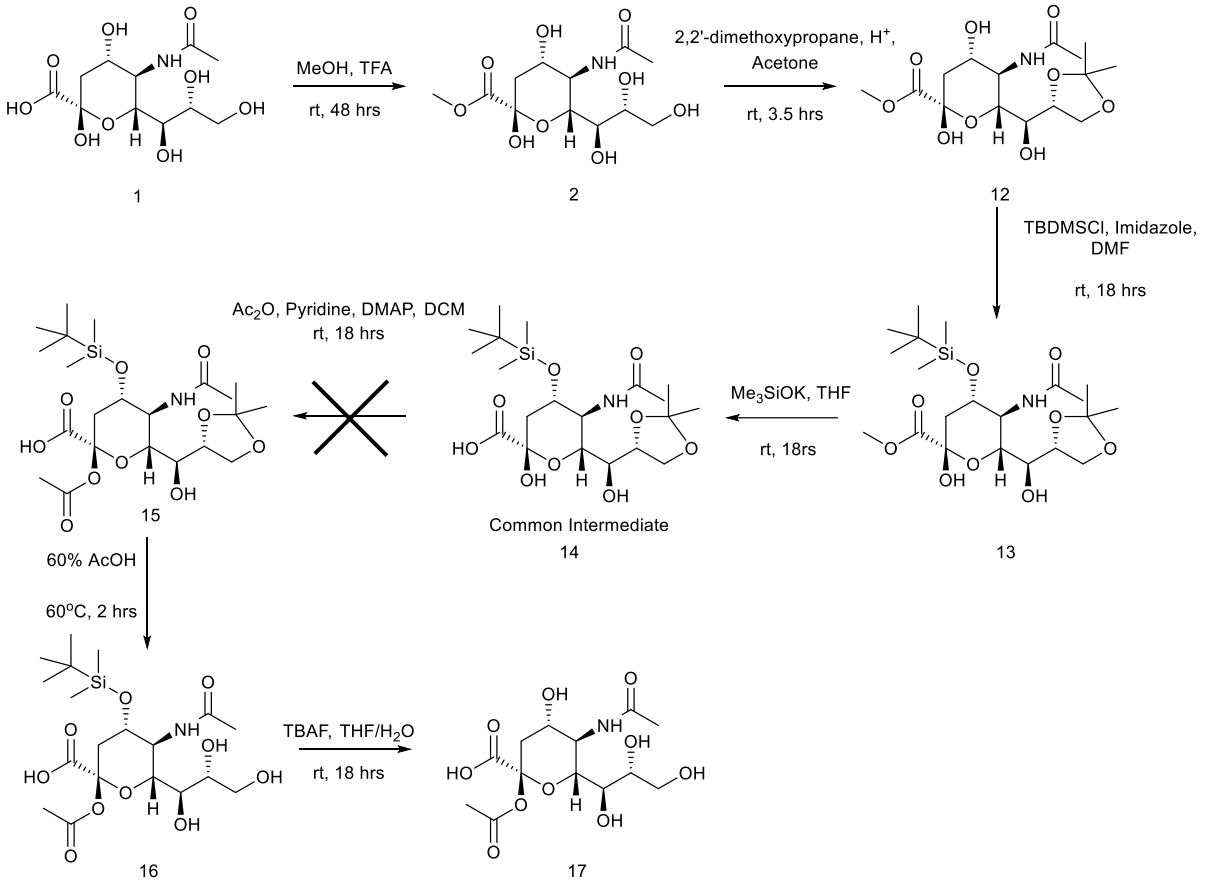


Figure 3: Synthesis of Neu2,5Ac$_2$

Synthesis of Neu5,7Ac$_2$ (21)

The synthesis of Neu5,7Ac$_2$ utilised a common intermediate **(17)** with the synthesis of Neu2,5Ac$_2$. Once the carboxylic acid **(17)** was synthesised, rather than attempting acetylation as in Figure 3 to give **(15)**, the addition of a further protecting group was attempted to give **(18)**. However, attempts to protect the C-2 position were unsuccessful, possibly due to poor reactivity of tertiary alcohols or steric hindrance from bulky protecting groups.

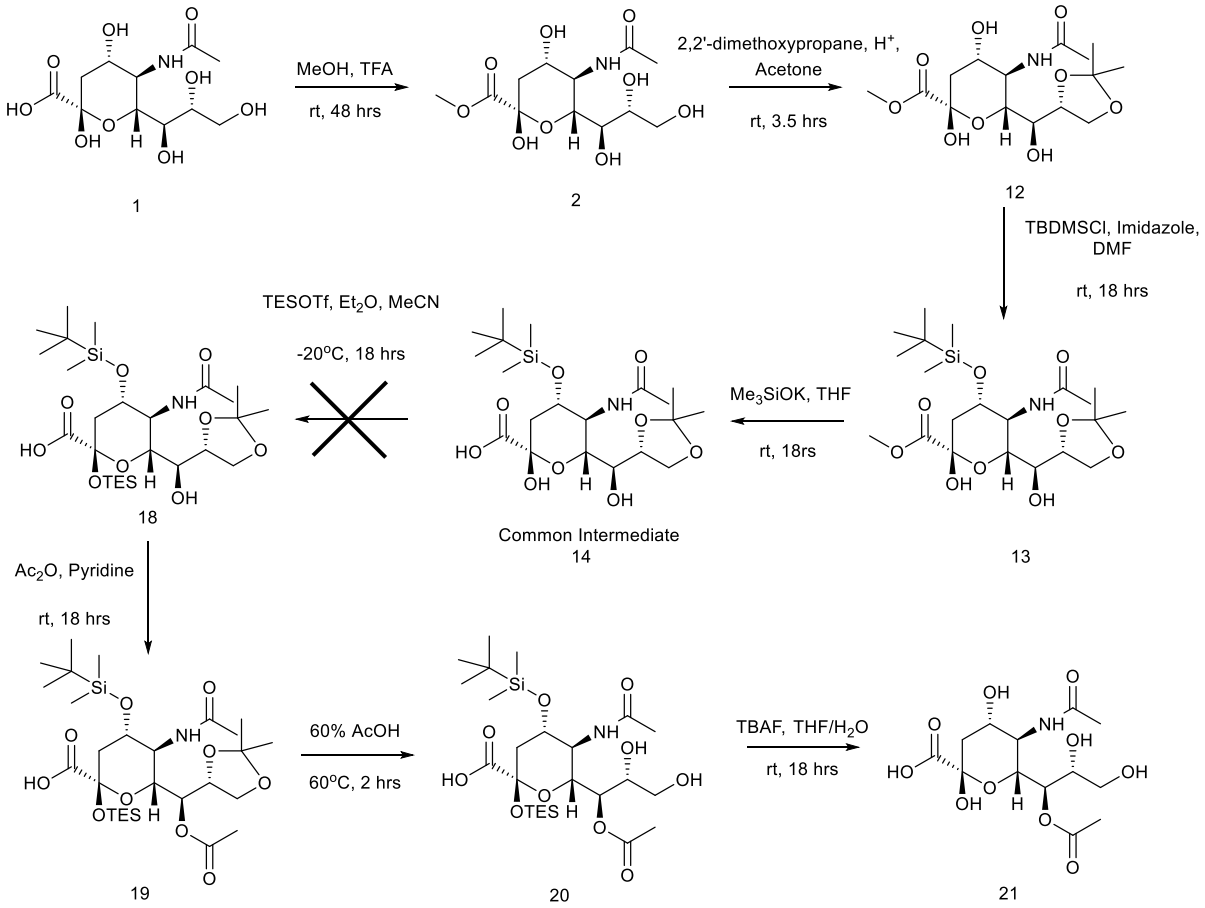


Figure 4: Synthesis of Neu5,7Ac_2

Synthesis of NeuGc (25)

Synthesis of Neu5Gc **(22)** was undertaken to attempt to access not only Neu5Gc itself but also acetylated Neu5Gc derivatives. The synthesis was started by de-*N*-acetylation of Neu5Ac **(1)** with simultaneous protection of the carboxylic acid and C-2 hydroxyl as a methyl ester and methyl ether respectively. This was performed using acetyl chloride in methanol at 100°C for 8 hours to give the de-*N*-acetylated compound **(22)** in 68% yield. The amine was then protected by the action of benzyloxyacetyl chloride and Et_3N to give the amide **(23)** in 25% yield. The methyl ester and methyl ether were deprotected using sodium hydroxide followed by acidic resin to give the deprotected carboxylic acid **(24)** in 89% yield. Two options were then available, deprotection of the benzyl group to yield Neu5Gc, or utilising further protecting group strategies to perform selective acetylation to yield acetylated Neu5Gc derivatives. Due to lack of available material and time constraints, synthesis of Neu5Gc was chosen at this stage. The benzyl group was deprotected using hydrogen and 10% Pd/C to give Neu5Gc **(25)** in quantitative yield.

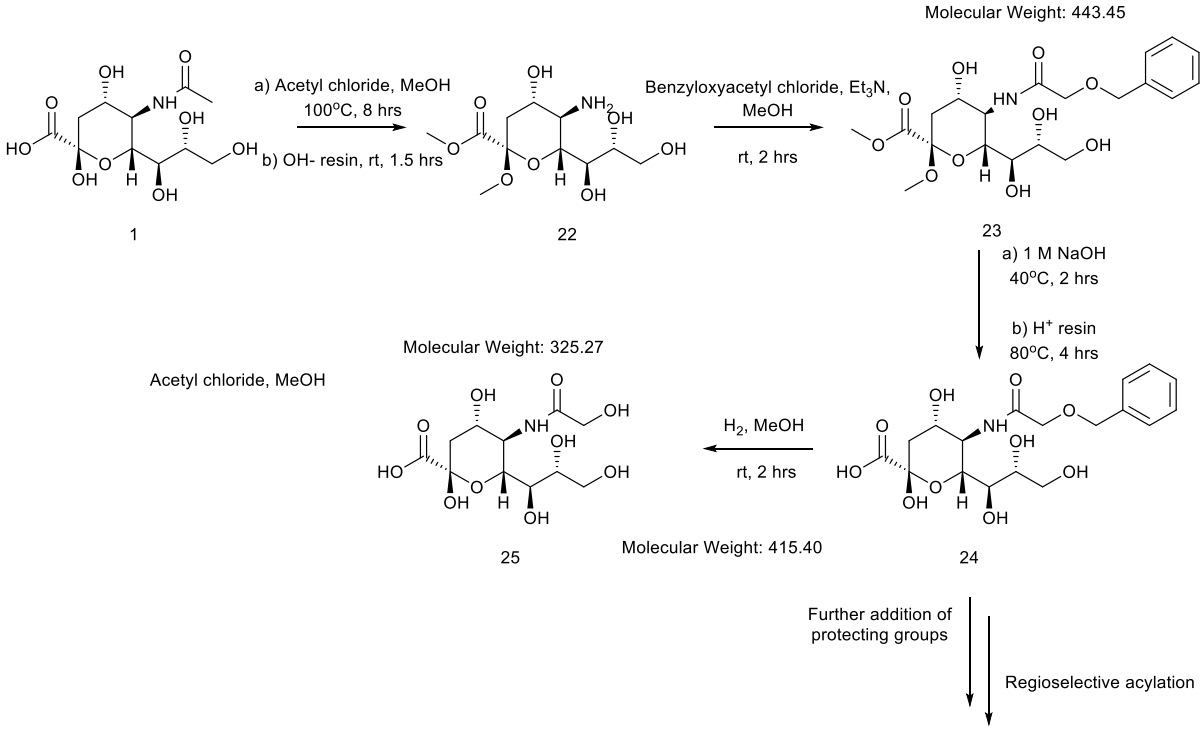


Figure 4: Synthesis of Neu5Gc

Methods

Methyl 5-Acetamido-3,5-dideoxy-7,9-*O*-benzylidene-D-glycero-D-galactononulopyranosonate (7)[4]

To a solution of methyl ester **4** (1.0 g, 3.09 mmol, 1 eq.) in DMF (20 mL) was added benzaldehyde dimethyl acetal (0.51 mL, 3.39 mmol, 1.1 eq.) and *p*-TsOH (50 mg). The reaction was stirred at 75°C for 18 hours in a Dean Stark apparatus. The solvent was removed under reduced pressure by co-evaporation with toluene (3 x 150 mL). Flash column chromatography MeOH:DCM 0-20% yielded the benzylidene **7** as a white powder (64 mg, 5% yield). Characterisation data matched that reported in the literature.[1]

Methyl 5-Acetamido-3,5-dideoxy-4,9-*O*-tert-butyldimethylsilyl-D-glycero-D-galactononulopyranosonate (13)[5]

To a solution of methyl ester **4** (1.0 g, 3.09 mmol, 1 eq.) in DMF (20 mL) at 0°C was added TBDMSCl (1.17 g, 7.73 mmol, 2.5 eq.) and imidazole (1.58 g, 23.18 mmol, 7.5 eq.). The solution was allowed to warm to room temperature after which it was stirred for 18 hours. The solvent was removed under reduced pressure by co-evaporation with toluene (3 x 150 mL). The resultant residue was dissolved in distilled water (100 mL) and extracted with diethyl ether (3 x 100 mL). The organics were combined, washed with brine (300 mL) and dried over Na_2SO_4. The diethyl ether was removed under reduced and the resultant white solid was purified by column chromatography petrol ether:EtOAc 1:1 to yield the silyl

protected compound **13** as a white powder (1.33 g, 78% yield). Characterisation data matched that reported in the literature. [5]

5-Acetamido-3,5-dideoxy-4,9-*O*-tert-butyldimethylsilyl-D-glycero-D-galactononulopyranosonate (14)[6]

To a suspension of the silyl protected compound **13** (0.5 g, 0.91 mmol, 1 eq.) in freshly distilled THF (25 mL) was added potassium trimethylsilanolate (0.14 g, 1.10 mmol, 1.2 eq.). The orange reaction mixture was stirred at room temperature for 18 hours before the solvent was removed under reduced pressure. The resultant residue was dissolved in distilled water (20 mL) and aq. 1 M HCl solution was added dropwise until a pH of 5-6 was achieved. The mixture was filtered, and the filtrate lyophilised to yield the methyl deprotected product **14** as a white solid (0.41 g, 83% yield). Characterisation data matched that reported in the literature. [5]

Methyl 5-acetamido, 3,5-dideoxy-8,9-*O*-isopropylidene-4-*O*-tert-butyldimethylsilyl-D-glycero-β-D-galactononulopyranosonate (16)[1]

To a solution of acetonide **5** (1.0 g, 2.75 mmol, 1 eq.) in DMF (20 mL) at 0^{o}C was added TBDMSCl (0.84 g, 5.50 mmol, 2 eq.) and imidazole (0.94 g, 13.75 mmol, 5 eq.). The solution was allowed to warm to room temperature after which it was stirred for 18 hours. The solvent was removed under reduced pressure by co-evaporation with toluene (3 x 150 mL). The resultant residue was dissolved in distilled water (100 mL) and extracted with diethyl ether (3 x 100 mL). The organics were combined, washed with brine (300 mL) and dried over Na_2SO_4. The diethyl ether was removed under reduced pressure and the resultant white solid was purified by column chromatography MeOH:DCM 0-5% to yield the silyl protected compound **16** as a white powder (1.15 g, 88% yield). Characterisation data matched that reported in the literature. [1]

5-acetamido, 3,5-dideoxy-8,9-*O*-isopropylidene-4-*O*-tert-butyldimethylsilyl-D-glycero-β-D-galactononulopyranosonate (17)[6]

To a suspension of the silyl protected compound **16** (0.5 g, 1.05 mmol, 1 eq.) in freshly distilled THF (20 mL) was added potassium trimethylsilanolate (0.16 g, 1.25 mmol, 1.2 eq.). The orange reaction mixture was stirred at room temperature for 18 hours before the solvent was removed under reduced pressure. The resultant residue was dissolved in distilled water (20 mL) and aq. 1 M HCl solution was added dropwise until a pH of 5-6 was achieved. The mixture was filtered, and the filtrate lyophilised to yield the methyl deprotected product **17** as

a white solid (0.37 g, 76% yield). Characterisation data matched that reported in the literature.[1]

Methyl 5-amino-3,5-dideoxy-α-D-galacto-2-nonulopyranosidonic acid methyl ester (22)[3]

A solution of dry HCl in methanol was prepared by dropwise addition of acetyl chloride (1.9 mL) to methanol (12.5 mL) in an ice bath at 0^{o}C. To this, a solution of Neu5Ac **1** (2.0 g, 6.46 mmol, 1 eq.) was added and refluxed at 100^{o}C for 8 hours. The resultant black solution was concentrated under reduced pressure and the crude product purified by column chromatography MeOH:DCM 10-20% to afford a beige foam. The foam was dissolved in methanol (10 mL) and Amberlyst A26 OH^- resin (1.0 g) was added. This was stirred at room temperature for 1.5 hrs before the resin was filtered out. The solvent was removed under reduced pressure to yield the de-*N*-acetylated product **25** as a beige foam (1.30 g, 68% yield). Characterisation data matched that reported in the literature. [3]

***N*-Benzyloxy-acetyl-2-*O*-methyl-β-neuraminic acid methyl ester (23)[3]**

To a stirring solution of amine **25** (0.5 g, 1.7 mmol, 1 eq.) in methanol (10 mL) containing Et_3N (1.7 mL, 10.2 mmol, 6 eq.) was added dropwise benzyl oxyacetylchloride (0.80 mL, 5.1 mmol, 3 eq.). The solution was stirred for 2 hrs at room temperature after which it was concentrated under reduced pressure, suspended in EtOAc (200 mL) and stirred for 20 minutes. The beige solid was filtered out. The filtrate was evaporated under educed pressure to yield a brown oil which was purified by column chromatography DCM:MeOH 0-10% to yield the product **26** (0.19 g, 25% yield). Characterisation data matched that reported in the literature.[3]

***N*-Benzyloxy-acetyl-β-neuraminic acid (24)[3]**

To a solution of **26** (100 mg, 0.23 mmol, 1 eq.) in distilled water (10 mL) was added 1 M NaOH solution (0.5 mL) and was stirred at 40^{o}C for 2 hours. After which, Amerlyst 15 H^+ resin (100 mg) was added, and the temperature was raised to 80^{o}C and stirred for 4 hours. The resin was filtered out and the filtrate lyophilised to give the product **27** as a white, fluffy solid (85 mg, 89% yield). Characterisation data matched that reported in the literature. [3]

***N*-Glycolyl neuraminic acid (25)[3]**

To a solution of **27** (50 mg, 0.12 mmol, 1 eq.) in methanol (20 mL) under argon was added 10% Pd/C (w/w, 50 mg). The flask was evacuated and backfilled with helium three times. The reaction mixture was stirred at room temperature for 1 hour after which it was filtered through

celite and concentrated under reduced pressure to give the final product **28** as a white powder (39 mg, Quant.). Characterisation data matched that reported in the literature. [3]

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Chapter 5:

The evaluation of sialic acid and 9-*O* acetyl sialic acid and their relationship to cardiovascular disease risk.

Chapter Summary: This chapter details the recruitment of a cohort of volunteers for the assessment of potential associations between sialic acid concentrations in biological fluids and cardiovascular risk (assessed via QRISK3), as well as cardiovascular risk factors. Potential associations were identified in women between urinary sialic acids and QRISK3, as well BMI.

