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The Impact of Sialidases on the Urogenital Tract

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The Impact of Sialidases on the Urogenital Tract

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Abstract

Sialidases are enzymes which modify carbohydrate chains on cell membranes and glycoproteins by removing sialic acid residues. Raised sialidase activity is associated with a condition of the female genital tract called bacterial vaginosis. Bacterial vaginosis is associated with various health problems, including infertility. In this study we sought to determine whether variations in sialidase activity could be detected in samples from the male urogenital tract and if so, whether changes could be linked to sexual health. Using a fluorogenic substrate we were able to measure the sialidase activity in semen samples taken from both fertile and infertile men. It was found that almost 20% of men visiting a fertility clinic had high sialidase activity in their semen, whilst this was the case for only around 10% of fertile men. It appeared that high sialidase activity had various effects on the semen, including reducing seminal viscosity and removing streaks from the semen. In addition, it appeared that sialidase activity was associated with an increased seminal abundance of *Prevotella*, a bacterial genus known to contribute to sialidase production in bacterial vaginosis. Finally, we attempted to determine the potential effect of raised sialidase activity on *Neisseria gonorrhoeae*, the causative agent of a known urogenital infection. It was found that removal of sialic acid from the surface of *N. gonorrhoeae* caused reduced sensitivity to antibiotics, potentially making the infection harder to treat in patients with raised urogenital sialidase activity.

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Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED:  ..... DATE: 22/08/2018

Contents

Introduction

It has been estimated that as many as 48.5 million couples are infertile[1]. Around 20% of cases involve both the male and female partner, whilst 30% of cases are caused by a male factor only, meaning factors affecting male fertility could be involved in 50% of all cases of infertility[2]. Despite this, factors affecting male infertility are not well understood and one male infertility clinic reported 32.6% of its cases as being idiopathic[3]. Difficulty conceiving can contribute to increased stress[4] which can result in further morbidity. It is important that the causes of infertility, particularly those affecting men, are better understood, so that more effective fertility treatments can be developed.

It is possible that bacteria may be involved in some cases of idiopathic male factor infertility, as bacterial influence on male fertility is currently not well understood. There have however, been numerous studies analysing the effect of bacteria on fertility in women. The findings from these studies can provide a starting point for investigating the influence of bacteria on male factor infertility.

1. Bacterial Vaginosis and Infertility

There are multiple clinical definitions for bacterial vaginosis[5–7] (BV), but it is often described as a disruption of the normal vaginal microflora. This disruption can lead to discomfort, discharge, elevated vaginal pH, and a fishy odour[8], but is asymptomatic in approximately 50% of cases[9]. Between 2001 and 2004 BV was found to affect 29.2% of women in the United States[10].

The vaginal microbiome was first described by Albert Doderlein in 1892[11]. “Doderlein’s bacilli” later determined to be predominantly lactobacillus, were found to be the primary constituent of a healthy vaginal microbiome. Lactobacillus help to maintain good vaginal health by preventing the urogenital tract from being colonised by potentially harmful organisms. Lactobacilli outcompete pathogens for nutrients and adhesion receptors, and produce bacteriocins actively killing harmful organisms[12]. It is also thought that lactic acid produced by lactobacilli plays an important role in maintaining low vaginal pH, providing potentially another means of reducing colonisation by harmful bacteria[13].

The disruption of this lactobacilli dominated flora during BV can lead to severe complications, many of which have an impact on fertility. BV during pregnancy has been linked to preterm birth and postpartum endometriosis[14], and there is evidence that it predisposes women to sexually transmitted infections. Additionally, it has been reported that BV is linked to pelvic

inflammatory disease[15], a serious condition that can leave women infertile. However, another study contradicts these findings, claiming there is no link[16].

There is a high prevalence of BV amongst infertile women. One study reported that 37.4% of women suffering from unexplained infertility tested positive for BV, compared to only 15.4% in the control group[17]. Using secnidazole to treat these women for BV increased their chances of getting pregnant, indicating that BV was the cause of their infertility, rather than merely being associated with it. The exact nature of the link between BV and infertility has not yet been established. It has been suggested that BV alters the environment required for successful embryo implantation, by changing the pH and increasing local prostaglandin production[18]. Another possible cause of BV associated infertility lies in the enzymes produced by BV associated bacteria. High levels of sialidase activity are detectable in the vaginal fluid from women with BV[19], and it appears to be bacterial in origin, produced by *Prevotella, Bacteroides*, and *Gardnerella vaginalis*, all of which are found in elevated levels in BV patients[20]. Sialic acid plays an important role in fertilisation (see section 2.3), and so it seems plausible that sialidases, which remove sialic acid, could interfere with fertility.

2. Sialic acid

2.1 Sialic Acid Structure

Sialic acid is present in every domain of life, playing a variety of different roles. The term sialic acid is a general term referring to molecules derived from neuraminic acid[21] (see Figure 1A) the most common of which is *N*-acetyl-5-neuraminic acid. The unique structure and negative charge of neuraminic acid allow it to play an important role in biological signalling. Many proteins can recognise and bind to sialic acid allowing it to act as a receptor[22], but sialic acid can also obscure other molecules, blocking protein ligand interactions[23]. This dual function provides a possible explanation for the abundance of sialic acid mediated processes found throughout nature. Sialic acid is often bound to a galactose residue at the end of a polysaccharide chain, and this bond can take the form of an α-2,3 or an α-2,6 ketosidic bond (shown in Figure 1C). The type of bond attaching the sialic acid to its substrate changes is orientation, affecting which proteins can bind to the sialic acid, further expanding its potential functions[24].

Figure 1- Sialic Acid Structure

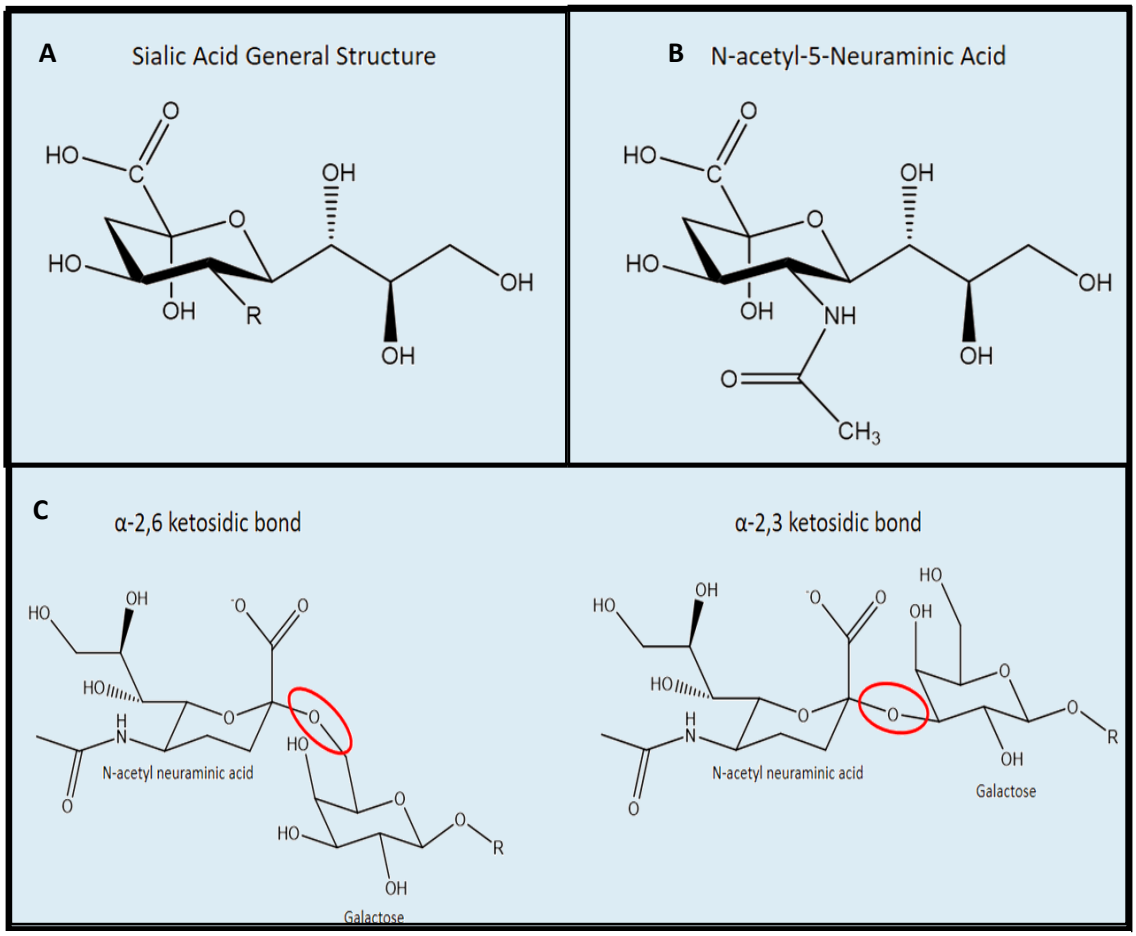

*Figure 1. Panel **A-** General structure of sialic acid. Panel **B-** the structure of N-acetyl neuraminic acid (right), the most common derivative in mammals. Panel **C-** Sialic acid linked to galactose via 2,3 and 2,6 linkages. Figure constructed using Chemdraw Professional.*

2.2 Role in immune regulation

A particularly well studied area of sialic acid function is its role in regulating the mammalian immune response. Terminal sialic acid residues on sugar chains attached to glycoproteins are recognised by the immune system as self-associated molecular patterns[25] and prevent immune activation against cells displaying them. Siglecs (short for sialic acid binding immunoglobulin type lectins) are a group of sialic acid binding proteins with various immune regulatory functions[26], that protect sialic acid expressing cells from both the innate and adaptive immune response. Siglec-7 is expressed by Natural Killer cells (NK cells), and inhibits cell killing by NK cells when it binds to sialic acid residues. CD22 (Siglec-2) is a siglec found on B-cells that inhibits their activation when it interacts with sialic acid[27], preventing a humoral response against sialic acid displaying cells.

Sialic acid also plays an important role in regulation of the complement system. The complement system is a component of the innate immune system, consisting of a number of serum proteins which, with appropriate activation, can kill target cells by forming a membrane attack complex, and can aid activation of other parts of the immune system, against the target[28]. Sialic acid acts as a cofactor along with glycosaminoglycans and complement protein C3b, for the C-terminal modules of factor H[29]. When factor H binds to a cell surface via sialic acid and other ligands, it adopts a conformation which allows it to inhibit activation of the alternative complement pathway (see Figure 2B), thereby preventing formation of the membrane attack complex and the death of the cell[30].