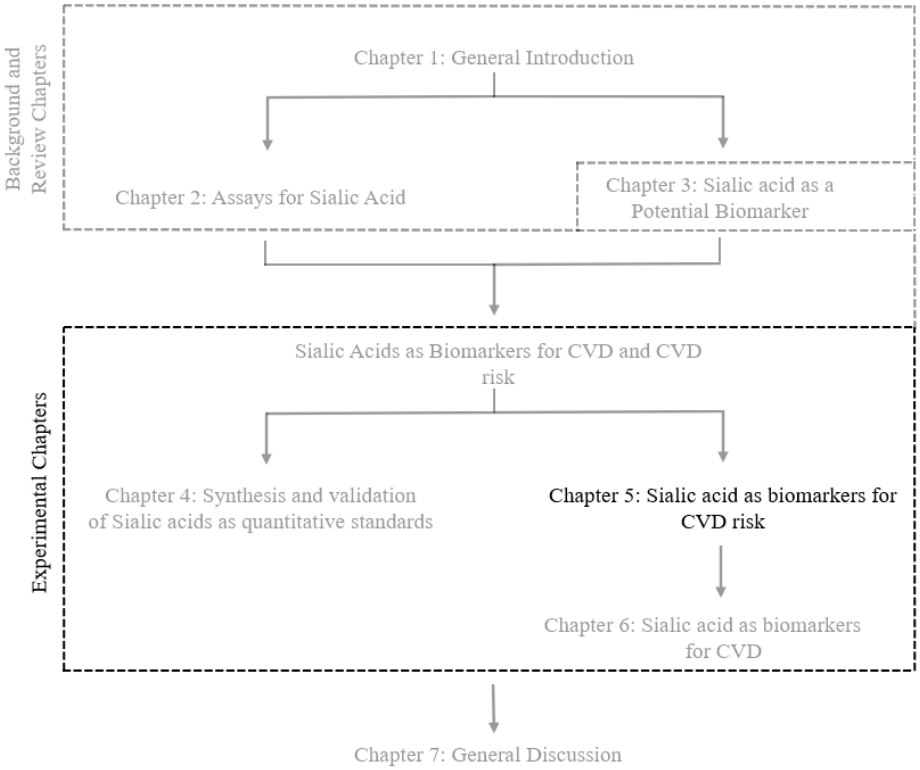
Bibliographic Details: The evaluation of sialic acid and 9-*O*-acetyl sialic acid and their relationship to cardiovascular disease risk. **J. Cheeseman**, C. Badia, K. Jackson, R. A. Gardner, D. I. R. Spencer, H. M. I. Osborn, G. Kuhnle. *Submitted to PLOS One, December 2021*.

Author Contributions: D.I.R.S, G.K and H.M.I.O designed the study, won funding for the programme and supervised the study. J.C designed the volunteer study with the assistance of G.K and K.J. The ethical approval application was prepared by J.C and G.K and was submitted by G.K for approval. J.C carried out recruitment of volunteers and volunteer study visits, samples were prepared for analysis with the aid of K.J. Analysis of samples was designed and carried out by J.C with the assistance of C.B and R.G. Statistical analysis was carried out by G.K and J.C. J.C prepared the first draft of the main manuscript text. Figure 3 was prepared by G.K, all other figures were prepared by J.C. The Manuscript was reviewed by all authors and J.C prepared the final draft for submission.

Appendices: Appendix 1 is part of the prepared journal article and details sensitivity analysis conducted as part of this work.

Appendix 2 contains the volunteer cohort characteristics in full, as well as the measured concentrations of Neu5Ac and Neu5,9Ac_2 in the collected plasma, serum, urine, and saliva samples.

Appendix 3 is the ethics application prepared for the volunteer study and analysis conducted as part of this project, as well as confirmation of ethical approval.


Background and Review Chapters
Chapter 1: General Introduction
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Sialic Acids as Biomarkers for CVD and CVD risk
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Chapter 6: Sialic acid as biomarkers for CVD
Chapter 7: General Discussion

The evaluation of sialic acid and 9-*O* acetyl sialic acid and their relationship to cardiovascular disease risk

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Keywords: Sialic acid, QRISK3, biomarker, cardiovascular disease

Abstract

Cardiovascular disease (CVD) accounted for 34% of global deaths in 2019 and poses an enormous healthcare burden with 523 million cases worldwide. Early treatment of CVD can improve outcomes, and thus sensitive diagnostic markers of CVD are crucial. Currently, risk prediction is based mainly on biomarkers such as blood lipids and blood pressure or using complex algorithms, for example QRISK3. A sensitive biomarker of emerging CVD could supplement risk prediction thereby supporting early interventions and better outcomes. Sialic acid has previously been shown to be a marker for the presence and pathogenesis of CVD, as well as CVD mortality risk. We investigated associations between *N*-Acetyl Neuraminic acid (Neu5Ac) and *N*-acetyl-9-O-acetyl neuraminic acid (Neu5,9Ac_2) concentration in plasma, serum, urine, and saliva samples and CVD risk in 80 volunteers aged 35-75. Associations between Neu5Ac and Neu5,9Ac_2 in urine and QRISK3 relative estimated risk score were significant in women. Sensitivity analyses showed a strong association between Neu5Ac and Neu5,9Ac_2 concentration in plasma and serum and BMI in women.

Introduction

Cardiovascular disease (CVD) is one of the main global health burdens, both in terms of morbidity and mortality and regarding healthcare expenditure. CVD accounts for more than one third of deaths worldwide(1), many of which could have been prevented (2). Biomarkers play a vital role in this early prediction and when combined with other measures such as risk calculation algorithms (QRISK3)(3), thereby, reducing prevalence of CVD and global healthcare burden. QRISK3 is the third iteration of an algorithm developed by Hippisley-Cox *et al*. that measures known CVD risk factors to determine the risk of a person developing CVD within the next 10 years. These risk factors include the total cholesterol to HDL ratio, systolic blood pressure (SBP), BMI, smoking status as health conditions such as chronic kidney disease, atrial fibrillation and rheumatoid arthritis. When using QRISK3, a risk score of > 10% is considered high risk.(4) In the case of this research, estimated relative risk score was utilised, whereby the risk score is compared with that of a healthy person of the same age, sex and ethnicity. For relative risk a score of 1.0 or less means that the person undergoing assessment has the same or lower risk than a healthy person. Whereas a risk of 1.1 or above means that the person has a higher risk than a healthy person.

Sialic acids are a family of nine-carbon backbone monosaccharides generally located as the terminating units of glycans which are in turn found as parts of glycoconjugates such as glycoproteins and glycolipids.(5) Sialic acids have been established to play many important biological functions such as cell-cell interaction and recognition by either acting as a receptor mask or receptor determinant.(6) Sialic acids boast a carboxylic acid functional group which contributes to the overall negative charge of cell surfaces and glycoproteins. The negative charge aids in cell-cell repulsion,(5) the prevention of erythrocyte aggregation(7) and the stability of circulating glycoproteins in blood.(8) Both Neu5Ac and Neu5,9Ac_2 have been shown to have an anti-inflammatory effect.(9–11)

The link between elevated Neu5Ac concentration in plasma and CVD was first observed by Linberg et al. in a large-scale (n = 54385) long term (21 years) study that indicated that elevated plasma Neu5Ac is associated with increased CVD mortality risk in both men and women.(12) The relative risk of volunteers in the highest quartile of plasma sialic acid concentration was 2.38 in men and 2.62 in women when compared to the lowest quartile. Neu5Ac is a marker that can be affected by factors external to CVD. Diseases that cause inflammation: arthritis,(13) type-2 diabetes(14) and chronic obstructive pulmonary disorder(15) can also cause increases in sialic acid concentration.(16) Sialic acid is not the only compound of interest here, for example, the action of esterases in human plasma has been shown to be reduced during an inflammatory state.(17) As such the presence of CVD may cause an increase in the concentration of acetylated sialic acid derivatives if these are not cleared by the action of acetylesterases. It is of interest to investigate the wide variety of different sialic acid derivatives to determine if they may be better markers for CVD. Expanding this research further, previous work has focussed on markers for CVD in plasma and serum. Urinary(18) and salivary(19) sialic acid have been investigated in the context of neurological disorders and oral malignancies respectively, but not for CVD. In this study, we investigated all four biological fluids (plasma, serum, urine and saliva) at once, this could help shed new light on this area of sialic acids as potential biomarkers.

Investigating sialic acid and its derivatives as biomarkers requires sensitive and specific quantitative analytical techniques. Many techniques have been employed since research into sialic acid began with many assays suffering from issues with poor specificity for sialic acids and therefore inaccurate results.(20) More modern assays such as labelling with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by HPLC analysis offers a robust method for the analysis of multiple derivatives in one assay with high specificity for sialic acids.(21,22) For accurate quantitation of sialic acid derivatives, this assay requires quantitative standards. Neu5Ac and Neu5Gc are commercially available but acetylated sialic acid derivatives,

Neu5,9Ac_2 for example, are not readily available and must be chemically synthesised. Neu5,9Ac_2 has previously been synthesised and analysed using quantitative nuclear magnetic resonance (QNMR) techniques(23) to allow for the usage of Neu5,9Ac_2 as a quantitative standard.

Neu5Ac and Neu5,9Ac_2 concentrations were measured in plasma, serum, urine and saliva from 80 volunteers aged 35-75 who were at risk of CVD or otherwise healthy with QRISK3 relative estimated risk scores ranging from 0.6-2.1 (38 with risk $\leq$ 1.0, 42 with risk > 1.0). These concentrations were correlated against QRISK3 estimated relative risk score to determine any potential associations between CVD risk and the concentrations of Neu5Ac and Neu5,9Ac_2.

Materials and Methods

Study Population

Between July and December 2019 80 volunteers were recruited from the community via the Hugh Sinclair Unit for Human Nutrition located within the University of Reading, Department of Food and Nutritional Sciences. The inclusion criteria for the study were: aged 35-75 years old, at risk of CVD (QRISK3 score > 1.0) or otherwise healthy, family history of CVD. Exclusion criteria included: a previous heart event (stroke or heart attack), pregnancy, smoking (current or former) and health conditions that affect sialic acid concentration independent of CVD including type-2 diabetes, cancer (all types), arthritis (all types), and chronic obstructive pulmonary disorder (COPD). All participants provided informed consent and the study was given a favourable opinion for ethical conduct by the University of Reading Research Ethics Committee (UREC 18/39). The study was conducted in adherence with the Declaration of Helsinki.

Sample and Volunteer Information Collection

Each volunteer was screened via e-mail or telephone, if successfully enrolled onto the study each volunteer attended a 30-minute visit to provide information for the calculation of QRISK3

relative estimated risk score which included a brief medical history, a family history of CVD (angina or heart attack in a first degree relative < 60 years of age) and measurements of height, weight, and SBP which was measured 3 times after a period of rest and then the data was averaged. A 2.5 mL unstimulated saliva sample collected by spitting, 10 mL spot urine sample, and two 5 mL venous blood samples were collected from each volunteer. A 5 mL 3.2% sodium citrate VACUETTE tube and a 5 mL serum gel VACUETTE tube was used for collection of blood samples. One blood sample was allowed to coagulate (the serum tube contained silica as a clot activator) at room temperature for 30 minutes. The blood and urine samples were centrifuged at 4^{o}C for 15 minutes at 3000 rpm (906 rcf) The saliva sample was centrifuged for 5 minutes at 15000 rpm (15093 rcf) at room temperature. The supernatant for each sample was collected. Aliquots (500 μL) of plasma, serum, urine and saliva were prepared and frozen at -20^{o}C. The specific gravity of urine was measured using a urinalysis test strip to enable normalisation of results obtained from the quantitative analysis of urine. Each serum sample was analysed for total cholesterol and high-density lipoprotein cholesterol (HDL-C) using a clinical chemistry analyser (RANDOX) using kits supplied by RANDOX. Total cholesterol was measured by the action of cholesterolesterase and cholesterol oxidase. Hydrogen peroxide formed in the reaction was reacted with 4-aminoantipyrine to give a chromophore with an intensity of 600 nm. HDL was measured by elimination of non-HDL cholesterol by the action of cholesterolesterase, cholesterol oxidase and catalase. Following release of HDL cholesterol with detergent, the reaction as for total cholesterol was repeated to give a chromophore.

Analytical Methods

Analysis of sialic acids was carried out using the DMB method. (23,24) The DMB method utilises a mild acidic release to cleave sialic acids from glycans whereupon the sialic acids are reacted with DMB to form a fluorophore. The resulting fluorophores of sialic acids were injected onto an LC system where they can be separated and quantitively analysed. Release of Neu5Ac and Neu5,9Ac$_2$ and DMB labelling of the samples was achieved using LudgerTagTM

DMB Sialic Acid (LT-KDMB-96). Five µL of plasma and serum and 10 µL of urine and saliva (for Neu5Ac analysis) or 20 µL of all samples for Neu5,9Ac_2 analysis were added to a 96-well plate. Each sample was subjected to acid release with 25 µL (for Neu5Ac) or 80 µL (for Neu5,9Ac_2) of 2M acetic acid. The samples were manually vortexed and centrifuged followed by incubation at 80^{o}C for 2 hours. The samples were cooled to room temperature before 5 µL (for Neu5Ac) or 20 µL (for Neu5,9Ac_2) of each released sample was transferred to a new 96-well plate. To this, 20 µL DMB labelling solution was added. For samples used to analysed Neu5,9Ac_2 values, 5 µL of Neu5Gc standard was added at this step. The samples were manually vortexed and centrifuged followed by incubation for 3 hours at 50^{o}C. The reaction was quenched by addition of water to make the volume up to 500 µL. Plasma and serum samples were then subjected to a 1 in 10 dilution, whereas neat urine and saliva were used for the analysis. All work was carried out using a Hamilton STARlet Liquid Handling Robot, apart from the initial dispensing of the samples into the 96-well plate. Analysis of the samples was performed in triplicate for Neu5Ac determination. For Neu5,9Ac_2 only one replicate of each was analyse in order to have all the samples analysed in the same 96 well plate, Neu5Gc internal standard was used in this case to assess intra assay variation.

Fetuin derived from fetal calf serum (GCP-Fet-50U), an A2G2S2 glycopeptide (BQ-GPEP-A2G2S2-10U) both from Ludger Ltd. and recombinant human plasma purchased from Sigma Aldrich were utilised as system suitability standards. These standards were subjected to the same release and labelling conditions as stated above for the samples containing Neu5Ac.

Standards of Neu5Ac, Neu5Gc and Neu5,9Ac_2 were also labelled using LudgerTagTM DMB Sialic Acid (LT-KDMB-96). 20 µL of labelled solution was added to each standard. The samples were vortexed and centrifuged before incubating for 3 hours at 50^{o}C. The labelling reaction was quenched with water to bring the final volume to 500 µL. Standards curves were prepared for each standard with points: 0.01-1 nmol.

The labelled sialic acids were analysed by LC-FLD (Figure 1). 5 µL of sample was injected into a UHPLC equipped with a fluorescence detector (λex = 373 nm, λem = 448 nm). For Neu5Ac analysis in plasma, serum and saliva an isocratic solvent system (7:9:84 %v/v MeOH:ACN:H_2O) was used. For Neu5Ac analysis in urine, and Neu5,9Ac_2 and Neu5Gc analysis in all samples a gradient solvent system was used 7:6:87 %v/v MeOH:ACN:H_2O for 6.5 minutes followed by 6:9:85 MeOH:ACN:H_2O for 11.5 minutes.

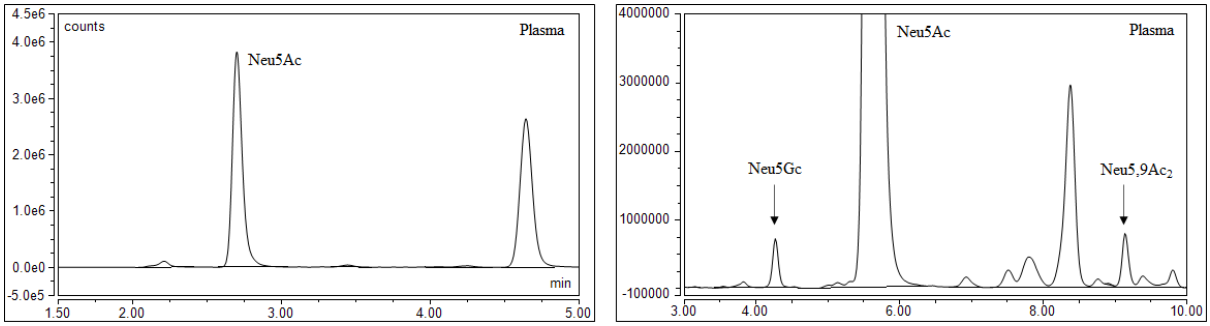

Figure 1: UHPLC traces: Neu5Ac analysis (left) and analysis of Neu5,9Ac_2 with Neu5Gc internal standard (right).

Neu5Ac analysis exhibited an intra-assay and inter-assay variation of < 10%. Neu5,9Ac_2 analysis exhibited an intra-assay and inter-assay variation of < 10%. The Neu5Gc internal standard variation was different between biological fluids: plasma (5%), serum (6%), urine (11%) and saliva (19%). Overall, these values show a low level of intra-assay and inter-assay variation apart from saliva which showed higher variation than the other biological fluids. Saliva also suffered from issues that while Neu5Ac sufficiently met the limit of detection in all saliva samples, 30 of the 80 samples had concentrations of Neu5,9Ac_2 that while they met the limit of detection, were below the limit of quantitation. As such, the full picture for Neu5,9Ac_2 and saliva may not be available. Neu5Ac was detectable and quantifiable in all samples whereas data for Neu5,9Ac_2 were only available in plasma, serum and urine.(23)

Statistical Analysis

The cohort was stratified according to sex. Results are presented as mean ± standard deviation. We used a=0.05 as threshold for statistical significance and did not adjust for multiple comparisons. Correlations between Neu5Ac or $Neu5,9Ac_2$ and QRISK3 or associated factors were estimated using multiple linear regression analysis without adjustment unless indicated otherwise. Analysis was conducted using R version 4.1.1.(25)

Results

Eighty volunteers were recruited based on inclusion/exclusion criteria to form the cohort studied in this research (Figure 2). The clinical characteristics of the cohort are shown in table 1. We investigated associations in men and women between Neu5Ac and $Neu5,9Ac_2$ concentrations in plasma, serum, urine, and saliva and QRISK3 relative estimated risk score (Table 2). Statistically significant associations were observed between urinary Neu5Ac and $Neu5,9Ac_2$ and QRISK3 relative estimated risk in women (Figure 3). Sensitivity analyses revealed a strong association between Neu5Ac and $Neu5,9Ac_2$ concentrations in plasma ($P = 0.007$) and serum ($P < 0.001$) and BMI in women (Appendix 1).

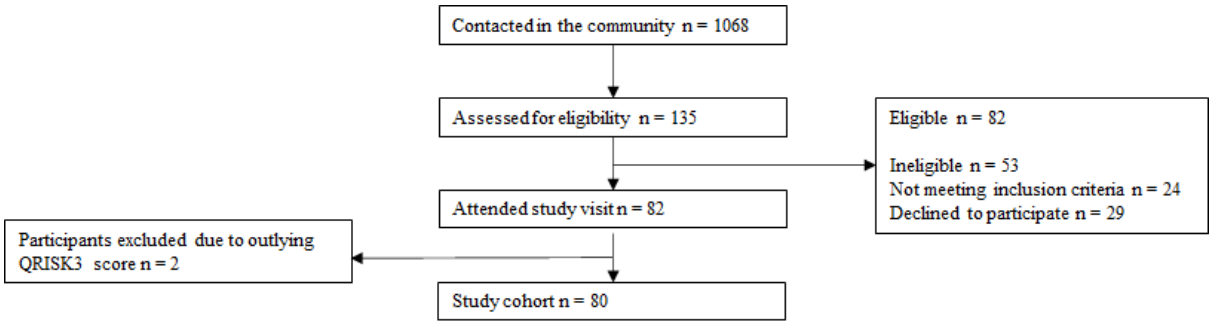

Figure 2: CONSORT flow diagram of cohort recruitment.