Figure 2- The Alternative Complement Pathway and its Regulation by Sialic Acid

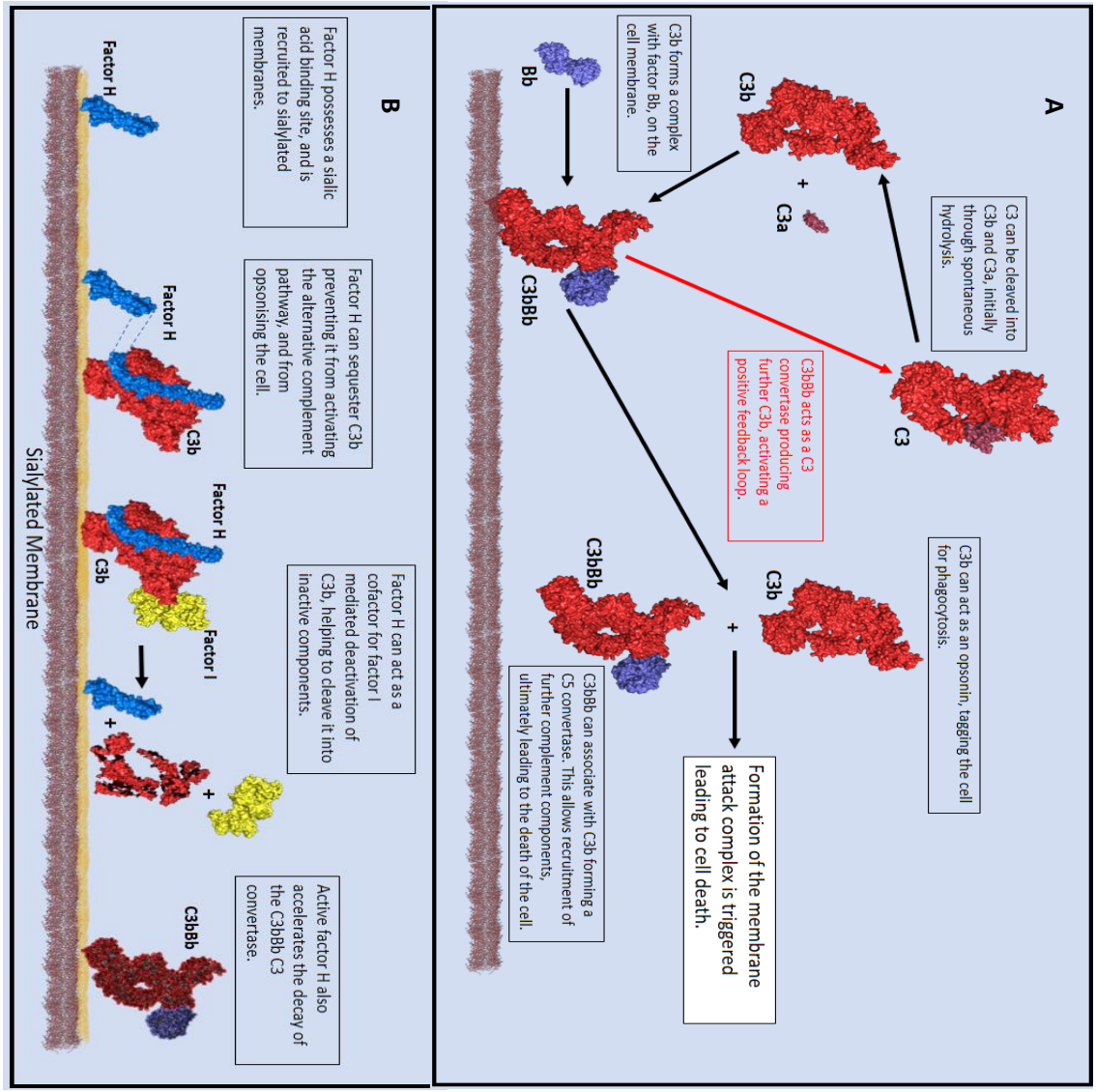

*Figure 2. Panel **A-** Diagram showing the role of C3b in activating the alternative pathway. C3b forms a C3 convertase with Factor B, leading to further C3b formation activating a positive feedback loop. C3bBb associates with another C3b molecule forming a C5 convertase which triggers the formation of the membrane attack complex and cell death (figure legend continued on next page).*

*Figure 2 (continued) Panel **B**- Diagram showing the multiple ways in which factor H prevents C3b from activating the alternative pathway after being recruited by sialic acid on the surface of the cell. Factor H on the cell membrane can sequester C3b, act with Factor I to deactivate C3b, and accelerate the decay of the C3bBb C3 convertase. Figure constructed using PyMol molecular visualisation software.*

2.3 Role in Fertilisation

Sialic acid also appears to play an important role in fertilisation. Sperm cells are coated in a glycocalyx which is rich in sialic acid[31]. This sialylation appears to play a key role in allowing the sperm cells to survive in the female genital tract, so that they can reach the ovum. The importance of sialic acid in sperm survival has been demonstrated in animal models. In 1991 Toshimori et al used sialidase to desialylate mouse sperm and measure the effect of sialic acid on phagocytosis by macrophages[32]. Sialic acid appeared to protect the sperm from phagocytosis, with more than 3 times the number of macrophages engulfing sperm cells being observed in the sialidase treated sperm than the untreated sperm. Ma et al found similar results in 2016, and also demonstrated the incremental addition of sialic acid to the sperm cells as they develop, indicating the importance of sialic acid in sperm function[33].