Table 1: Cohort clinical characteristics

	Men	Women	All
n	38	42	80
Age (ears)	57 ± 11	60 ± 9	58 ± 10
Height (cm)	176 ± 7.8	163 ± 7.8	170 ± 10
Weight (kg)	78.8 ± 11.0	65.9 ± 14.4	72.6 ± 14.2
BMI (kg/m^2)	25.5 ± 3.6	24.8 ± 4.8	25 ± 4.3
SBP	128 ± 11	130 ± 15	129 ± 13
Total Cholesterol (mmol/ L)	5.5 ± 0.97	6.0 ± 1.12	5.72 ± 1.07
HDL-C data	2.09 ± 0.59	1.73 ± 0.72	1.90 ± 0.67
Cholesterol/HDL-C ratio	3.46 ± 1.02	3.17 ± 1.11	3.29 ± 1.08
QRISK3 score (range)	1.1 (0.6-1.9)	1.1 (0.6-2.1)	1.1 (0.6-2.1)
Neu5Ac (mg/100 mL)			
Plasma	45.0 ± 6.6	47.9 ± 8.6	46.4 ± 7.7
Serum	60.4 ± 8.7	62.7 ± 9.2	61.5 ± 8.9
Urine	4.41 ± 2.80	3.55 ± 2.54	4.00 ± 2.72
Saliva	2.63 ± 1.49	2.45 ± 2.31	2.55 ± 1.91
$Neu5,9Ac_2$ (mg/100 mL)			
Plasma	0.37 ± 0.043	0.38 ± 0.04	0.38 ± 0.04
Serum	0.39 ± 0.07	0.41 ± 0.06	0.40 ± 0.07
Urine	0.68 ± 0.60	0.38 ± 0.32	0.54 ± 0.50
Saliva	0.38 ± 0.69	0.17 ± 0.14	0.30 ± 0.55

Table 2: Associations between Neu5Ac, Neu5,9Ac$_2$ and QRISK3 with correlation coefficients (R^2) for the associations

Factor (Material)	Marker	Men (P-value)	R^2	Women (P-value)	R^2
QRISK3 (Plasma)	Neu5Ac	0.071	0.127	0.881	0.007
QRISK3 (Serum)	Neu5Ac	0.142	0.097	0.686	0.021
QRISK3 (Urine)	Neu5Ac	0.638	0.023	0.009*	0.234
QRISK3 (Saliva)	Neu5Ac	0.738	0.016	0.189	0.099
QRISK3 (Plasma)	Neu5,9Ac$_2$	0.856	0.008	0.118	0.115
QRISK3 (Serum)	Neu5,9Ac$_2$	0.312	0.059	0.143	0.105
QRISK3 (Urine)	Neu5,9Ac$_2$	0.261	0.269	0.005*	0.068
QRISK3 (Saliva)	Neu5,9Ac$_2$	0.917	0.006	0.128	0.186

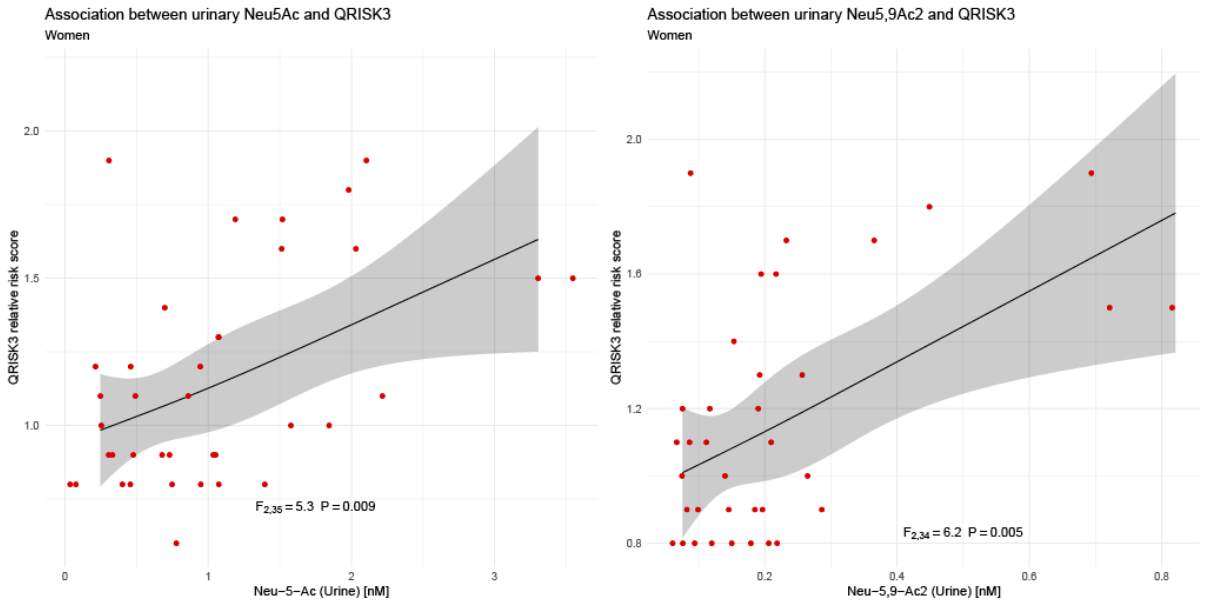

Figure 3: Regression analysis plots for the association between QRISK3 relative estimated risk and Neu5Ac and Neu5,9Ac$_2$ concentrations

Discussion

In this study, we have investigated the associations of sialic acid CVD risk. Our results suggest that at least in women, there are strong associations between sialic acid and QRISK3 estimated relative risk score in urine.

Elevated urinary sialic acid in relation to QRISK3 score may be caused by several factors. Overexpression of mucins may be one possible explanation for the increase in Neu5Ac and Neu5,9Ac_2 observed. The bladder is lined with epithelial cells which produce mucins which have been indicated to be overexpressed as a response to inflammation.(26) Mucins are a family of large complex glycoproteins that are decorated with a wide array of *O*-glycans which comprises more than 70% of their mass.(27) The *O*-glycans identified are highly sialylated and exhibit an array of mono, di and tri-acetylated neuraminic acid derivatives and therefore may account for the observed increase in urinary Neu5Ac and Neu5,9Ac_2. Urinary Neu5Ac concentration reflects serum Neu5Ac concentration as the increased renal clearance of serum glycoproteins leads to an increase of sialylated glycans in urine.(28) Increased concentrations of serum, and therefore urinary, sialylated glycans may be linked to inflammation which is associated with increased CVD risk wherein the acute-phase response causes an increase in concentrations of certain glycoproteins such as: alpha-1-acid glycoprotein, alpha-1-antitrypsin, fibrinogen, alpha-2-macroglobulin and hemopexin.(29). These proteins are decorated with a variety of sialylated glycans and are likely to contain acetylated sialic acid derivatives in small quantities. Clerc *et al.* identified A2G2S2 as the main glycan present in human plasma especially in relation to alpha-1-antitrypsin and hemopexin. Alpha-1-acid glycoprotein is particularly interesting in that it acts as the source for nearly all highly sialylated glycans (three or four sialic acid units).(30) Given that these glycoproteins contain a large quantity of sialic acids an increase in glycoproteins would result in higher total sialic acid concentrations in serum and therefore urine. During an inflammatory state it has been observed that there is a decrease in the activity of plasma esterase. The decrease observed would result in reduced

conversion of Neu5,9Ac_2 to Neu5Ac by cleavage of the acetyl group. This may lead to a higher concentration of Neu5,9Ac_2 to be observed when inflammation is present.

The association between Neu5Ac and Neu5,9Ac_2 in plasma and serum concentrations and BMI in women revealed by sensitivity analyses (Appendix 1) may be able to be explained similarly. Elevated BMI (> 25 kg/m^2) has been associated with low-grade inflammation characterised by increased levels of C-reactive protein (CRP).(31) Inflammation in turn leads to an acute-phase response which as discussed above may result in increased concentrations of sialic acids.

Strengths and Limitations

The sample size for this study, especially in the male and female groups was small. The current range of QRISK relative estimated risk scores (0.6-2.1) is quite small also, as such it would be of interest to expand this range of QRISK3 scores. Recruiting volunteers at a much higher risk of CVD (QRISK3 relative estimated risk score > 5) with higher blood pressure, BMI and cholesterol/HDL ratio would aid in the validation of this marker at both low and high risk of CVD.

Conclusions

Neu5Ac and Neu5,9Ac_2 concentrations were positively associated with increased QRISK3 relative risk score in urine in women. The observed associations are possibly related to overexpression of sialylated glycoproteins and mucins as a result of inflammation and the subsequent acute-phase response. Neu5Ac and Neu5,9Ac_2 concentrations appear to be affected by inflammation and the acute-phase response and as such may act as potential markers for this. Other risk factors may also affect concentrations of Neu5Ac and Neu5,9Ac_2 but only in volunteers who exhibit higher risk of CVD. The cohort studied here however, while having a range of QRISK3 scores, did not contain volunteers with a high enough risk score to sufficiently determine further relationships between sialic acid concentrations and QRISK3, or factors such as BMI, SBP and HDL-C ratio. To further this research, recruitment of volunteers

with higher QRISK3 scores (> 5) may be necessary to elucidate any further correlations between elevated CVD risk or CVD risk factors and elevated Neu5Ac or Neu5,9Ac_2 concentrations in biological fluids. This would aid in the establishment of whether sialic acids could act as markers for elevated CVD risk.

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Appendix 1

Associations between QRISK3 parameters and Neu5Ac concentrations in plasma, serum, urine, and saliva

Factor (Material)	Men (P-value)	R^2	Women (P-value)	R^2
SBP (Plasma)	0.566	0.139	0.094	0.157
SBP (Serum)	0.196	0.190	0.837	0.038
SBP (Urine)	0.328	0.174	0.112	0.148
SBP (Saliva)	0.064	0.228	0.082	0.164
Cholesterol/HDL-C ratio (Plasma)	0.46	0.132	0.494	0.069
Cholesterol/HDL-C ratio (Serum)	0.109	0.194	0.671	0.051
Cholesterol/HDL-C ratio (Urine)	0.557	0.119	0.163	0.129
Cholesterol/HDL-C ratio (Saliva)	0.414	0.143	0.614	0.098
Total Cholesterol (Plasma)	0.416	0.081	0.956	0.258
Total Cholesterol (Serum)	0.757	0.047	0.831	0.265
Total Cholesterol (Urine)	0.26	0.106	0.55	0.283
Total Cholesterol (Saliva)	0.136	0.137	0.439	0.297
BMI (Plasma)	0.92	0.047	0.005*	0.331
BMI (Serum)	0.19	0.125	0.004*	0.342
BMI (Urine)	0.709	0.059	0.06	0.220
BMI (Saliva)	0.806	0.047	0.115	0.206

SBP: Systolic Blood Pressure; HDL-C ratio: high density lipoprotein cholesterol.

Associations between QRISK3 parameters and Neu5,9Ac_2 concentrations in plasma, serum, urine, and saliva

Factor (Material)	Men (P-value)	R^2	Women (P-value)	R^2
SBP (Plasma)	0.877	0.118	0.18	0.124
SBP (Serum)	0.336	0.165	0.462	0.072
SBP (Urine)	0.433	0.162	0.081	0.169
SBP (Saliva)	0.687	0.138	0.138	0.372
HDL-C ratio (Plasma)	0.782	0.106	0.95	0.031
HDL-C ratio (Serum)	0.178	0.172	0.975	0.029
HDL-C ratio (Urine)	0.35	0.167	0.191	0.125
HDL-C ratio (Saliva)	0.058	0.287	0.267	0.222
Total Cholesterol (Plasma)	0.709	0.054	0.08	0.362
Total Cholesterol (Serum)	0.579	0.061	0.302	0.309
Total Cholesterol (Urine)	0.006*	0.278	0.586	0.281
Total Cholesterol (Saliva)	0.677	0.058	0.228	0.297
BMI (Plasma)	0.15	0.136	0.007*	0.318
BMI (Serum)	0.213	0.120	< 0.001*	0.395
BMI (Urine)	0.101	0.158	0.133	0.199
BMI (Saliva)	0.006*	0.324	0.184	0.271

SBP: Systolic Blood Pressure; HDL-C ratio: high density lipoprotein cholesterol ratio.

Appendix 2

Table 1: Volunteer characteristics and calculate QRISK3 estimated relative risk score (QRISK3 score)

Volunteer ID	Sex	Ethnicity	Age (Years)	BMI (kg/m^2)	BP Average (mm/Hg)	Cholesterol (mmol/ L)	HDL (mmol/ L)	Cholesterol/ HDL ratio	QRISK3 score
1	Male	White British	35	27.4	137.7	5.41	1.29	4.19	1.4
2	Male	White British	49	25.5	143.3	6.55	1.74	3.76	1.2
3	Male	White British	45	26.2	127.0	6.08	1.44	4.22	1.0
4	Female	White British	60	34.7	142.3	5.79	1.21	4.79	1.9
5	Male	White British	62	25.1	125.3	4.51	1.07	4.21	1.1
6	Male	White British	52	25.7	115.7	3.11	1.03	3.02	1.6
7	Female	White British	64	20.7	111.3	6.74	2.25	3.00	0.8
8	Male	White British	57	25.4	135.3	6.34	1.82	3.48	1.5
9	Male	White British	40	21.0	115.7	4.45	1.46	3.05	0.7
10	Female	White British	58	31.1	136.3	5.63	1.88	2.99	1.5
11	Male	White British	41	25.5	130.0	5.02	1.46	3.44	1.0
12	Female	White British	69	25.1	134.0	5.19	2.27	2.29	1.0
13	Female	White British	56	19.1	132.3	5.10	2.81	1.81	0.9
14	Male	White British	56	26.6	147.0	6.54	1.94	3.37	1.2
15	Female	White British	37	27.7	126.7	7.38	1.24	5.95	5.9
16	Female	White British	67	25.4	155.3	8.66	3.77	2.30	1.8
17	Female	White British	64	27.2	143.0	4.92	1.32	3.73	1.5
18	Female	White British	43	24.3	139.3	4.42	1.78	2.48	1.1
19	Male	White British	58	29.8	131.7	6.29	1.13	5.57	1.5
20	Male	White British	48	21.3	135.0	6.01	1.52	3.95	1.2
21	Male	White British	61	26.1	128.3	4.70	2.26	2.08	1.1
22	Female	White British	63	17.5	116.7	6.95	2.57	2.70	0.8

23	Male	White British	50	21.0	111.7	5.28	1.65	3.20	0.8
24	Female	White British	50	26.7	146.7	5.07	1.42	3.57	1.3
25	Female	White British	53	30.0	125.7	6.76	2.60	2.60	1.2
26	Male	White British	46	30.8	134.0	6.04	1.53	3.95	1.6
27	Male	White British	55	25.2	126.7	6.28	1.29	4.87	1.3
28	Female	White British	66	27.7	118.3	4.72	1.70	2.78	1.6
29	Male	White British	58	30.1	139.7	5.51	1.40	3.94	1.2
30	Male	White British	57	24.2	139.7	6.02	1.96	3.07	1.2
31	Male	White British	56	29.6	136.0	6.62	1.67	3.96	1.1
32	Female	White British	68	22.6	111.3	4.82	1.90	2.54	0.9
33	Female	White British	72	22.7	110.7	6.51	2.11	3.09	0.8
34	Male	White British	37	22.7	128.0	3.46	1.27	2.72	0.8
35	Male	White British	38	26.7	128.3	4.74	1.25	3.79	1.1
36	Female	White British	65	26.1	146.0	7.86	2.01	3.91	1.2
37	Female	White British	59	21.4	161.3	6.87	3.35	2.05	1.0
38	Female	White British	59	23.3	101.7	4.95	2.67	1.85	0.8
39	Female	White British	64	26.4	132.0	7.04	1.76	4.00	1.1
40	Male	White British	47	22.6	127.0	5.03	1.39	3.62	0.9
41	Male	White British	64	21.8	135.7	5.65	2.13	2.65	0.9
42	Male	White British	73	25.6	126.7	4.04	1.80	2.24	0.8
43	Male	White British	63	28.1	109.0	4.41	1.38	3.20	1.4
44	Male	White British	58	23.6	128.3	5.37	2.52	2.13	0.9
45	Male	White British	71	38.0	129.7	4.87	1.59	3.06	0.9
46	Female	White British	64	26.5	142.3	6.11	1.46	4.18	1.9
47	Female	White British	56	24.1	115.3	5.36	1.64	3.27	1.3
48	Female	Arab	53	21.2	124.7	5.45	2.71	2.01	0.8
49	Female	White British	36	22.4	118.7	4.75	1.71	2.78	0.8

50	Female	White British	75	21.8	119.0	6.27	1.97	3.18	0.9
51	Male	White British	44	19.5	106.7	5.61	3.26	1.72	0.6
52	Male	White British	46	24.0	140.3	6.27	1.97	3.18	1.7
53	Male	White British	62	30.0	125.7	6.20	1.10	5.64	2.1
54	Male	White British	67	20.3	139.7	4.48	1.27	3.53	1.1
55	Male	White British	70	28.7	144.3	7.66	2.24	3.42	1.1
56	Male	White British	68	31.0	133.7	4.38	1.06	4.13	1.3
57	Female	White British	65	24.1	101.3	8.36	1.53	5.46	1.4
58	Male	White British	75	19.9	135.3	7.20	3.03	2.38	0.9
59	Female	White British	57	24.1	116.7	5.67	2.19	2.59	1.1
60	Male	Indian	63	29.5	156.0	5.32	0.80	6.65	3.0
61	Female	White British	68	20.1	110.3	6.39	2.42	2.64	0.8
62	Female	White British	50	18.9	123.7	5.19	1.83	2.84	0.8
63	Male	White British	56	24.0	138.3	5.05	2.32	2.18	0.9
64	Female	White British	40	20.2	108.0	4.67	1.86	2.51	0.6
65	Male	White British	68	26.6	124.0	6.50	2.90	2.24	0.9
66	Female	White British	51	32.8	133.0	4.14	1.51	2.74	1.1
67	Male	White British	73	25.5	134.0	4.71	1.75	2.69	0.9
68	Female	White British	63	23.9	137.0	6.68	2.81	2.38	0.9
69	Female	White British	48	28.1	122.3	5.02	1.02	4.92	1.7
70	Male	White British	74	25.1	147.3	5.88	2.02	2.91	1.2
71	Female	White British	51	39.0	120.0	5.61	1.37	4.09	1.2
72	Female	White British	62	19.0	136.3	7.26	2.86	2.54	1.0
73	Male	White British	64	26.4	132.0	5.00	1.56	3.21	1.2
74	Female	White British	53	21.7	123.7	5.80	1.99	2.91	0.9
75	Male	White British	59	20.9	122.3	4.34	1.66	2.61	0.9
76	Female	White British	69	25.1	151.3	6.16	1.28	4.81	1.7