Despite the apparent importance of the sperm sialome, it is possible that sperm need to lose their sialome for fertilisation to occur, although evidence supporting this is conflicting. In 2007 Velásquez et al treated bovine sperm with various bacterial sialidases, and measured the effect this had on oocyte binding[34]. The result was a dose dependent loss of sperm binding to the outer membrane of the oocyte (the zona pellucida), indicating that in addition to protection from the female immune system, the sialic acid is needed for binding to the oocyte (at least in cattle). The opposite seemed to be true in a 2012 study by Ma et al[35]. They measured a release of *N*-acetyl neuraminic acid during capacitation (a process in which the sperm glycoprotein layer is shed to allow for binding to the oocyte), in both mouse and human sperm. Furthermore, they found that treatment with a sialidase inhibitor during capacitation had a negative impact on oocyte binding by the sperm. The release of sialic acid coupled with the need for sialidase activity during capacitation seemed to indicate a need for sperm cells to shed their sialome to be able to successfully bind the oocyte. This is supported by a 1988 study showing the induction of capacitation and enhancement of fertility by sialidase treatment of guinea pig and rabbit sperm[36]. It is possible that the role of sialic acid in mammalian fertilisation varies between species, with it being required for oocyte binding in cattle, whilst inhibiting it in other species. Alternatively, it is possible that an outer layer of sialic acid is shed during capacitation, to reveal other sialylated structures beneath which are required for oocyte binding. A summary of the