77	Male	White British	65	26.0	120.3	6.04	3.20	1.89	0.7
78	Female	White British	75	22.1	138.7	6.20	3.67	1.69	0.9
79	Female	White British	73	20.3	135.7	6.51	3.64	1.79	0.9
80	Female	White British	52	34.4	119.7	7.46	1.27	5.87	1.6
81	Male	White British	75	24.2	155.0	6.10	2.06	2.96	0.9
82	Male	White British	49	23.8	104.0	5.79	1.38	4.20	0.9

Table 2: Neu5Ac concentrations measured using the DMB assay in plasma, serum, urine and saliva

Volunteer ID	Neu5Ac (mg/100 mL) plasma	Neu5Ac (mg/100 mL) serum	Neu5Ac (mg/100 mL) urine	Neu5Ac (mg/100 mL) saliva
1	47.72	65.33	5.16	2.99
2	39.15	62.42	3.38	1.45
3	44.24	59.85	3.32	1.87
4	48.08	79.95	6.50	1.46
5	40.79	62.42	9.07	1.98
6	46.34	52.35	3.60	2.08
7	43.28	62.92	2.31	1.14
8	36.64	51.35	2.77	1.78
9	35.45	38.25	6.19	4.84
10	56.62	69.54	10.96	N/A
11	45.36	58.61	2.80	3.75
12	57.55	71.17	4.87	1.73
13	50.25	60.25	2.26	1.87
14	39.46	60.13	7.43	2.02
15	65.81	90.29	14.89	2.52
16	48.84	56.68	6.12	1.47
17	48.56	69.35	10.21	0.84

18	44.94	57.45	6.85	2.39
19	43.35	N/A	1.27	1.64
20	35.31	49.24	2.30	3.04
21	40.28	52.85	6.22	5.51
22	47.28	60.48	1.41	7.32
23	43.64	52.96	4.89	2.72
24	41.87	60.26	3.32	2.02
25	43.92	64.55	1.42	2.71
26	33.64	61.92	2.60	2.82
27	54.99	58.48	2.31	3.77
28	46.96	61.47	4.68	1.33
29	40.38	65.45	0.00	1.73
30	39.41	63.64	7.72	7.03
31	40.78	56.05	3.81	0.78
32	36.28	56.79	3.20	3.60
33	53.26	65.64	4.31	1.01
34	48.86	59.86	1.08	5.40
35	55.65	61.20	1.70	5.95
36	44.63	70.75	0.66	1.06
37	45.94	63.51	0.78	N/A
38	43.00	68.07	1.24	1.06
39	55.02	58.23	1.52	1.84
40	50.25	62.97	9.82	2.54
41	52.69	73.52	2.08	2.43
42	51.63	52.23	1.00	1.82
43	36.43	59.53	5.62	2.91
44	41.96	58.08	3.61	1.84

45	52.43	73.28	5.98	1.86
46	48.71	58.65	0.95	2.05
47	35.33	53.05	3.31	1.34
48	43.57	60.47	0.11	4.83
49	49.56	50.43	0.23	4.13
50	51.09	57.24	3.25	0.92
51	40.87	56.42	5.48	4.13
52	40.48	50.42	3.52	1.17
53	58.95	58.19	5.07	N/A
54	38.96	47.52	3.43	1.09
55	41.42	58.01	7.09	0.78
56	42.71	69.74	11.10	3.69
57	34.77	59.31	2.15	0.43
58	49.31	72.83	5.11	2.10
59	40.52	52.35	0.76	2.30
60	49.93	64.62	6.50	2.22
61	49.31	75.96	3.32	0.98
62	32.27	55.28	2.93	1.28
63	50.71	69.34	2.52	1.89
64	47.18	50.48	2.40	12.79
65	41.55	45.86	8.07	3.86
66	44.78	50.91	2.66	2.66
67	56.03	65.61	2.71	1.35
68	63.82	74.22	2.09	1.38
69	38.31	52.53	3.67	2.41
70	36.18	65.11	1.32	1.09
71	79.47	95.81	2.92	2.56

72	44.01	54.06	5.70	0.92
73	52.87	74.70	3.43	2.57
74	40.57	62.59	0.94	1.38
75	54.87	71.17	0.69	2.70
76	54.67	60.79	4.69	N/A
77	49.54	79.02	2.83	0.83
78	52.78	56.68	1.02	3.29
79	40.40	55.42	1.47	1.69
80	53.28	62.15	6.28	5.49
81	49.03	64.32	3.46	1.86
82	43.81	53.76	11.54	N/A

Table 2: Neu5Ac concentrations measured using the DMB assay in plasma, serum, urine and saliva

Volunteer ID	Neu5,9Ac2 (mg/100 mL) plasma	Neu5,9Ac2 (mg/100 mL) serum	Neu5,9Ac2 (mg/100 mL) urine	Neu5,9Ac2 (mg/100 mL) saliva
1	0.48	0.58	0.94	0.15
2	0.39	0.45	0.63	N/A
3	0.43	0.51	0.16	0.45
4	0.46	0.53	1.22	N/A
5	0.39	0.46	1.65	1.11
6	0.38	0.46	0.23	0.07
7	0.34	0.41	0.31	N/A
8	0.36	0.40	0.35	0.16
9	0.31	0.32	0.27	0.08
10	0.45	0.57	1.43	N/A
11	0.37	0.36	0.41	0.12
12	0.46	0.54	0.25	N/A

13	0.44	0.46	0.50	0.08
14	0.38	0.42	1.04	0.09
15	0.41	0.48	0.35	N/A
16	0.40	0.44	0.79	0.04
17	0.44	0.49	1.27	N/A
18	0.39	0.41	0.15	N/A
19	0.37	N/A	0.90	2.76
20	0.29	0.32	0.37	0.10
21	0.35	0.39	0.37	0.16
22	0.35	0.43	0.21	0.32
23	0.36	0.36	0.30	0.30
24	0.35	0.39	0.34	0.04
25	0.39	0.46	0.21	0.13
26	0.34	0.34	0.72	0.06
27	0.33	0.36	0.21	0.21
28	0.38	0.44	0.38	0.05
29	0.39	0.35	0.57	0.04
30	0.38	0.40	0.98	0.38
31	0.38	0.43	0.55	N/A
32	0.37	0.42	0.33	0.13
33	0.40	0.39	0.26	N/A
34	0.39	0.43	3.40	0.61
35	0.36	0.34	0.21	0.17
36	0.36	0.43	0.13	N/A
37	0.42	0.40	0.13	N/A
38	0.39	0.41	0.13	N/A
39	0.38	0.37	0.20	N/A

40	0.38	0.41	1.40	0.03
41	0.48	0.49	0.31	N/A
42	0.40	0.40	0.52	N/A
43	0.35	0.38	0.50	0.11
44	0.36	0.32	0.31	N/A
45	0.45	0.48	0.60	2.84
46	0.41	0.38	0.15	N/A
47	0.34	0.34	0.45	N/A
48	0.41	0.43	0.11	0.39
49	0.35	0.35	0.17	0.20
50	0.35	0.40	0.35	N/A
51	0.36	0.36	0.70	0.12
52	0.30	0.27	0.52	N/A
53	0.41	0.46	0.55	N/A
54	0.29	0.27	0.32	N/A
55	0.35	0.31	0.94	N/A
56	0.36	0.40	1.67	0.17
57	0.36	0.34	0.27	N/A
58	0.43	0.46	0.57	0.23
59	0.38	0.33	0.12	0.08
60	0.40	0.43	0.77	0.41
61	0.40	0.39	0.38	N/A
62	0.33	0.32	0.36	N/A
63	0.39	0.38	N/A	N/A
64	0.36	0.34	0.33	0.28
65	0.33	0.33	0.97	0.28
66	0.42	0.42	0.37	0.08

67	0.38	0.39	0.37	0.07
68	0.39	0.40	0.26	0.06
69	0.41	0.40	0.41	0.18
70	0.33	0.31	0.23	0.16
71	0.46	0.55	0.33	0.03
72	0.33	0.35	0.46	N/A
73	0.37	0.34	0.52	0.08
74	0.34	0.31	N/A	N/A
75	0.34	0.34	0.12	0.08
76	0.41	0.38	0.64	0.09
77	0.39	0.35	0.24	N/A
78	0.39	0.39	0.14	0.29
79	0.27	0.32	0.17	0.23
80	0.33	0.40	0.34	0.51
81	0.37	0.37	0.52	0.07
82	0.33	0.37	1.60	N/A

Appendix 3: Ethics application and notifications of approval for project UREC 18/39: Sialic Acids as Biomarkers for Cardiovascular Disease

UREC 18/39

Sialic Acids as Biomarkers for Cardiovascular disease.

Dr Gunter Kuhnle, CFP

SCHOOL OF CHEMISTRY, FOOD AND PHARMACY (SCFP)

ETHICS APPLICATION INTERNAL REVIEW COVER SHEET
UREC APPLICATIONS

Submissions to the University Ethics and Research Committee cannot be made without completion of this form

Title of Project …Sialic Acids as Biomarkers for Cardiovascular disease ……..

Investigator(s) …Gunter Kuhnle, Helen Osborn, Jack Cheeseman

Department ……Food & Pharmacy

1. Has this application been read by your Supervisor/PI ? (where applicable)

(YES)
NO
NOT APPLICABLE

2. Has your Group Internal Reviewer read the application and any suggested revisions been undertaken?

(YES)
NO

Internal Reviewer Signature ..... Date 13/2/18

Head of Department Signature ... Date 15/9/18

Ethics Administrator Signature .... Date 14/9/18

Application Form for UREC Applications

SECTION 1: APPLICATION DETAILS

1.1

Project Title: Sialic Acids as Biomarkers for Cardiovascular disease

Date of Submission: Proposed start date: Proposed End Date:

1.2

Principal Investigator: Gunter Kuhnle

Office room number: Harry Nursten 2-14 Internal telephone: 7723

Email address: g.g.kuhnle@reading.ac.uk Alternative contact telephone:

Other applicants

Name: Helen Osborn Staff
Institution/Department: Food and Nutritional Science
Email: h.m.i.osborn@reading.ac.uk

Name: Jack Cheeseman.Student
Institution/Department Pharmacy
Email: rv015433@reading.ac.uk

1.3

Project Submission Declaration

I confirm that to the best of my knowledge I have made known all information relevant to the School Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I understand that it is a legal requirement that both staff and students undergo Criminal Records Checks when in a position of trust (i.e. when working with children or vulnerable adults).

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed Principal Investigator) Date:13/9/15

Form

..... (Student) Date: 13/09/18

.. (Other named investigators) Date: 13.9.18

..... (Other named investigators) Date:.....

1.4

University Research Ethics Committee Applications

Projects expected to require review by the University Research Ethics Committee must be reviewed by a member of the School research ethics committee and the Head of School before submission.

Signed.: . (~~Chair~~/Deputy Chair of School Committee) Date: 13/9/18

Signed. . (Head of Department) Date: 13/9/18

Signed..... (School Ethics Administrator) Date: 14/9/18

SECTION 2: PROJECT DETAILS

2.1

Cardiovascular disease causes millions of deaths worldwide each year, being the leading cause of death in a majority of countries in Europe. Detecting cardiovascular disease at this current moment can be difficult, inconsistent and is based on a number of factors such as age, weight and non-pathological factors such as smoking and alcohol consumption. It is thought that a better, more specific way of detecting cardiovascular disease is through that of biomarkers. Biomarkers are molecules, genes or other characteristics that can be associated with a disease state, in this case it is hypothesised that sialic acid and its many derivatives are associated with cardiovascular disease. The disease states, such as inflammation of blood vessels, associated with cardiovascular disease appears to raise the levels of sialic acid in the blood plasma. Detecting this rise in sialic acid, or a specific derivative of sialic acid, could lead to better detection of cardiovascular disease, potential for a heart event in the future associated with this, and could lead to better detection that then current method which is known as the QRISK score.

We will therefore compare a sialic acid-based risk marker with the current QRISK algorithm. Blood, urine and saliva samples will be collected from apparently health free living individuals and analysed for sialic acid (and other glycans) using LC-MS. We will also measure blood lipids, blood pressure and BMI to obtain a CVD risk using the QRISK algorithm and compare results.

2.2

Procedure

Participants will be invited for a single visit to the Hugh Sinclair Unit of Human Nutrition. After giving consent, we will collect blood, urine and saliva samples to measure sialic acid and blood lipids. We will also measure blood pressure, height, weight and waist circumference, and will collect blood, urine and saliva sample. These data will be used to estimate CVD risk using the QRISK algorithm, and compare estimated CVD risk with sialic acid biomarker.

2.3

Where will the project take place? Hugh Sinclair Unit of Human Nutrition

If the project is to take place in Hugh Sinclair Unit of Human Nutrition, projects must be reviewed and approved by the Hugh Sinclair Manager (Kelly Jarrett, Kelly.jarrett@reading.ac.uk)

Signed.... ..... (Hugh Sinclair Unit Manager)
Date:..14.09.18.

2.4

Funding
Is the research supported by funding from a research council or other *external* sources (e.g. charities, business)? Yes

If Yes, please give details: MRC Industrial CASE award

2.5

Ethical Issues
Could this research lead to any risk of harm or distress to the researcher, participant or immediate others? Please explain why this is necessary and how any risk will be managed.

This research will involve the taking of blood, saliva and urine samples. Participants will be informed of their QRISK score and CVD risk.

2.6

Deception
Will the research involve any element of intentional deception at any stage (i.e. providing false or misleading information about the study, or omitting information)?
[If so, this should be justified. You should also consider including debriefing materials for participants, which outline the nature and the justification of the deception used]

It will not include any deception.

2.7

Payment
Will you be paying your participants for their involvement in the study? No
If yes, please specify and justify the amount paid

Note: excessive payment may be considered coercive and therefore unethical. Travel expenses need not to be declared.

2.8

Data protection and confidentiality
What steps will be taken to ensure participant confidentiality? How will the data be stored?

Each subject will be assigned a six-digit random alpha-numerical code. Identifying data will be stored separately from other information and sample ID in a locked cupboard in the PI's office and only PIs will have access to these data.

2.9

Consent

Please describe the process by which participants will be informed about the nature of the study and the process by which you will obtain consent

Participants will attend the Hugh Sinclair Unit of Human Nutrition for a screening visit. At this visit, the nature of the study will be explained to them, they will be given the study information sheet and the opportunity to ask question. They will then be asked to sign the consent form (attached).

Please note that a copy of consent forms and information letters for all participants must be appended to this application.

2.10

Genotyping

Are you intending to genotype the participants? Which genotypes will be determined?

No

Please note that a copy of all information sheets on the implications of determining the specific genotype(s) to be undertaken must be appended to this application.

SECTION 3: PARTICIPANT DETAILS

3.1

Sample Size

How many participants do you plan to recruit? Please provide a suitable power calculation demonstrating how the sample size has been arrived at or a suitable justification explaining why this is not possible/appropriate for the study.

This is a pilot study and therefore a power calculation is not possible. We aim to recruit 40 participants.

3.2

Will the research involve children or vulnerable adults (e.g. adults with mental health problems or neurological conditions)

No

3.3 Will your research involve children under the age of 18 years? No Will your research involve children under the age of 5 years? No
3.4 Will your research involve NHS patients, Clients of Social Services or will GP or NHS databases be used for recruitment purposes? No Please note that if your research involves NHS patients or Clients of Social Services your application will have to be reviewed by the University Research Ethics Committee and by an NHS research ethics committee.
3.5 **Recruitment** Please describe the recruitment process and append all advertising and letters of recruitment. We will use the usual recruitment routes, i.e. posters on campus and outside (e.g. supermarkets - attached), the Hugh Sinclair Unit mailing lists and social media such as the Hugh Sinclair Facebook page.

Important Notes

1. The Principal Investigator must complete the Checklist in Appendix A to ensure that all the relevant steps and have been taken and all the appropriate documentation has been appended.

2. If you expect that your application will need to be reviewed by the University Research Ethics Committee you must also complete the Form in Appendix B.

3. For template consent forms, please see Appendices C.

Appendix A: Application checklist

This must be completed by an academic staff member (e.g. supervisor)

Please tick to confirm that the following information has been included and is correct. Indicate (N/A) if not applicable:

Information Sheet

Is on headed notepaper	☒	
Includes Investigator's name and email / telephone number	☒	
Includes Supervisor's name and email / telephone number	☐	
Statement that participation is voluntary	☒	
Statement that participants are free to withdraw their co-operation	☒	
Reference to the ethical process	☒	
Reference to Disclosure	☐	N/A ☒
Reference to confidentiality, storage and disposal of personal information collected	☒	

Consent form(s) ☐

Other relevant material

Questionnaires	☒	N/A ☐
Advertisement/leaflets	☒	N/A ☐
Letters	☐	N/A ☒
Other (please specify)	☐	N/A ☒

Expected duration of the project (months) 24

Name (print) ..... Signature

V8 06.06.2018 UREC Ethics Application Form

Appendix B

Project Submission Form

Note All sections of this form should be completed. Please continue on separate sheets if necessary.

Principal Investigator: Gunter Kuhnle

School: SCFP

Title of Project: Sialic acid

Proposed starting date: 1/10/2018

Brief description of Project: CVD risk markers

I confirm that to the best of my knowledge I have made known all information relevant to the School Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed.. (Investigator) Date 13/9/18

.. (Head of Department) Date 13/9/18

.. (Student) Date 13/09/18

(Where applicable)

Checklist

V8 06.06.2018 UREC Ethics Application Form

1. This form is signed by my Head of Department ☑

2. The Consent form includes a statement to the effect that the project has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee, and has been allowed to proceed ☑

3. I have made, and explained within this application, arrangements for any confidential material generated by the research to be stored securely within the University and, where appropriate, subsequently disposed of securely. ☑

4. I have made arrangements for expenses to be paid to participants in the research, if any, OR, if not, I have explained why not. ☑

5. Tick EITHER **(a) OR (b) - Head of School to sign if (b) ticked**

   (a) The proposed research does **NOT** involve the taking of blood samples; ☐

   OR

   (b) For anyone whose proximity to the blood samples brings a risk of Hepatitis B, documentary evidence of protection prior to the risk of exposure will be retained by the Head of School. ☑

   Signed.. ..(Head of Department) Date..15/9/18..

6. Tick **EITHER (a) OR (b)**

   (a) The proposed research does **NOT** involve the storage of human tissue, as defined by the Human Tissue Act 2004; ☐

   OR

   (b) I have explained within the application how the requirements of the Human Tissue Act 2004 will be met. ☒

7. Tick **EITHER (a), (b) OR (c)**

   (a) The proposed research will not generate any information about the health of participants; ☐

   OR

   (b) In the circumstance that any test reveals an abnormal result, ☒

I will inform the participant and, with the participant's consent, also inform their GP, providing a copy of those results to each; ☒

OR

(c) I have explained within the application why (b) above is not appropriate. ☐

8. Tick **EITHER (a) OR (b) - Head of School to sign if (b) ticked**

(a) the proposed research does not involve children under the age of 5; ☑

OR

(b) My Head of School has given details of the proposed research to the University's insurance officer, and the research will not proceed until I have confirmation that insurance cover is in place. ☐

Signed..... (Head of Department) Date.....

This form and further relevant information (see Sections 5 (b)-(e) of the Notes for Guidance) should be returned to, School Ethics Administrator. You will be notified of the Committee's decision as quickly as possible, and you should not proceed with the project until then.

University of Reading

Department of Food and Nutritional Sciences

Whiteknights, PO Box 226
Reading RG6 6AP

Consent Form- Sialic acid as biomarkers for cardiovascular disease?

Please read the following carefully and initial and date each box. If you have any questions, please do not hesitate to contact the study team (see below)

		Initial & Date
1	I have received the study and diet information sheets relating to the 'Sialic acids as biomarkers for cardiovascular disease?' study and I have read and understood the information.	☐
2	I understand that my participation is entirely voluntary and that I have the right to withdraw from the project any time without giving a reason. Withdrawing from the project will not cause me any disadvantage.	☐
3	I understand that all samples and information I provide will be anonymised. I agree that the samples and information I provide can be used for research on nutritional biomarkers at the University of Reading and collaborating institutions.	☐
4	'I understand that the data collected from me in this study will be preserved and made available in anonymised form, so that they can be consulted and re-used by others.	☐
5	I agree that my personal details can be stored on the volunteer database of the *Hugh Sinclair Human Nutrition Unit* and that I can be contacted with information of future studies.	☐ Yes ☐ No

This study has been reviewed and given a Favourable Opinion for conduct by the University Research Ethics Committee (UREC).

Please sign below and return using the envelope provided. You will receive a copy of this form, signed by the study PI, at your first visit to the Hugh Sinclair Human Nutrition Unit.

Participant		Researcher	
Name	……..	Name	……..
Date of Birth	……..	Signature	……..
Signature	……..	Date:	……..
Date	……..		

Department of Food and Nutritional Sciences

University of Reading

Screening questionnaire – Biomarkers

Volunteer ID: _____ Date: _____

To be completed by study team. Please circle answer.

Sex/Gender assigned at birth	Male Female Other Prefer not to say
Year of birth	
What, if any, medications are you currently taking?	
Do you currently have any health conditions? (Please select from the following options)	☐ Arthritis ☐ Osteoporosis ☐ Multiple Sclerosis ☐ Conditions affecting the thyroid ☐ Cancer ☐ Neurodegenerative diseases (e.g. Alzheimers, dementia) ☐ Other _____
Do you smoke?	
Do you currently have cardiovascular disease or any related condition? (e.g. heart disease, heart failure, angina).	
Have you ever suffered from a heart event? (e.g. stroke, heart attack)	
Has your family ever had a history of cardiovascular disease?	
Post Code	
Are you pregnant? Or likely to be pregnant?	
Have you recently travelled to a tropical country or got a tattoo?	

University of Reading

UREC 18/39

Department of Food and Nutritional Sciences

Whiteknights, PO Box 226
Reading RG6 6AP

Participant Information Sheet

Sialic Acids as Biomarkers for Cardiovascular Disease

Thank you very much for your interest in our study. Before you decide to take part, it is important for you to understand why this study is undertaken and in particular what it involves for you. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. If you have any questions, please do not hesitate to contact the study team – the contact details are at the end of this document.

What is the purpose of this study?

Cardiovascular disease is a major disease affecting a large amount of the world population and is the leading cause of death in Europe. It can be difficult to detect in good time, to allow for successful intervention. It is hoped that through the use of a biomarker (a molecule that is associated with a disease and appears when the disease is affecting the body) we could detect cardiovascular disease earlier and more easily. The biomarker we have chosen is sialic acid, a sugar molecule that is found all over the human body, but when it is found in the blood, urine and saliva, it has been shown to be associated with cardiovascular disease. This study hopes to show a correlation between sialic acid and cardiovascular disease and to look more specifically at different types of sialic acid to see if a more accurate biomarker can be found.

Do I have to take part?

Your participation is entirely voluntary. It is up to you to decide whether or not to take part. If you do decide to take part, you are still free to withdraw at any time and without giving a reason. This will not cause you any disadvantage.

Can I take part?

You can take part in this study if you are over 18 years of age. Depending on the nature of the study, there might be further restrictions such as age, sex and dietary preferences or restrictions.

Do I have to make any changes to my lifestyle?

No

What will happen to me if I take part?

Will be asked to attend the Hugh Sinclair Unit for Human Nutrition once to provide a blood sample, a saliva sample and a urine sample. We will also measure your blood pressure, height, weight and waist circumference.

What happens to my samples and data?

The data and samples we collect are important for our research, but we do not need any personal information after the study is completed. For this reason, we will assign each participant a random alphanumeric study ID which will be linked to personal information only for the duration of the study. We are also unable to provide you with any information about your samples.

Your samples will be processed using a variety of analytical techniques to give data on the levels of sialic acid in blood plasma, urine and saliva. Your samples will be processed and stored according to the Human Tissue Act 2004.

What are the possible disadvantages and risks of taking part?

You will need to visit the *Hugh Sinclair Human Nutrition Unit*. Some people find collecting urine samples to be inconvenient. The saliva taking can be tedious and the blood samples will require needles to be used.

What are the possible benefits of taking part in this study?

You will get an estimate of your 10-year CVD risk based on an algorithm used by NHS. Your participation will also help to develop new methods for the assessment of diet and to investigate links between diet and health.

Will my taking part in this study be kept confidential?

All information collected about you during the course of this study will be kept strictly confidential. After sample collection is completed, all data will be anonymised and it will be impossible to link results with individuals. Personal data (including consent forms) will be stored securely in the Office of the Principal Investigator (Gunter Kuhnle); all other data will be encrypted for storage.

What will happen to the results of the research study?

The results of this study are very important for future research on the health effect of diet. Results from this study will be published in scientific journals and presented at national and international conferences. They will also be used in applications for research grants.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The complaints procedure is explained below.

The University has the appropriate insurances in place. Full details are available on request.

Complaints procedure

This study has been independently reviewed and been given a favourable opinion for conduct by the School of Chemistry, Food and Pharmacy Research Ethics Committee

If you have any concerns about this study, or any complaint about the way you have been dealt with during the study or any possible harm you might suffer, please contact the researchers who will do their best to address your questions (see below for contact details). If you remain unhappy and wish to make a formal complaint, you should contact the Head of the Department of Food and Nutritional Sciences, Professor Richard Frazier.

Contact information of the study organiser

Gunter Kuhnle
Department of Food and Nutritional Sciences
University of Reading
Whiteknights PO Box 226
Reading RG6 6AP

Email: g.g.kuhnle@reading.ac.uk
Phone: 0118 378 7723

Contact information of the Head of the Department of Food and Nutritional Sciences

Professor Richard Frazier
Head of Department
Department of Food and Nutritional Sciences
University of Reading
Reading RG6 6AP

Email: r.a.frazier@reading.ac.uk

University of Reading

Are you interested in your 10-year risk of heart disease?

Are you interested to know your 10-year CVD risk? We are looking for volunteers to participate in a study to develop new risk markers for heart disease which could allow an earlier diagnosis and faster treatment.

What do you need to do?

If you are interested, you need to attend the Hugh Sinclair Unit of Human Nutrition, answer a few questions about your lifestyle and provide a blood, urine and saliva sample.

Why should I take part?

By taking part in this study, you provide invaluable support for the development of a new risk marker to detect heart disease early. We will use the information you give us to estimate your 10-year heart disease risk using the method recommended by the NICE and used by the NHS.

If you are interested in taking part in this study, please contact Jack at j.a.cheeseman2@pgr.reading.ac.uk.

University of Reading

Coordinator for Quality Assurance in Research
Dr Mike Proven, BSc(Hons), PhD

Academic and Governance Services

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Reading RG6 6AH

phone +44 (0)118 378 7119
email urec@reading.ac.uk

Dr Gunter Kuhnle
School of Chemistry, Food and Pharmacy
University of Reading
RG6 6UR

11 January 2019

Dear Gunter,

UREC 18/39: Sialic Acids as Biomarkers for Cardiovascular disease. *Favourable opinion*

Thank you for the response (your email, dated 7 January 2019, from Jack Cheeseman refers) addressing the issues raised by the UREC Sub-committee at its October 2018 meeting (*my Provisional Opinion email of 19 October including attachments refers*). On the basis of these responses, I can confirm that the Chair is pleased to confirm a favourable ethical opinion.

Please note that the Committee will monitor the progress of projects to which it has given favourable ethical opinion approximately one year after such agreement, and then on a regular basis until its completion.

Please also find attached Safety Note 59: Incident Reporting in Human Interventional Studies at the University of Reading, to be followed should there be an incident arising from the conduct of this research.

The University Board for Research and Innovation has also asked that recipients of favourable ethical opinions from UREC be reminded of the provisions of the University Code of Good Practice in Research. A copy is attached and further information may be obtained here:

http://www.reading.ac.uk/internal/res/QualityAssuranceInResearch/reas-RSqar.aspx.

Yours sincerely

Dr M J Proven
Coordinator for Quality Assurance in Research (UREC Secretary)
cc: Dr John Wright (Chair); Mrs Barbara Parr (Ethics Administrator); Professor Helen Osborn (Researcher); Jack Cheeseman (Research Student);

University of Reading

Coordinator for Quality Assurance in Research
Dr Mike Proven, BSc(Hons), PhD

Academic and Governance Services

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email urec@reading.ac.uk

Dr Gunter Kuhnle
School of Psychology and Clinical
Language Sciences
University of Reading
RG6 6UR

25 July 2019

Dear Gunter,

UREC 18/39: Sialic Acids as Biomarkers for Cardiovascular disease. *Amendment favourable opinion. AM011839*

Thank you for your application (email dated 18 July 2019 and including attachments refers from Jack Cheeseman) requesting and detailing amendments to the above project (*amendment to procedure to include a payment (£10) for participant*). I can confirm that the UREC Chair has reviewed that request and is happy for the project to continue.

Yours sincerely

Dr M J Proven
Coordinator for Quality Assurance in Research (UREC Secretary)
cc: Dr John Wright (Chair); Mrs Barbara Parr (SREC Administrator); Jack Cheeseman (PhD Student);

Appendix 4: References in the style of the Future Medicine journals

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Chapter 6:

Elevated concentrations of Neu5Ac and Neu5,9Ac$_2$ in human plasma: Potential Biomarkers of Cardiovascular Disease.

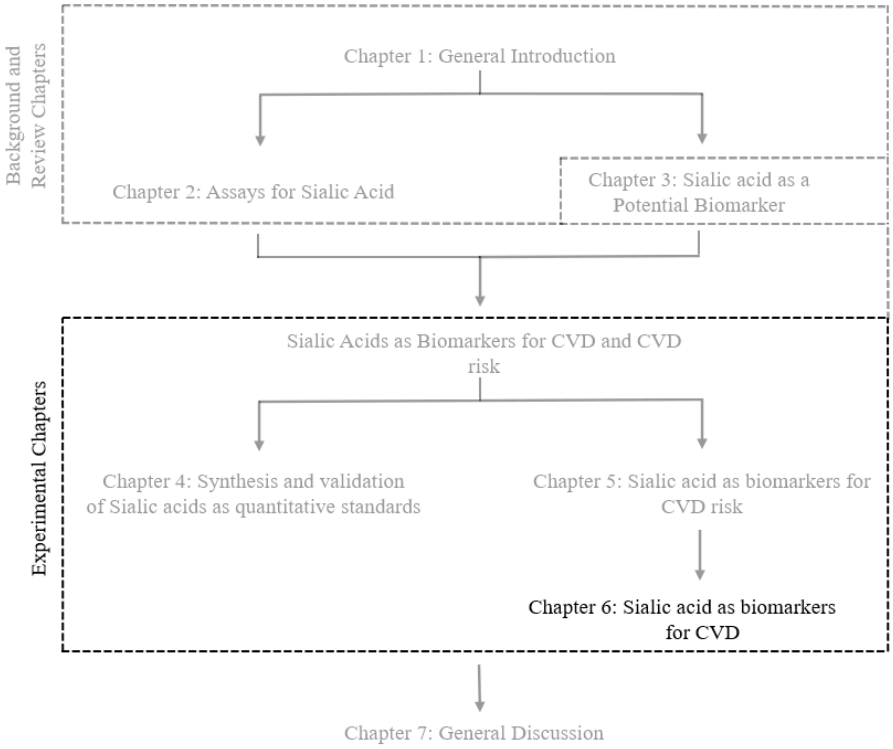
Chapter Summary: This chapter involves the analysis of samples to determine the biomarker potential of Neu5Ac and Neu5,9Ac$_2$ for advanced cardiovascular disease. Samples from patients with cardiovascular disease and healthy controls were purchased from BioIVT. These samples were analysed for Neu5Ac and Neu5,9Ac$_2$ after which the concentrations in the two cohorts were compared to determine the statistical significance of the biomarker. ROC analysis was then utilised to determine the predictive power of the marker.

Bibliographic Details: Elevated concentrations of Neu5Ac and Neu5,9Ac$_2$ in human plasma: Potential Biomarkers of Cardiovascular Disease. **J. Cheeseman**, C. Badia, G. Elgood-Hunt, R. A. Gardner, D. I. R. Spencer, G. Kuhnle, H. M. I. Osborn *Submitted to Scientific Reports, December 2021.*

Author Contributions: D.I.R.S, G.K and H.M.I.O designed the study, won funding for the programme, and supervised the study. J.C carried out the selection of samples with the assistance of G.K, D.I.R.S and H.M.I.O. J.C designed and carried out the analysis with the assistance of C.B. and R.G. G.K. was responsible for statistical analysis, G.EH performed ROC analysis and modelling. J.C wrote the first draft of the main manuscript text and prepared all figures except for figure 1 that was prepared by G.EH. All authors reviewed the manuscript and J.C prepared the final draft for submission.

Appendices: Appendix 1 details the samples from patients with cardiovascular disease chosen for purchase from BioIVT.

Appendix 2 contains a flow chart which outlines the selection process of CVD samples from BioIVT, ensuring to exclude samples with health conditions that may affect sialic acid concentration measurements independently of cardiovascular disease.


Background and Review Chapters
Chapter 1: General Introduction
Chapter 2: Assays for Sialic Acid
Chapter 3: Sialic acid as a Potential Biomarker
Experimental Chapters
Sialic Acids as Biomarkers for CVD and CVD risk
Chapter 4: Synthesis and validation of Sialic acids as quantitative standards
Chapter 5: Sialic acid as biomarkers for CVD risk
Chapter 6: Sialic acid as biomarkers for CVD
Chapter 7: General Discussion

Elevated concentrations of Neu5Ac and Neu5,9Ac$_2$ in human plasma: Potential Biomarkers of Cardiovascular Disease

Jack Cheeseman[a], Concepcion Badia[b], Georgia Elgood-Hunt[b] Richard Gardner[b], Daniel I.R. Spencer[b], Gunter Kuhnle*[c], Helen M.I. Osborn*[a]

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Keywords: Sialic acid, biomarker, cardiovascular disease, inflammation, ROC analysis

Abstract

Cardiovascular disease (CVD) is a group of health conditions affecting the heart and vascular system, that poses a global health issue with very high prevalence and mortality. The presence of CVD is characterised by high levels of inflammation which is associated with overexpression of *N*-Acetyl neuraminic acid (Neu5Ac) and reduced plasma esterase activity possibly affecting 5-*N*-acetyl-9-*O*-acetyl neuraminic acid (Neu5,9Ac$_2$) concentrations. Thirty plasma samples from patients with diagnosed CVD, and thirty age and sex-matched healthy controls were assayed for Neu5Ac and (Neu5,9Ac$_2$) concentrations. Mean Neu5Ac and Neu5,9Ac$_2$ concentrations were significantly elevated in CVD patients compared to healthy controls (Neu5Ac: $P < 0.001$; Neu5,9Ac$_2$: $P < 0.04$). Receiver operator curve (ROC) analysis indicated that both Neu5Ac and Neu5,9Ac$_2$ had good predictive power for the presence of CVD (Neu5Ac AUC: 0.95; Neu5,9Ac$_2$ AUC: 0.84). However, while Neu5Ac had both good sensitivity (0.88) and specificity (0.9), Neu5,9Ac$_2$ had equivalent specificity (0.9) but poor sensitivity (0.5). A combination marker of Neu5Ac/Neu5,9Ac$_2$ showed improvement over Neu5Ac alone in terms of predictive power (AUC: 0.96), sensitivity (0.88) and specificity (1.00). Thus, Neu5Ac and Neu5Ac/Neu5,9Ac$_2$ would appear to be good predictive markers for the presence of CVD.

Introduction

CVD is highly prevalent, affecting populations in both developed and developing countries, and hence poses a major healthcare burden. CVD is associated with high morbidity and mortality rates, indeed it was the leading cause of death worldwide, accounting for 34% (20.1 million) of all deaths in 2019 with an estimated 500 million active cases.[1] CVD accounted for the loss on average of 4860 disability adjust life years (DALY) per 100,000 population in

2019.[2] Accurate diagnosis of CVD to allow for earlier intervention and treatment could help lower the global healthcare burden and mortality rates associated with CVD. CVD is associated with increased levels of inflammation, especially in the vascular endothelium. This inflammation may contribute to the progression of CVD and cause myocardial and vascular damage.[3] Markers for inflammation such as high sensitivity c-reactive protein (hs-CRP)have previously been studied in the context for CVD,[4] and it has been shown that hs-CRP may underestimate inflammation and therefore have lower predictive power than required for diagnosis or prediction of CVD.[5]

Neu5Ac has many important biological functions primarily as a receptor mask or receptor determinant which aids in cell-cell recognition and immune response.[6] The carboxylic acid functional group conveys an overall negative charge to the cell surface and the endothelium which aids in cell repulsion[7] and the prevention of cell aggregation, especially among erythrocytes.[8] Neu5Ac and Neu5,9Ac_2 expression of the surface of glycoproteins improves their circulating half-life in the blood.[9] These sialic acid derivatives have also been shown to have an anti-inflammatory effect, protecting the cardiovascular system from inflammatory damage. This may explain the upregulation of sialic acid during an inflammatory state.[10–12] Neu5,9Ac_2 concentrations may also appear upregulated due to the reduced esterase activity in plasma during an inflammatory state. Hubbard *et al.* reported significant associations between elevated inflammatory markers hs-CRP, interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-alpha) and reduced plasma esterase activity.[13] This effect may reduce the conversion of Neu5,9Ac_2 to Neu5Ac by the action of acetylesterase resulting in elevated concentrations of Neu5,9Ac_2 when inflammation is present.

Previous studies have also identified *N*-acetyl neuraminic acid (sialic acid, Neu5Ac) as a marker for CVD.[14] Neu5Ac is a monosaccharide with a nine-carbon backbone that is generally located as the terminating unit of glycans which are parts of glycoproteins and glycolipids.[7] Neu5Ac is one of a family of over 50 sialic acids. The main derivatives present in humans are Neu5Ac and Neu5,9Ac_2[15] with human plasma exhibiting a Neu5,9Ac_2 (0.29 mg/100 mL)/Neu5Ac (45.49 mg/100 mL) ratio of 0.006.[16] Quantitation of sialic species in breast cancer cell lines have yielded ratios of Neu5,9Ac_2/Neu5Ac of 0.005-0.09 depending on the cell line.[17] Neu5Ac is ubiquitous in the body, while Neu5,9Ac_2 has been identified in biological fluids (blood, urine, saliva) and in the brain, lungs, kidneys, intestines and pancreas.[18,19]

Elevated Neu5Ac concentration has been linked to increased cardiovascular mortality risk, with the risk associated with the highest quartile of sialic acid concentration versus the lowest quartile being 2.38 in men and 2.62 in women.[20] Increased plasma and serum concentrations

of sialic acid have also been linked to the presence and pathogenesis of CVD and associated inflammation could potentially be utilised to diagnose the disease.[14] Neu5Ac concentrations can be elevated by other diseases that cause inflammation however, such as arthritis[21] and type-2 diabetes.[22] Therefore, investigating other derivatives of sialic acid may reveal a potential biomarker that is more specific for CVD.