hypothetical role of sialic acid in fertilisation, based on review of the literature, can be seen in Figure 3.

Figure 3- Hypothetical Role of Sialic Acid in Fertilisation

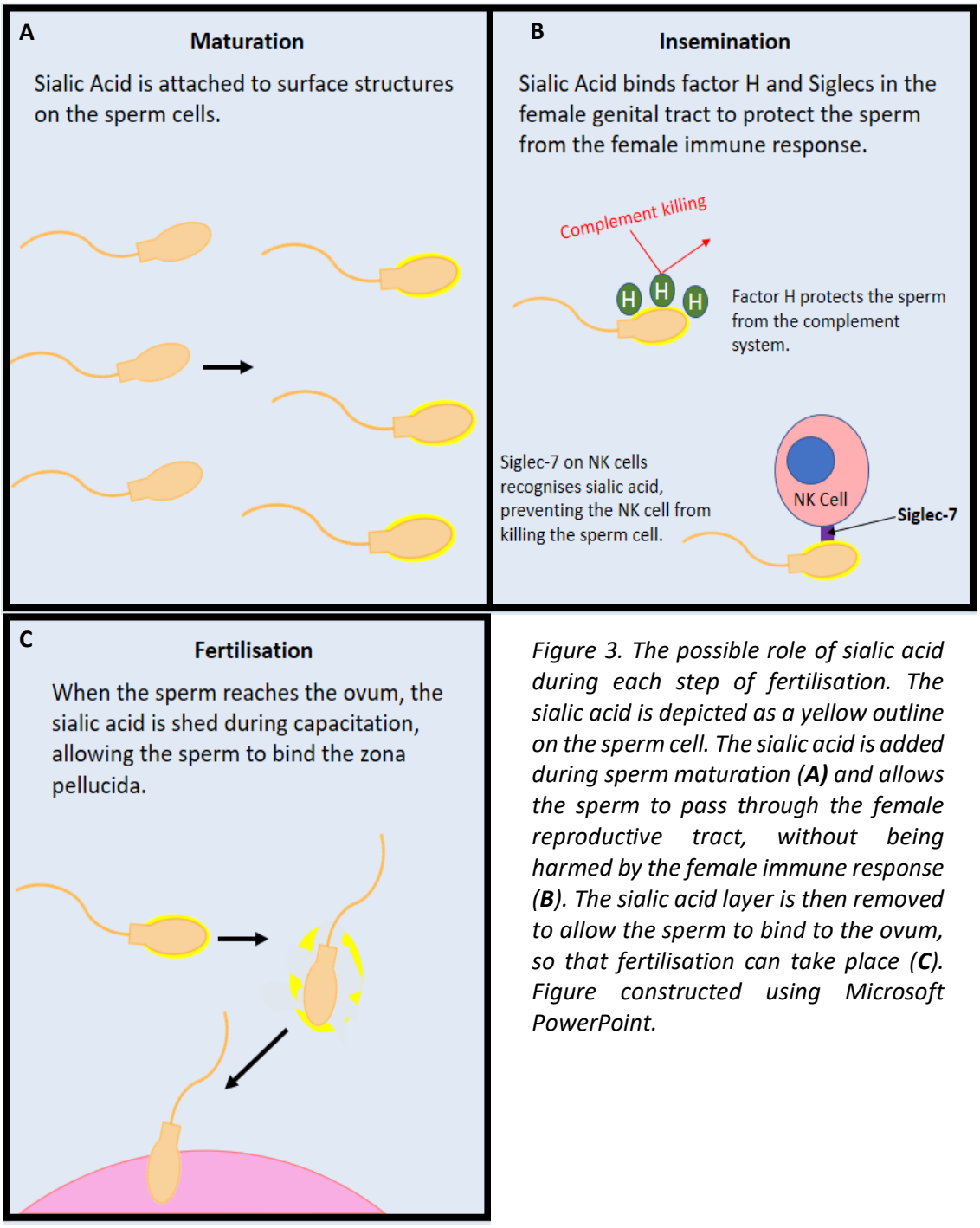

*Figure 3. The possible role of sialic acid during each step of fertilisation. The sialic acid is depicted as a yellow outline on the sperm cell. The sialic acid is added during sperm maturation (**A**) and allows the sperm to pass through the female reproductive tract, without being harmed by the female immune response (**B**). The sialic acid layer is then removed to allow the sperm to bind to the ovum, so that fertilisation can take place (**C**). Figure constructed using Microsoft PowerPoint.*

With maintaining the correct sialylation status of the sperm cell being so important for successful oocyte contact, it is likely that anything influencing the sialylation status of sperm cells could impede fertilisation. If the sialic acid is removed too early from the sperm cells, before they have successfully penetrated the cervical mucous and reached the zona pellucida, it could result in the cell being destroyed by the female immune system. Yet it would appear that if the sialic acid is not removed at all, the spermatozoa cannot bind to the ovum. This provides a possible explanation for the association between sialidases, which remove sialic acid, and infertility.

3. Sialidases

3.1 Structure and Catalytic Mechanism

Just as sialic acid is involved in a wide range of biological processes, so are sialidases. Sialidases are used by eukaryotes, prokaryotes, and viruses, and are involved in processes ranging from cell-cell signalling, to nutrient acquisition, to facilitating the spread of viral particles.