Quantitative analysis of these sialic acid derivatives as biomarkers for CVD requires a sensitive and specific analytical technique with a sufficiently low limit of detection. This is because while Neu5Ac is present in sufficiently large quantities (45.49 mg/100 mL), Neu5,9Ac_2 is generally present in much smaller quantities (0.29 mg/100 mL).[16] Labelling of sialic acids with 1,2-Diamino-4,5-methylenedioxybenzene Dihydrochloride (DMB) followed by HPLC analysis was chosen in this instance, it allows for the analysis of multiple sialic acids in the same assay while exhibiting high specificity for sialic acids and a low limit of detection (< 0.01 nmol).[23,24] Previous research in this area has utilised assays which suffer from poor specificity for sialic acid which can lead to inaccurate results.[25] Like all quantitative techniques, the DMB assay requires quantitative standards for effective and accurate quantitation of sialic acids. Neu5Ac is commercially available in sufficiently large quantities but Neu5,9Ac_2 is not and therefore must be chemically synthesised. For the accurate quantitation of Neu5,9Ac_2 in plasma samples, we have previously synthesised Neu5,9Ac_2 and analysed the standard using quantitative nuclear magnetic resonance (QNMR) techniques.[16] The presence of CVD and the resulting inflammatory state has been shown to result in an overexpression of Neu5Ac and therefore an elevation of Neu5Ac in blood. Following this, blood Neu5,9Ac_2 may also be affected and as such it is of interest as a potential marker for CVD that may be more specific. Neu5Ac and Neu5,9Ac_2 concentrations were measured in plasma samples from both healthy controls and CVD cohorts (n = 60). Analysis was carried to determine if the elevation of concentrations of Neu5Ac and Neu5,9Ac_2 between the healthy and disease cohorts was statistically significant. ROC curves were prepared to determine the sensitivity and specificity of each marker for the prediction of CVD to allow for a comparison to existing markers.

Materials and Methods

Study Population

30 plasma samples from patients (16 female; 14 male) with an average age of 65 with CVD were selected and purchased from BioIVT along with 30 age and sex matched healthy controls. Samples were chosen from volunteers that had one or multiple diagnosed CVDs but no other

conditions that could otherwise affect plasma sialic acid concentration such as type-2 diabetes,[26] arthrtiis[21] and chronic obstructive pulmonary disorder (COPD).[27]

Our study was approved by the University of Reading Ethics Committee (UREC 18/39) and all samples were collected by BioIVT with informed consent in accordance with Declaration of Helsinki. All experimental procedures were carried out in accordance with institutional guidelines.

Analytical Methods

Analysis of sialic acids was carried out using the DMB method.[16,24,28] Release of sialic acids and DMB labelling of the samples was achieved using LudgerTagTM DMB Sialic Acid (LT-KDMB-96). 5 µL of each sample was added to a 96-well plate. Each sample was subjected to acid release with 25 µL of 2M acetic acid. The samples were vortexed and centrifuged (RCF 1677) followed by incubation at 80°C for 2 hours. The samples were cooled to room temperature before 5 µL of each released sample was transferred to a new 96-well plate. To this, 20 µL DMB labelling solution was added. The samples were vortexed and centrifuged (RCF 1677) followed by incubation for 3 hours at 50°C. The reaction was quenched by addition of water to make-up the volume to 500 µL. Neu5Ac samples were then subjected to a 1 in 10 dilution, Neu5,9Ac_2 samples were not. All work was carried out using a Hamilton STARlet Liquid Handling Robot, apart from the initial dispensing of the samples into the 96-well plate. Analysis of the samples was performed in triplicate for Neu5Ac and Neu5,9Ac_2 determination.

Fetuin derived from fetal calf serum (GCP-Fet-50U), an A2G2S2[29] glycopeptide (BQ-GPEP-A2G2S2-10U) both from Ludger Ltd. and a human plasma standard purchased from Sigma Aldrich were utilised as system suitability standards. These standards were subjected to the same release and labelling conditions as stated above for the samples containing Neu5Ac.

Standards of Neu5Ac and Neu5,9Ac_2[16] were also labelled using LudgerTagTM DMB Sialic Acid (LT-KDMB-96). 20 µL of labelling solution (3.5 mg DMB dye, 2.2 mL mercaptoethanol (1.4 M), 20 mg sodium dithionite) was added to each standard. The samples were vortexed and centrifuged (RCF 1677) before incubating for 3 hours at 50°C. The labelling reaction was quenched with water to bring the final volume to 500 µL. Standards curves were prepared for each standard with points: 0.01-1.0 nmol.

The labelled sialic acids were analysed by LC-FLD. 5 µL of sample was injected into a U3000 UHPLC equipped with a fluorescence detector ($\lambda ex = 373$ nm, $\lambda em = 448$ nm, Thermo, UK). For Neu5Ac analysis an isocratic solvent system (7:9:84 %v/v MeOH:ACN:H_2O) was used.

For Neu5,9Ac_2 analysis a gradient solvent system was used 7:6:87 %v/v MeOH:ACN:H_2O for 6.5 minutes followed by 6:9:85 %v/v MeOH:ACN:H_2O for 11.5 minutes.

Statistical Analysis

Data are presented as mean ± standard deviation. P=0.05 was used as the threshold for statistical significance. Difference in mean values were estimated using a two-sided t-test. Analysis was performed using R version 4.1.1.[30] ROC curves were prepared using a Support Vector Classifier model[31] where the model was trained on 70% of the data and tested on the remaining 30% after data was standardised such that it followed a normal distribution. No outliers were identified. The outcomes were whether a given person had a diagnosed CVD (CVD cohort) or whether they had no diagnosed CVD (control cohort).

Results

The DMB assay employed for the analysis of Neu5Ac and Neu5,9Ac_2 exhibited low levels of inter and intra-assay variation (< 10%). Neu5Ac and Neu5,9Ac_2 concentrations in all samples met the criteria to overcome the limit of detection and limit of quantitation (3 times and 9 times signal to noise ratio respectively).

The cohort characteristics are shown in Table 1. The average concentration of Neu5Ac in healthy controls was 43.73 ± 17.49 mg/100 mL and in patients with CVD it was 63.55 ± 8.46 mg/100 mL. The average concentration of Neu5,9Ac_2 in healthy controls was 0.32 mg/100 mL and in the CVD patients was 0.40 mg/100 mL. Values for Neu5Ac and Neu5,9Ac_2 were higher in the CVD patients when compared to healthy controls. Neu5Ac and Neu5,9Ac2 concentrations were significantly higher in patients when compared with healthy controls (two-sided t-test, $p<0.001$ for Neu5A, $p<0.04$).

Table 1: Cohort characteristics

	Healthy Controls	CVD Patients
n	30	30
Age (years)	65	65
Male:Female	14:16	14:16
Mean Plasma Neu5Ac (mg/100 mL)	43.73 ± 17.49	63.55 ± 8.46
Mean Plasma Neu5,9Ac_2 (mg/100 mL)	0.32 ± 0.19	0.40 ± 0.06

ROC curves were prepared to determine the predictive power of Neu5Ac and Neu5,9Ac_2 for the presence of CVD (Figure 1). The specificity, sensitivity, precision, F-score and area under the curve (AUC) for each marker are provided in Table 2.

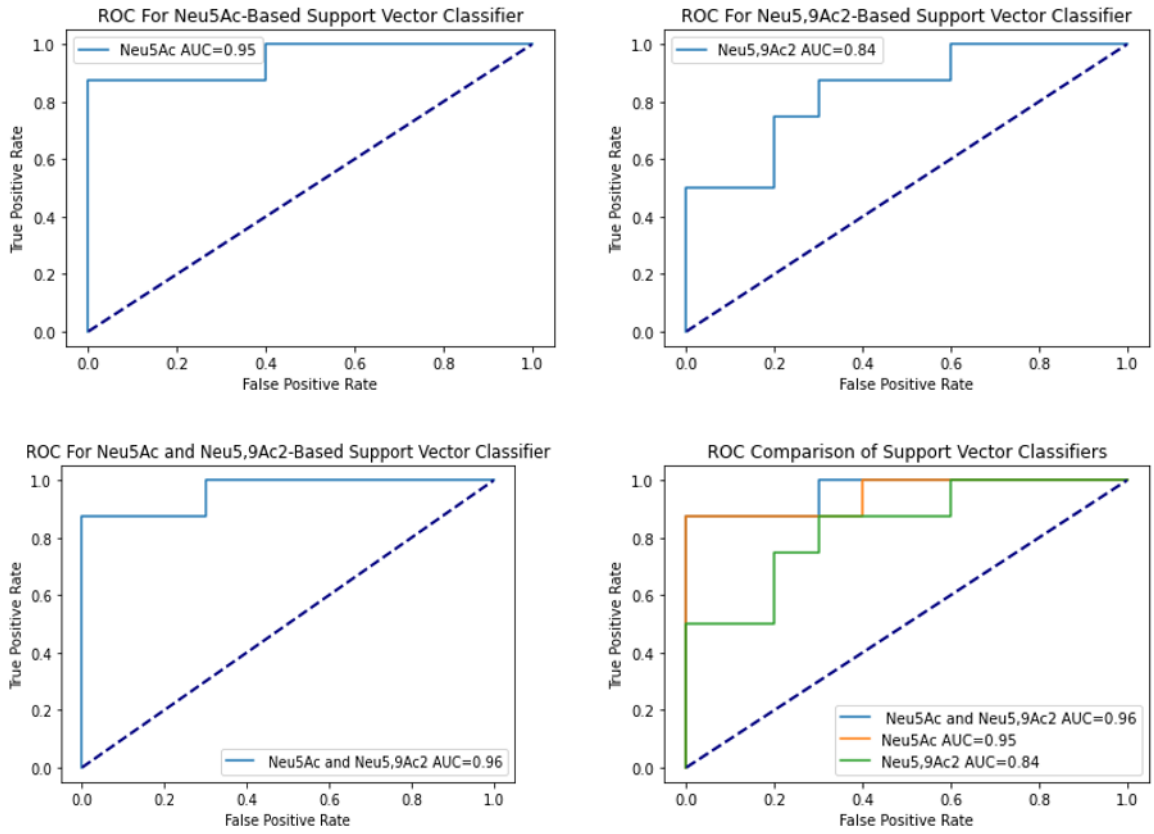

Figure 1: ROC curves for Neu5Ac, Neu5,9Ac$_2$ and Neu5Ac/Neu5,9Ac$_2$ as well as a comparison of the three plots

Table 2: ROC analysis data

	Sensitivity	Specificity	Precision	F-score	AUC [Confidence Interval]
Neu5Ac	0.88	0.90	0.88	0.88	0.95 [0.57; 1.0]
Neu5,9Ac$_2$	0.50	0.90	0.80	0.62	0.84 [0.57; 0.84]
Neu5Ac + Neu5,9Ac$_2$	0.88	1.00	1.00	0.93	0.96 [0.73; 1.00]

Discussion

The statistically significant elevation of Neu5Ac between healthy controls and disease patient cohorts ($P < 0.001$) in this study is supported by previous work. The presence of atherosclerosis, hypertension, coronary heart disease and heart failure have all previously been associated with increased Neu5Ac concentrations.[14] Neu5,9Ac$_2$ has not been previously

investigated as a marker in the context of CVD. A statistically significant elevation in Neu5,9Ac$_2$ concentration between healthy controls and CVD is reported here ($P < 0.04$), although the significance here may be borderline give the value is close to 0.05.

ROC analysis (Figure 1, Table 2) revealed the predictive power of Neu5Ac and Neu5,9Ac$_2$ for advanced levels of CVD progression and potential utility as biomarkers. The AUC, or predictive power for CVD cases, was calculated for plasma concentrations of Neu5Ac (0.95), Neu5,9Ac$_2$ (0.84) as well as Neu5Ac/Neu5,9Ac$_2$ as a combined marker (0.96). Neu5Ac and Neu5,9Ac$_2$ were both indicated to have good predictive power. Neu5Ac showed better predictive power than Neu5,9Ac$_2$ which indicated that it may be a better predictor of CVD over Neu5,9Ac$_2$. Interestingly, the combined marker for Neu5Ac/Neu5,9Ac$_2$ had a marginally better AUC than Neu5Ac indicating a similar predictive power. Comparing the markers in more depth revealed more about the potential of each marker when comparing sensitivity and specificity. An ideal marker would exhibit a sensitivity and specificity of 1.0, meaning the marker had a 100% true positive (sensitivity), 100% true negative (specificity) and 0% false positive rate (1 – specificity). Neu5Ac exhibited high specificity (0.9) and sensitivity (0.88), Neu5,9Ac$_2$ exhibited equivalent specificity (0.9) but low sensitivity (0.5). Both markers have a low false positive rate Neu5,9Ac$_2$ poorly discriminates the positive results and as such would not be a good marker in this context. Neu5Ac however performs very well in this cohort. The combined marker Neu5Ac/Neu5,9Ac$_2$ is interesting in this context in that it offers sensitivity (0.88) comparable to that of Neu5Ac, but also very high specificity (1.0) indicating a zero false positive rate in this cohort.

Inflammation is an important aspect of CVD that can cause myocardial and endothelial damage.[32,33] . An increase in inflammation related to CVD can lead to an acute-phase response which is characterised by upregulation of specific proteins: alpha-1-acid glycoprotein, alpha-1-antitrypsin, fibrinogen, alpha-2-macroglobulin and hemopexin.[34] A variety of sialylated glycans decorate the surface of these proteins. A2G2S2 is the main glycan present in human plasma and is present in large quantities on alpha-1-antitrypsin and hemopexin. Alpha-1-acid glycoprotein has been reported to account for nearly all highly sialylated *N*-glycan species in plasma circulation.[35] These glycoproteins contain large quantities of sialic acids and an increase in the concentrations of these proteins during an acute-phase response would account somewhat for elevated Neu5Ac and Neu5,9Ac$_2$ concentrations in plasma. Further to this, elevated sialic acid concentrations might be such that the sialic acid can act as a substrate for the resialylation of low-density lipoprotein (LDL) and erythrocytes. Desialylated LDL and erythroctyes have been found to aggregate more than unmodified variants thus leading to the

build-up of atherosclerotic plaques.[36,37] This is perhaps supported by evidence of an increase in the activity of sialyltransferase during an inflammatory state, which is perhaps an attempt to resialylate these structures and prevent cardiovascular damage occurring.[38] On the other hand, downregulation of the activity of plasma esterases has been observed with increased levels of inflammation.[39] This may reduce the quantity of acetylated derivatives, such as Neu5,9Ac_2, in plasma cleared by activity of these enzymes, leading to an observed increase in their concentrations.

Comparison of the markers investigated here with well-established CVD biomarkers indicates that Neu5Ac and Neu5,9Ac_2 may provide better discrimination and overall predictive power in patients with advanced CVD versus healthy controls. Kimmenade *et al.* investigated markers for the presence of heart failure (HF), an advanced CVD, using ROC analysis: pro-brain natriuretic peptide (NT-proBNP) (AUC: 0.94, sensitivity:specificity – 0.90:0.85) and galectin-3 (gal-3) (0.72, sensitivity:specificity – 0.80:0.52).[40] While only Neu5Ac and the combination marker Neu5Ac/Neu5,9Ac_2 performed similarly to NT-proBNP in terms of predictive power (AUC) for advanced CVD, all of the markers in this research performed better than gal-3. The three sialic acid markers also appeared to show better predictive power than carotid intima-media thickness (IMT) (AUC: 0.72, sensitivity:specificity – 0.71:0.60).[41] In terms of sensitivity and specificity, Neu5,9Ac_2 offered higher specificity than NT-proBNP, gal-3 and carotid IMT but much lower sensitivity. On the other hand, Neu5Ac and the combination marker Neu5Ac/Neu5,9Ac_2 showed better sensitivity than gal-3 and carotid IMT but performed similarly to NT-proBNP in this aspect. Neu5Ac showed higher specificities than gal-3 and carotid IMT but was only marginally higher in comparison to NT-proBNP. Neu5Ac/Neu5,9Ac_2 showed much better specificity than all three markers (NT-proBNP, gal-3, carotid IMT) with specificity of 1.0. Given this information, sialic acids may act as better markers for the presence of CVD with research described herein indicating a combined marker of Neu5Ac/Neu5,9Ac_2 offering the best predictive power for the presence of CVD with overall better specificity and sensitivity than previously investigated markers.

Strengths and Limitations

The samples size for this study was small and may have resulted in an underpowered study, potentially explaining the borderline significance ($P < 0.04$) of plasma Neu5,9Ac_2 concentrations between CVD cases and healthy controls. A higher-powered future study may supplement this data.

Conclusions

Neu5Ac and Neu5,9Ac_2 concentrations were determined in samples from 30 healthy controls and 30 patients with diagnosed CVD. Statistically significant elevations of concentrations for both Neu5Ac and Neu5,9Ac_2 were observed. Neu5Ac was shown to be a good marker for the discrimination of some classes of CVD patients from healthy controls, Neu5,9Ac_2 however suffered from low sensitivity and as such poor prediction of CVD cases. Interestingly, a combination marker of Neu5Ac/Neu5,9Ac_2 offered better predictive power than just Neu5Ac alone. The markers identified here offer an improvement on previously studied markers such as hs-CRP which can suffer from poor discrimination of CVD. Neu5Ac and Neu5Ac/Neu5,9Ac_2 could act as markers for the presence of CVD with excellent predictive power. This may allow for more accurate diagnosis of CVD, allowing for treatment to be administered earlier, thus reducing CVD healthcare burden.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors based at Ludger Ltd work in commercializing analytical assays for use in the field of glycomics and the analysis of biopharmaceuticals. The remaining authors do not have competing interests to declare.

Acknowledgements

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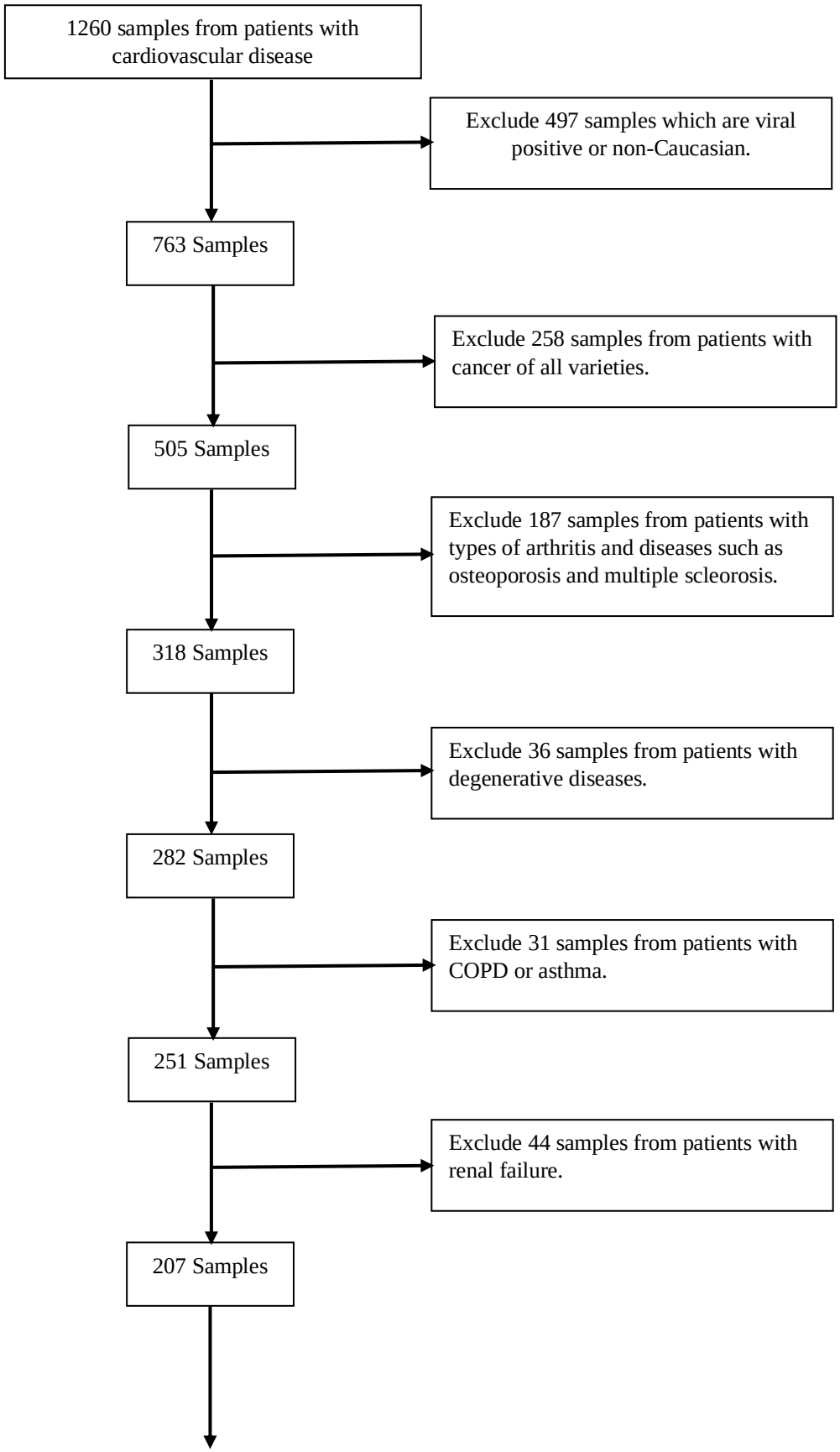
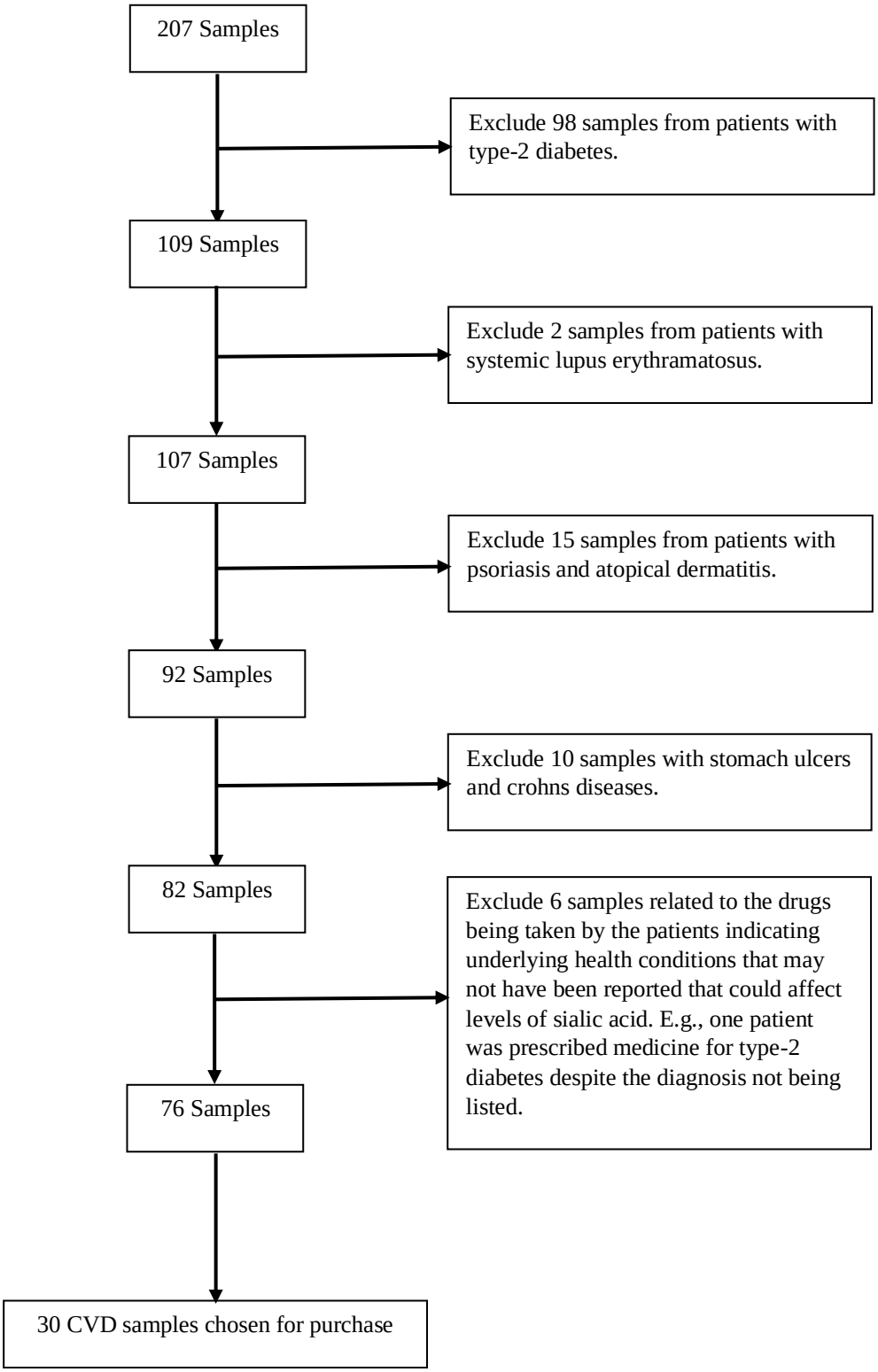
Appendix 1: BioIVT CVD case samples

Sex	Age (Years)	Race	Diagnosis
Male	40	Caucasian	Hypertension, Intestinal Obstruction, Vitamin D Deficiency, Anemia
Male	49	Caucasian	Hypercholesterolemia, Hypertension
Male	49	Caucasian	Hypertension
Male	52	Caucasian	Hypertension
Male	57	Caucasian	Atrial Fibrillation, Hypertension
Male	61	Caucasian	Bipolar Disorder, Hypertension
Male	67	Caucasian	Hypertension, Atrial Fibrillation
Male	70	Caucasian	Congestive Heart Failure
Male	72	Caucasian	Hypertension, Hyperlipidemia
Male	73	Caucasian	Congestive Heart Failure, Vertigo, Hypertension, Hyperlipidemia, Ventricular Tachycardia, Ischemic Cardiomyopathy
Male	75	Caucasian	Congestive Heart Failure, Hypertension, Anemia, Hyperlipidemia
Male	77	Caucasian	Congestive Heart Failure
Male	80	Caucasian	Congestive Heart Failure, Hypertension
Female	41	Caucasian	Hypertension
Female	51	Caucasian	Hypertension
Female	51	Caucasian	Hypertension, Hyperchlolesterolemia
Female	52	Caucasian	Cardiovascular Disease, Hypertension
Female	55	Caucasian	Hyperuricemia, Hypertension
Female	56	Caucasian	Hypercholesterolemia, Hypertension
Female	58	Caucasian	Hypertension, Hypertriglyceridemia, Vitamin D Deficiency
Female	59	Caucasian	Hypertension, Hypercholesterolemia
Female	63	Caucasian	Hypertension
Female	69	Caucasian	Congestive Heart Failure

Female	72	Caucasian	Congestive Heart Failure, Sleep Apnea, Fibromyalgia, Gastroesophageal Reflux Disease, Hypertension, Hyperlipidemia, Aortic Stenosis, Paroxysmal Atrial Fibrillation, Pulmonary Hypertension
Female	75	Caucasian	Coronary Artery Disease, Hypertension
Female	83	Caucasian	Hypercholesterolemia, Hypertension, Hyponatremia, Pneumonia
Female	84	Caucasian	Congestive Heart Failure
Female	87	Caucasian	Hypertension
Female	88	Caucasian	Congestive Heart Failure, Atrial Fibrillation, Pulmonary Hypertension, Cardiovascular Disease
Female	91	Caucasian	Congestive Heart Failure, Atrial Fibrillation, Gastroesophageal Reflux Disease, Hyperlipidemia, Insomnia, Cerebrovascular Attack, Coronary Artery Disease

Appendix 2: BioIVT CVD samples selection process




207 Samples
Exclude 98 samples from patients with type-2 diabetes.
109 Samples
Exclude 2 samples from patients with systemic lupus erythramatosus.
107 Samples
Exclude 15 samples from patients with psoriasis and atopical dermatitis.
92 Samples
Exclude 10 samples with stomach ulcers and crohns diseases.
82 Samples
Exclude 6 samples related to the drugs being taken by the patients indicating underlying health conditions that may not have been reported that could affect levels of sialic acid. E.g., one patient was prescribed medicine for type-2 diabetes despite the diagnosis not being listed.
76 Samples
30 CVD samples chosen for purchase

Appendix 3: References in the style of the Future Medicine journals

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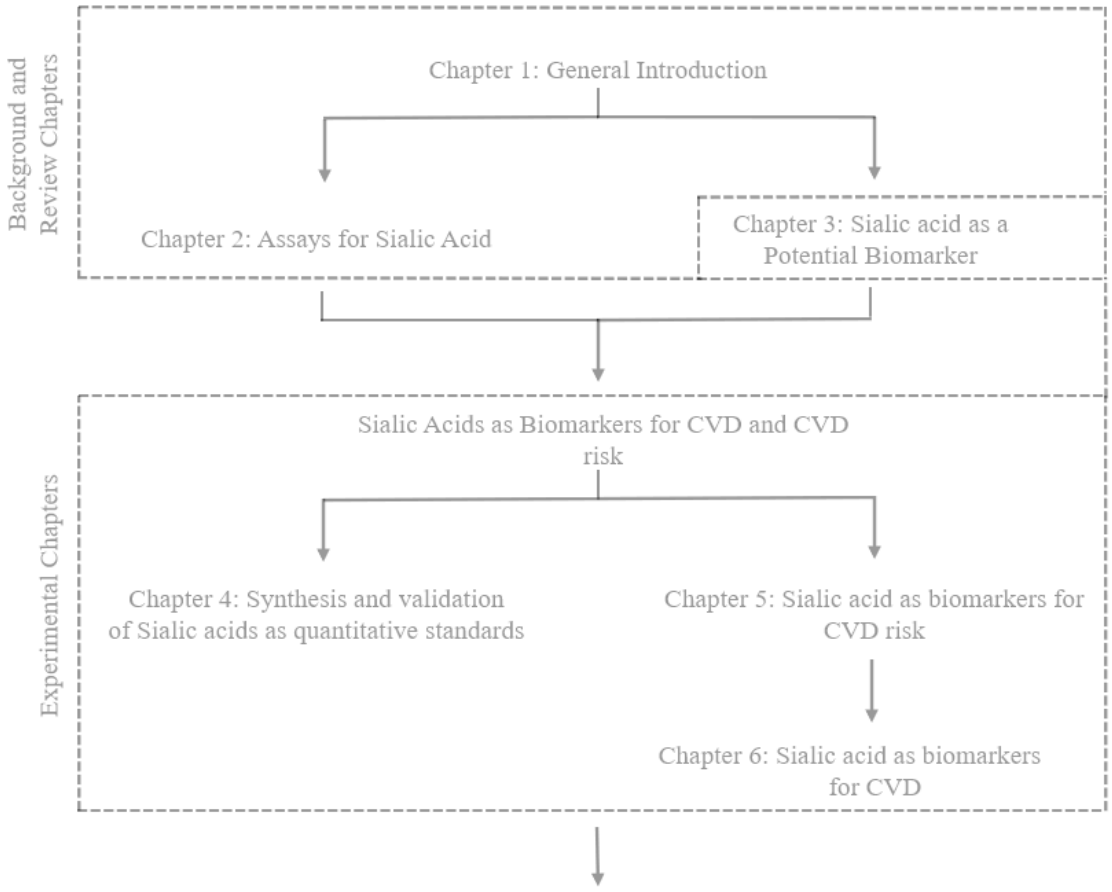
Chapter 7:

General Discussion



7.1 Introduction

Many biomarkers for CVD have been established and are utilised in medicine for the diagnosis and assessment of CVD.[1] Use of these biomarkers has resulted in a marked improvement in clinical outcomes in the last 30 years.[2] Despite this, CVD accounts for the highest percentage of mortality globally equal to 34% (20.1 million) of deaths in 2019.[3] This is expected to increase over the next few years as CVD risk factors increase worldwide. Early diagnosis of CVD, or prediction of CVD before it occurs is currently difficult. Risk prediction algorithms such as QRISK3 have been developed and validated to enable assessment of CVD risk,[4] but these have been shown to be unreliable in older populations.[5] As such, more reliable marker for CVD risk, or early CVD, may be useful for the reduction of CVD mortality. Sialic acid has been established as a marker for CVD,[6] although other isomers of sialic acid have not been investigated despite being implicated in many processes associated with CVD and inflammation.[7]

In this chapter, critical evaluation of the work presented in this thesis is presented. This chapter is therefore comprised of **section 7.2** outlining the key findings of the work outlined in this thesis. Following this, **section 7.3** offers a critical evaluation of this project and **section 7.4** provides insight into potential areas for future investigation.

7.2 Key Findings

The work outlined in this thesis tackled two main aims: firstly, to access acetylated derivatives of Neu5Ac via synthetic chemistry techniques; secondly, to investigate the potential of Neu5Ac and its acetylated derivatives as biomarkers for CVD and CVD risk by analysing plasma, serum, urine, and saliva samples.

Assays for the qualitative and quantitative analysis of sialic acids (Chapter 2)[8]

By reviewing the literature related to the analysis of sialic acids, it has been demonstrated that many assays for sialic acids have been developed since their discovery in the 1930's. Challenges were encountered with specificity in earlier assays for sialic acids, whereby other compounds present in complex biological mixtures can cause inaccurate quantitation. More modern developments have led to highly specific and accurate methods for the analysis of sialic acids, with the added ability to analyse multiple derivatives at once. Future scope was identified for the use of plate-based assays for high-throughput analysis of sialic acids using fluorescence detection.

Sialic acid as a potential biomarker for CVD, diabetes and cancer (Chapter 3)[6]

The literature currently available in biomarker research has indicated that *N*-acetyl neuraminic acid (Neu5Ac, sialic acid) has potential as a biomarker for different cardiovascular diseases, type-2 (but not type-1) diabetes and diabetic complications, and different types of cancer. Elevated concentrations of plasma and serum Neu5Ac were associated with the presence of these diseases when compared with healthy controls. Unfortunately, using Neu5Ac as a biomarker may be difficult as concentrations in biological fluids can be affected by all the aforementioned disease states.[9–15] Further to this, differentiating between the disease, especially is they are co-morbid may be difficult. Neu5Ac did offer potential to determine disease severity in cases such as coronary artery disease[16,17] or staging in the case of cancers.[18,19] There was also scope for Neu5Ac to be utilised as a biomarker for tracking the progression of treatment, with decreased concentrations in plasma/serum indicating a successful response to treatment.[20] Future scope was identified for Neu5,9Ac_2 as a biomarker for breast and oral cancer.[21,22]

Synthesis and utilisation of quantitative standards of Neu5,9Ac_2 and Neu4,5Ac_2 (Chapter 4)[23]

A distinct lack of quantitative standards of acetylated sialic acid derivatives has been noted by reviewing the literature related to quantitative analysis of sialic acids. Two acetylated sialic acid derivatives, Neu5,9Ac_2 and Neu4,5Ac_2, were synthesised by employed protecting group strategies. Yields were improved from those reported in the literature and the synthesis of Neu4,5Ac_2 was improved by reducing the number of synthetic steps by one.[24] The synthesised derivatives, as well as commercially available Neu5Ac and Neu5Gc, were utilised as quantitative standards for the analysis of sialic acid derivatives in plasma and serum using the DMB method outlined in Chapters 1 and 2**.** A method was developed that could qualitatively and quantitatively analyse multiple sialic acid derivatives in one assay while exhibiting extremely low limits of detection and quantitation in line with those previously reported in the literature.[25] Work was also carried out towards the synthesis of acetylated derivatives of Neu5Gc,[26] and towards the synthesis of Neu5,8Ac_2, Neu2,5Ac_2 and Neu5,7Ac_2.[27]

Potential associations between Neu5Ac and Neu5,9Ac_2 concentrations, and CVD risk (Chapter 5)

Previous studies have indicated an association between elevated concentrations of Neu5Ac in biological fluids and CVD mortality risk. The scope of these studies was limited however to only plasma, and only Neu5Ac. Potential associations between plasma, serum, urine, and saliva

concentrations of Neu5Ac and Neu5,9Ac_2, and QRISK3 score were investigated. Sensitivity analysis was also performed to determine any associations between the sialic acid markers and CVD risk factors that form part of QRISK3 assessment. Associations were observed between urinary Neu5Ac and QRISK3 as well as urinary Neu5,9Ac_2 and QRISK3 in women. Sensitivity analysis revealed a further association in women between Neu5Ac/Neu,9Ac_2 concentrations in urine and BMI, potentially a driving force behind QRISK3 associations. No associations were observed in men, however. Associations in plasma for CVD mortality risk have been identified previously in the literature which did not match the findings here. It was identified however that the cohort studied herein was relatively healthy and as such further work on a cohort with greater CVD risk may be required.

Neu5Ac and Neu5,9Ac_2 as potential biomarkers for advanced CVD (Chapter 6)

Neu5Ac has been well researched as a potential marker for CVD (Chapter 3). Preliminary research has been carried out for Neu5,9Ac_2 as a biomarker for malignant tumours,[21,28] but no research on Neu5,9Ac_2 in the context of CVD has been reported. Plasma concentrations of Neu5Ac and Neu5,9Ac_2 were determined using the DMB labelling method outlined in Chapters 2, 4 and 5. Neu5Ac and Neu5,9Ac_2 concentrations were significantly elevated in CVD cases versus healthy controls (Neu5Ac: $P < 0.001$; Neu5,9Ac_2: $P < 0.04$). Elevated Neu5Ac concentrations were as expected.[6] Elevated Neu5,9Ac_2 concentrations have not previously been reported in the context of CVD, although the significance is borderline. ROC analysis was carried out to determine the predictive power (AUC), as well as sensitivity and specificity of both markers. Neu5Ac and Neu5,9Ac_2 were both shown to have good ability to discriminate between CVD cases and healthy controls (Neu5Ac AUC: 0.95; Neu5,9Ac_2 AUC: 0.84). Both markers exhibited good specificity (Neu5Ac: 0.90; Neu5,9Ac_2: 0.90), but while Neu5Ac offered good sensitivity (0.88), Neu5,9Ac_2 had very low sensitivity (0.50) indicated a high false positive rate. Interestingly, a combined marker of Neu5Ac/Neu5,9Ac_2 was found to have similar predictive power (AUC: 0.96) to both markers, and similar sensitivity to Neu5Ac (0.88), but also offered excellent specificity (1.00) indicating a 0% false positive rate.