Most sialidases conform to a six bladed β-propeller fold, with the active site located in the centre of the enzyme (see Figure 4). Sialidases are categorised based both on the position of their sialic acid substrate, and the mechanism by which the bond cleavage occurs. The majority of discovered sialidases are referred to as exo-sialidases[37], as they catalyse the removal of terminal sialic acid from the end of carbohydrate chains. Endo-sialidases instead cleave bonds within polysialic acid chains. Exo-sialidases can be further grouped into three distinct classes: hydrolytic exo-sialidases, trans-sialidases, and intramolecular trans-sialidases (IT-sialidases). All exo-sialidases are thought to act through the classical Koshland retaining glycosidic hydrolase mechanism[38] in which a glycosyl enzyme intermediate is formed after nucleophilic attack of the ketosidic bond[39], which is then hydrolysed, freeing the sialic acid and retaining its anomeric configuration. Sialidases are unusual in that tyrosine acts as a nucleophile, rather than the typical glutamic or aspartic acid, although it is thought that a proximal glutamic acid activates the tyrosine to allow this. The hydrolysis of the sialic acid-enzyme covalent intermediate is part of what differentiates hydrolytic, trans, and IT sialidases. In hydrolytic sialidases water attacks the covalent intermediate, in IT-sialidases it is the O7-hydroxyl group in the sialic acid that does this which leads to the release of 2,7 anhydro N-acetlyneuraminic acid instead of neuraminic acid. In trans-sialidases this step appears to be catalysed by the presence of lactose, allowing the ketosidic bond to be transferred to another carbohydrate structure.