7.3 Critical evaluation

7.3.1 Synthesis of acetylated sialic acid derivatives

The synthesis of acetylated sialic acid derivatives was undertaken as these are the most abundantly available in humans and animals after Neu5Ac and Neu5Gc.[29] As such, they are of interest in many capacities such as biomarker research. They have also been associated with

many disease states,[6,30–32] and play roles in infections caused by bacteria, viruses, and parasites.[33–35]

Most attempts at the synthesis of sialic acid derivatives have utilised protecting groups to facilitate regioselective addition of desired functional groups at specific positions. These techniques were utilised effectively in Chapter 4 to yield acetylated derivatives of sialic acid, specifically Neu5,9Ac_2 and Neu4,5Ac_2, with improvements upon previous work.[24] Work was also carried out towards other acetylated sialic acid derivatives. Successful synthesis of these derivatives is reported in the literature.[27] However, low yields for some steps, such as protection of the C-7 and C-9 hydroxyls with a benzylidene protecting group were encountered which prevented access to some synthetic routes. Further to this, some further challenges with hydroxyl group reactivity, such as the C-2 position, were encountered when attempting to carry out acetylation or protection with a triethylsilyl protecting group. This is interesting however in the context of sialic acid, as the C-2 hydroxyl is stated to be more reactive than the C-7 hydroxyl despite being a tertiary alcohol.[27,36] The encountered reduced reactivity of the C-2 position may possibly be due to steric hindrance from bulky functional groups such as the acetonide or *tert*-butyl dimethylsilyl protecting groups. Synthesis of Neu5Gc was successful, although it was not taken forward as a quantitative standard as these are readily available commercially. Only a small quantity was synthesised however, even starting from gram scale quantities of Neu5Ac, which may cause challenges in future work with accessing acetylated derivatives of Neu5Gc. This is because while acetylating Neu5Gc at the C-9 position requires just one further synthetic step, other acetylated derivatives require the use of synthetic routes ranging from four-eight steps.

Given the issues encountered here with protecting group strategies, other approaches may be necessary to realise the synthesis of the full complement of acetylated sialic acids. Park *et al.* utilised microwave techniques to synthesise multiple sialic acid derivatives at the same time, this posed its own issues, however.[37] The technique resulted in the synthesis of mixtures of sialic acids rather than pure compounds and these derivatives are difficult to separate from one another, especially using conventional chromatographic techniques. Chemoenzymatic techniques may be more promising, with the ability to yield pure compounds, with reduced synthetic steps. Previous research in this area has demonstrated the synthesis of acetylated derivatives of Neu5Ac and Neu5Gc such as: Neu5,9Ac_2, Neu5Gc,9Ac and Neu5Gc4Ac.[38–41] This is generally carried out by first derivatising a substrate such as *N*-acetyl mannosamine and converting it to the desired sialic acid compound. This highlights the potential utility of

enzymes for the synthesis of these derivatives and perhaps demonstrates that by further derivatising mannosamine, different acetylated sialic acid derivatives can be synthesised.

7.3.2 Analysis of sialic acids via DMB labelling with LC-FLD

Analysing sialic acids in complex biological mixtures requires the utilisation of specific and sensitive methods. This is to avoid interference from endogenous compounds in the sample, and to accurately detect and quantify derivatives that may only be present in very small quantities. Combining methods such as 1,2-diamino-4,5-methyleneoxybenzene (DMB) labelling with separation *via* liquid chromatography and subsequent detection using fluorescence detection provides a method with excellent ability to detect and importantly separate sialic acids from one another (Figure 1).[42,43]

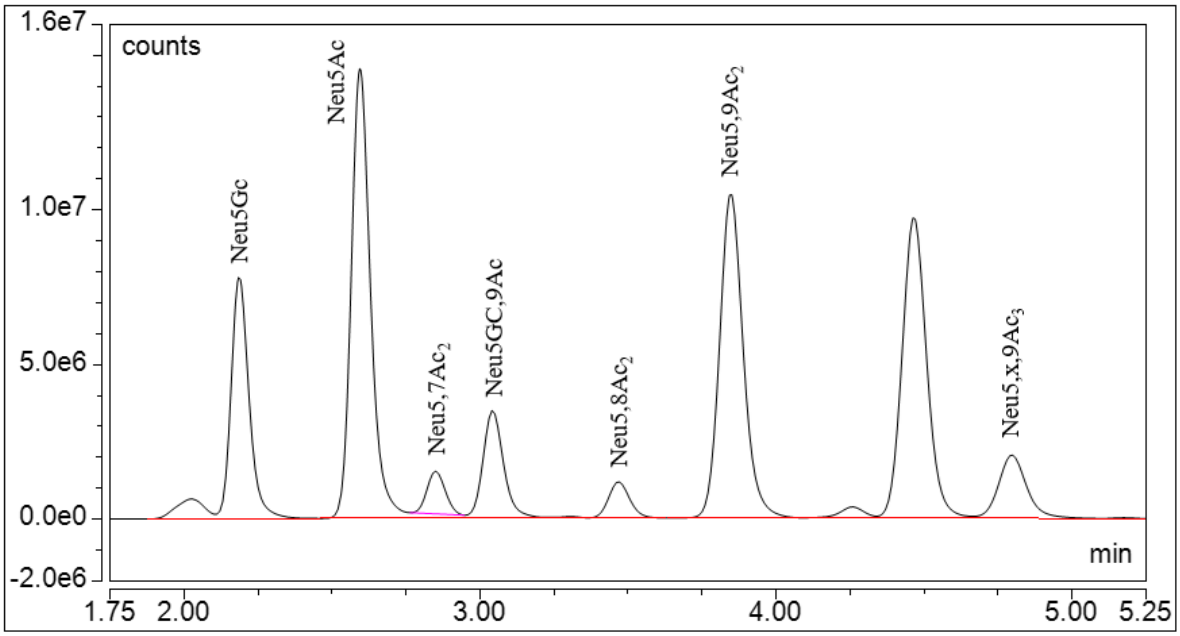


Figure 1: Sialic acids present in bovine mucin labelled with DMB and analysed via LC-FLD

The DMB method offered many advantages. Firstly, it is highly specific for sialic acids, with the fluorescent label reacting with a diketone species formed from the reaction of sialic acids with acid. This diketone species is not native to biological fluids and as such is not prone to suffer from interferences as is the case with many other assays (Chapter 2). Secondly, the method exhibited very low limits of detection (LOD) and limits of quantitation (LOQ) which were in line with previous literature.[44,45] Finally, the method was able to be utilised to analyse multiple derivatives with just one assay, this reduces the quantity of sample required and reduces the time required for sample analysis (Chapter 4).

In this research, Neu5,9Ac$_2$ proved relatively difficult to analyse (Chapters 5 and 6). Neu5,9Ac$_2$ was found to suffer from overlapping impurities in many of the chromatographic conditions tested, and as such much considerable method development was required. Neu5,9Ac$_2$ was also found to be present in only extremely small quantities in human samples (100-200 times less than Neu5Ac) and as such the samples required concentrating to a great degree to ensure that the concentrations of Neu5,9Ac$_2$ overcame the LOD and LOQ. Combining this with the need to adjust chromatographic conditions for this specific derivative, this resulted in the need for each sample to be analysed twice: once for Neu5Ac and once for Neu5,9Ac$_2$. While this did not affect the results, it greatly increased the amount of time required for analysis (2 x 15 minutes per sample) and impacts the high-throughput nature of the DMB method. Investigation of future prospects for analysis of sialic acids identified plate-based assays as a potential area of research with the ability overcome the issues outlined here. These are high-throughput methods, with the capacity to measure up to 96 samples at one time and can be performed using older methods such as the resorcinol assay[46] or more modern methods such as an enzymatic assay to measure the levels of sialylation and galactosylation.[47]

7.3.4 Sialic as a biomarker for CVD risk and the presence of CVD

Sialic acid has been identified as a biomarker for both increased CVD risk[10] and the presence of diseases such as CVD, type-2 diabetes and cancer (Chapter 3).[6] The literature suggests that elevated plasma concentrations of Neu5Ac in both men and women are associated with increased risk of CVD mortality risk.[48]

Chapter 5 aimed to test this hypothesis not only for Neu5Ac but also for Neu5,9Ac2 expanding the scope of previous research. The scope was also expanded by investigating serum, urine, and saliva alongside the plasma samples. A volunteer study recruited volunteers between the ages of 35-75 from the community who were either at risk of CVD or were otherwise healthy. The study successfully recruited 80 volunteers who provided blood, urine and saliva samples, as well as information for the evaluation of QRISK3 estimated relative risk score.[4] Statistical analysis was performed to determine any potential associations between concentrations of sialic acids in plasma, serum, urine, and saliva and QRISK3 estimated relative risk score, as well as CVD risk factors (cholesterol/high density lipoprotein cholesterol ratio, systolic blood pressure, body-mass index (BMI)) in both men and women. Associations were observed in women only, in urine, as shown in Chapter 5. Possible explanations may be related to overexpression of mucins in the bladder epithelium[49] or upregulation of highly sialylated acute-phase proteins as a result of increased levels of inflammation.[50,51] While these

associations have not previously been reported, the expected associations between QRISK3 estimated relative risk score or CVD risk factors, and sialic acid concentrations in plasma were not observed. The main potential cause of this was that the cohort was relatively healthy compared to the general population, for example average BMI and systolic blood pressure were only slightly above levels considered to be unhealthy. This resulted in a low range of QRISK3 risk scores (0.6-2.1). While volunteers with low risk were an integral part of the study and are easy to recruit, volunteers with higher risk scores (> 5) may be required to further validate the findings outlined in Chapter 5 and to determine any further associations. Recruitment of volunteers with higher risk scores may not be possible in the community, and as such it may be necessary to pursue other avenues for recruitment, such as through hospitals or other clinical settings. This would potentially allow for access to volunteers with higher BMI and blood pressure, as well as those with certain health conditions such as chronic kidney disease that greatly increased CVD mortality risk.

Chapter 6 provided a robust analysis of Neu5Ac and Neu5,9Ac_2 as biomarkers for the presence of advanced CVD. Elevated concentrations of both sialic acids were observed in plasma samples from CVD patients compared to healthy controls, with the elevations indicated to be statistically significant (Neu5Ac: $P < 0.001$; Neu5,9Ac_2: $P < 0.04$). It should be noted that while the P-value for Neu5Ac indicates high significance, the P-value for Neu5,9Ac_2 indicates that the significance is borderline, and care should be taken in interpreting this. Further research, in the form of a higher-powered study,[52] would be pertinent to confirm whether Neu5,9Ac_2 is in fact a marker for advanced CVD. Chapter 6 did not only evaluate the statistical significance of elevations in concentrations but also the predictive power of the two markers, that is the ability to discriminate between CVD and non-CVD cases. This was performed using receiver operator curve analysis (ROC) wherein it was found that Neu5Ac performed extremely well as a biomarker for CVD with excellent ability to discriminate between CVD and non-CVD cases. Neu5,9Ac_2 performed less well, with poor ability to discriminate between CVD and non-CVD cases. Interestingly however, a combination marker of Neu5Ac and Neu5,9Ac_2 performed better than Neu5Ac as a biomarker in terms of discrimination and showed an ideal false positive rate of 0%. These results were found to be better than previously established markers for CVD that were also analysed via ROC analysis. However, this research was performed on a relatively small cohort and as such, future studies on a larger cohort with more of both CVD and non-CVD cases should be undertaken to confirm these findings.

Overall, Neu5Ac and Neu5,9Ac_2 show some promise as markers for both the risk of CVD mortality and the presence of advanced CVD. One issue identified during the course of this

research however is that sialic acid concentrations in biological fluids can be affected by a number of other health conditions. Health conditions associated with increased sialic acid concentrations include: arthritis,[31,53] sepsis,[30] COPD,[32] cancer,[6] diabetes[6] and thyroid conditions.[54] Volunteers with any of these health conditions had to be excluded from the studies carried out in this thesis. If attempts were made to expand this research into wider clinical settings, the fact that these health conditions affect sialic acid concentrations would preclude the use of the biomarker in a large subset of the population. This may require further research to determine if sialic acid concentrations are able to be used discriminate between CVD and non-CVD cases, or predict CVD risk, when comorbidities that also affect sialic acid concentrations are present. Sialic acid may have greater utility as a marker for general inflammation. The current literature appears to point towards inflammatory processes, and the associated acute-phase response, as a potential explanation as to why overexpression of sialic acid is observed during the presence and pathogenesis of many, mostly inflammatory, health conditions.[32,50,55,56]

As established in this work, Neu5Ac is not the only sialic acid derivative of interest as a biomarker not only for CVD but also cancer. Neu5,9Ac_2 is of growing interest in cancer research due to the expression of acetylated glycolipids such as GD2 and GD3 on the surface of cancer cells.[57] Neu5,8Ac_2 has also been identified in breast cancer cell lines[58] which linking back to section 7.3.1 highlights the need for the synthesis of more sialic acid derivatives for their quantitation in a wide range of contexts.

7.4 Future Work

The work in this thesis highlighted potential associations between Neu5Ac and Neu5,9Ac_2, and QRISK3 (Chapter 5). While also confirming Neu5Ac as a marker for the presence of advanced CVD, and demonstrating that Neu5,9Ac_2 may tentatively be a marker for the same (Chapter 6). However, applying these markers in a clinical/diagnostic setting may require further investigation. This is not only to confirm whether these compounds are true markers for CVD and CVD risk, but also whether other inflammatory health conditions interfere with measurements. This requires further studies on larger cohorts with patients with higher QRISK3 scores and larger CVD case cohorts. Given the high global healthcare burden of CVD, further research may aid in providing methods for earlier diagnosis of CVD and reduction of the CVD healthcare burden.

There is also scope to improve upon the quantitative analysis of sialic acids outlined in this thesis, and to access further acetylated derivatives of sialic acid through chemoenzymatic

synthetic routes. Accessing a wider array of sialic acid derivatives has implications for not only biomarker research but also in areas such as *in vitro* protein binding studies and the analysis of glycosylation patterns in biopharmaceuticals. Oligosaccharides can be synthesised by employed both synthetic chemistry or chemoenzymatic strategies to produce a range of natural and unnatural oligosaccharides.[59–61] Incorporation of these oligosaccharides into glycan arrays can allow for the determination of protein binding mechanisms possibly highlighting protein function or even potential treatment routes for certain diseases.[62,63] Following this, analysis of biopharmaceuticals is extremely important, as well as a regulatory requirement. Expanding the repertoire of *N*-glycans standards may allow for easier quantification of *N*-glycans as part of complex biopharmaceuticals.[64]

7.4.1 Synthesis of acetylated sialic acid derivatives

The chemical synthesis using protecting group strategies is well reported in the literature.[24,36,65] These strategies can be difficult however with limitations including low overall yields from many synthetic steps and poor reactivity due to many bulky protecting groups. The number of synthetic steps, and therefore the number of protecting groups required, may be reduced by attempting chemoenzymatic synthesis of sialic acid derivatives.

Neu5Ac can be synthesised using enzymes such as sialic acid aldolase, sialic acid synthase, and *N*-acetyl neuraminate lyase generally using *N*-acetyl mannosamine (ManNAc) as the substrate.[66–68] Neu5,9Ac_2 can be synthesised with Neu5Ac as the substrate by the action of pancreas lipase or subtilisin.[38,39] Neu5,9Ac_2 has also been synthesised using 6-*O*-acetyl ManNAc as the substrate.[40] This points towards the utility of enzymes for the synthesis of acetylated sialic acid derivatives. It may be possible to chemically synthesise different acetylated ManNAc derivatives, and then utilise enzymes to produce acetylated Neu5Ac derivatives, possibly reducing the number of synthetic steps required. Progress has been made towards the synthesis of Neu5Ac derivatives that have been functionalised at the C-7 position using a chemoenzymatic approach, indicating the promise of this area of research.[69] This work can also be applied to Neu5Gc and its derivatives, with the ability to chemoenzymatically synthesise not only Neu5Gc itself but also its acetylated derivatives, especially Neu5Gc9Ac and Neu5Gc4Ac.[41] These strategies would form the basis for a strategy to access a wide array of acetylated Neu5Ac and Neu5Gc derivatives.

Synthesis of these sialic acid derivatives is important, a wider range of quantitative standards can be readily utilised for the analysis of sialic acids not only in humans, but also animals and pharmaceuticals. Analysis of Neu5Gc content of biotherapeutics is an important area in

ensuring that these treatments work effectively and do not contribute to further disease exacerbation.[70] These derivatives are not only useful as quantitative standards but in other areas as well such as the synthesis of both natural and unnatural oligosaccharides.[59–61] These can be utilised for investigating areas such as enzymatic activity and protein binding efficiency.

7.4.2 Development of improved quantitative assays for sialic acids

The DMB assay outlined in this thesis is a robust method for the analysis of sialic acids with high sensitivity and specificity (Chapter 4). However, due to the chromatographic nature of the method, impurities that overlapped the sialic acid peaks were identified and problems with ensuring samples were sufficiently concentrated for analysis were encountered. Some of this may be overcome by utilising plate-based assays. These are high-throughput methods, able to analyse up to a whole plate of samples (96) at once possibly cutting down time for analysis of a plate of samples from days to minutes.[47] The potential speed of plate-based assays comes at the expense of the ability to separate sialic acids from one another. It may be possible however, to analyse total acetylation by comparing fluorescence values before and after a de-*O*-acetylation reaction with labels such as acetylacetone which was discussed in detail in Chapter 2.[8]

7.4.3 Further evaluation of Neu5Ac and Neu5,9Ac_2 as biomarkers

Neu5Ac and Neu5,9Ac_2 showed some promise as biomarkers for CVD risk and the presence of advanced CVD. Limited associations were observed however in the case of CVD risk, and while Neu5Ac was established as a good biomarker for advanced CVD, Neu5,9Ac_2 was only tentatively established as a CVD marker.

To further confirm associations for CVD risk, a larger cohort with a greater range of QRISK3 estimated relative risk scores would be required. Recruitment of volunteers with high QRISK3 scores (> 5) can be difficult to carry out in the community where the recruitment was performed in this research. Accessing alternative recruitment routes through hospitals, clinics or GP surgeries may be the best option for finding volunteers who match the desired criteria of higher QRISK3 scores. This will be best achieved by recruiting volunteers with characteristics such as: high BMI (>25 kg/m^2), high systolic blood pressure (> 125), high cholesterol ratio (> 4.00), chronic kidney disease, atrial fibrillation, erectile dysfunction and a family history of CVD. Similarly, to confirm Neu5Ac and Neu5,9Ac_2 as biomarkers for advanced CVD, as well as their ability to discriminate between CVD and non-CVD cases, a larger cohort is required. Recruitment of healthy controls in the community and accessing clinical routes for advanced

CVD cases could yield a larger cohort to allow for confirmation of the findings outlined in this thesis.

Studies to determine whether health conditions that increase concentrations of sialic acids as comorbidities to CVD affect the utilisation of sialic acid as biomarkers for CVD could be carried out. It may also be of interest to determine if acetylated sialic acids are biomarkers for other diseases. Breast cancers have been associated with increased concentrations of Neu5,9Ac_2,[58] with the authors also reporting the presence of Neu5,8Ac_2 in the breast cancer samples analysed. It should be noted, however, that quantitation was not carried out, perhaps due to lack of availability of quantitative standards, once again highlighting the need to access these standards through synthetic chemistry and chemoenzymatic techniques to fully determine the biomarker potential of sialic acid and its acetylated derivatives.

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