Figure 4- Exo-sialidase Structure

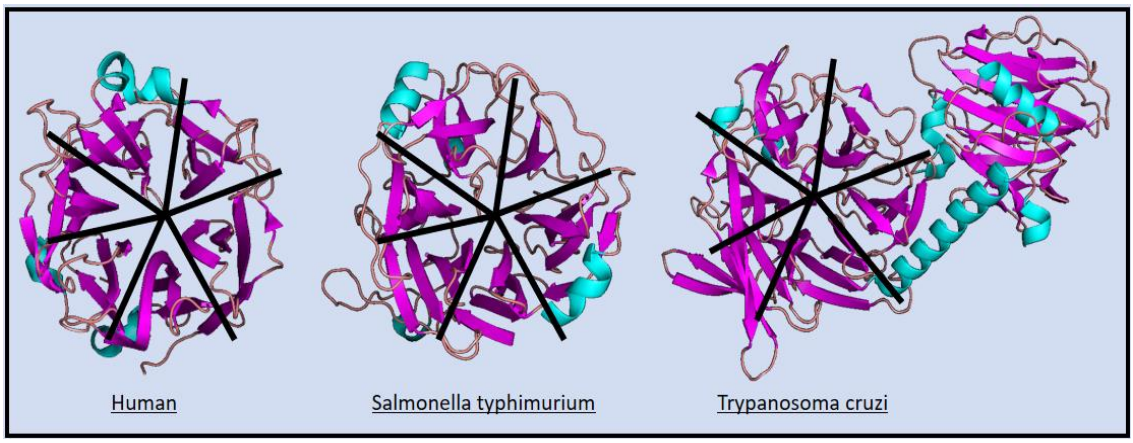

Figure 4. Three different exo-sialidases each sharing the six bladed β-propeller fold. The β-sheets making up the folds have been separated with black lines to highlight their conformation. Figure constructed using PyMol molecular visualisation software.

3.2 Bacterial Sialidases

Many different species of bacteria produce exo-sialidases, and they often play a role in processes related to pathogenicity. Some bacteria use sialic acid as a source of nutrients and produce sialidases as a means of scavenging host sialic acid, an example of which can be seen in *Vibrio cholerae* the causative agent of Cholera, which produces a sialidase to scavenge host sialic acid (NanH), machinery for uptake of sialic acid (SiaPQM), and enzymes for catabolising sialic acid (NanA and NanEK), allowing it to use sialic acid as a carbon source[40].

Other bacteria use sialidases to uncover receptors for adhesion. Mucosal surfaces are often home to a vast number of different bacterial species, all of which compete for space and nutrients. Bacteria such as *Tannerella forsythia* (often found in the mouth) have adapted to this competition by using sialidases to uncover novel receptors normally obscured by sialic acids[41], allowing them to bind in places sialidase deficient bacteria would not be able to. Some bacteria use sialic acid itself as an adhesion factor, and it has been demonstrated that sialidase producing bacteria are able to reduce colonisation of other bacteria that need the sialic acid to adhere to host cells[42] possibly providing another competitive function of sialidases.

Bacteria also use sialidases to acquire sialic acid as a means of protecting themselves from the innate immune response. Pathogenic bacteria such as *Haemophilus influenzae*[43], and *Neisseria gonorrhoeae*[44,45] often use sialic acid to protect themselves from the immune response, by using sialyltransferase enzymes to attach the sialic acid to their LPS, allowing sialic acid dependent

immune regulatory mechanisms such as Siglecs and factor H, to protect them. Sialidases may allow some bacteria to acquire sialic acid for this purpose.

Streptococcus pneumoniae is a pathogenic bacterium for which sialidases play an important role during infection. *S. pneumoniae* produces three different sialidases, NanA, NanB, and NanC. Each one having a slightly different mechanism of action and carrying out a different function. NanA is a hydrolytic sialidase able to cleave free α2,3, α2,6, and α2,8 linked sialic acid whilst retaining anomeric configuration, which appears to be involved in epithelial cell adherence[46], perhaps by unmasking hidden receptors. NanB is an IT-sialidase, catalysing the release of 2,7 anhydro N-acetlyneuraminic acid rather than neuraminic acid, the expression of which is downregulated in response to iron[47], possibly indicating a role in nutrient acquisition or metabolism. NanC is unusual in that rather than releasing N-acetyl neuraminic acid, it releases 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA) which is a sialidase inhibitor[48], although it can also further hydrolyse DANA to produce N-acetyl neuraminic acid, depending on how much substrate is present, which could allow it to play a role in regulating the activity of NanB and NanC.

3.3 Mammalian Sialidases

Mammalian sialidases are often involved in cellular signalling, removing sialic acid from sialylated glycoproteins as needed, to allow access to underlying receptors. Four sialidases have been identified in humans: NEU1, NEU2, NEU3, and NEU4[49]. Each of the human sialidases varies in its subcellular localisation and its substrate specificity. Mammalian sialidases have a six bladed β-propeller fold, as seen in bacterial sialidases (shown in Figure 4), and are thought to use a mechanism of action similar to that of other hydrolytic exo-sialidases. The differing subcellular localisation of each neuraminidase allows each enzyme to carry out a different function.

NEU1 is mainly located within lysosomes, where it appears to play a role in catabolising glycoconjugates, although it is also involved in a huge range of signalling pathways, such as that of TOLL-like receptor 4, a component of the innate immune system, which it activates by desialylating key residues[50]. NEU2 is mainly found in the cytosol and appears to be involved in muscle and neuronal cell differentiation in rats and mice[51]. However, NEU2 is not expressed at high levels in human cells and often cannot be detected at all[52], making its role in humans unclear. NEU3 is associated with the plasma membrane and (in humans) specifically hydrolyses gangliosides. Association with the plasma membrane allows NEU3 to regulate a range of cell surface processes[53], and it has been demonstrated to play a role in neuronal differentiation and

apoptosis, by activating various cell surface receptors[54]. NEU4 has been reported to be localised both to lysosomes and mitochondria[55] Like NEU3, NEU4 appears to play a role in neuronal cell differentiation, although whilst NEU3 positively regulates neurite formation, NEU4 seems to negatively regulate it[56]. Mammalian sialidases seem to play a far more complex role than bacterial sialidases, with each enzyme being involved in regulating a range of different processes and signalling pathways.

3.4 Other Sialidases

Sialidases are not just limited to bacteria and mammals. They are also found in various species of protozoa, fungi, and even viruses. A particularly well studied sialidase is produced by *Trypanosoma cruzi,* the parasitic protozoan responsible for causing Chaga's disease. *T. cruzi* produces a trans-sialidase, which transfers sialic acid from one residue to another, rather than releasing free sialic acid[57]. It is thought that *T. cruzi* uses trans-sialidase activity to transfer sialic acid from the host to its own surface glycoconjugates. This coating of sialic acid allows it to evade the immune system and survive in the host[58].

The influenza virus also produces a sialidase[59]. Influenza binds to sialic acid on the surface of the target host cell before entering and infecting the cell. The outer shell of the influenza virus contains the clusters of the sialidase NA, which cleaves sialic acid to allow mature viral particles to leave the cell after replication[60]. Some viral sialidases have been demonstrated to have endo-sialidase activity, able to hydrolyse residues within chains of poly-sialic acid. An example of endo-sialidase activity can be seen in the bacteriophage K1F which uses endo-sialidase activity to degrade the polysialic acid coat on *Escherichia coli* cells, allowing the phage access to the cell surface for infection[61].

4. Sialidases in Semen

4.1 Rationale for Predicting the Presence of Sialidases in Semen

As mentioned in section 1, bacterial vaginosis is associated with subfertility, and also with raised sialidase activity. These two observations may be linked, given the important role sialic acid plays in fertilisation. Abnormally high sialidase activity in the genital tract could trigger the premature removal of the sperm sialome through sialidase activity, allowing the female innate immune system to destroy the sperm cells before they reach the ovum. The sialidase activity in bacterial vaginosis patients has been demonstrated to be bacterial in origin, meaning it is possible that raised sialidase levels may not just be limited to the female urogenital tract. Studies have demonstrated that the urogenital microbiome of sexual partners are shared to

some degree[62], meaning the sialidase producing organisms in bacterial vaginosis patients may also inhabit the urogenital tract of their male partners. It is possible that the association between bacterial vaginosis and infertility, may be in part, due to bacterial vaginosis being an indication of sialidase producing bacteria in the male urogenital tract, which may be desialylating sperm before they enter the female genital tract. If sialidase producing bacteria in the urogenital tract are linked with infertility, bacteria in both the male and female genital tract could be involved.

4.2 The Seminal Microbiome

Whilst the male urogenital microbiome is not as well characterised as the vaginal microbiome, there have been a number of studies analysing the bacteria present within the male urogenital tract either using 16S rRNA sequencing[62–64] or culturing of semen samples[65,66]. The apparent composition of the seminal microbiome seems to vary from study to study and is likely affected by factors such as the whether the microbiome was analysed by culturing or sequencing, geographical variation, and (if sequencing was used) the part of the 16S rRNA gene targeted by the sequencing primers. However, all studies find potential sialidase producers to be present within semen samples.

Two studies which cultured organisms from semen samples found an abundance of *Enterococcus faecalis,*[65,66] which has a sialidase annotated in its genome, possibly allowing it to desialylate sperm cells. In 2014, Hou et al used 16S rRNA sequencing to analyse the bacteria present within semen samples, and they found that several samples (almost 20% of the total sample population) contained *Prevotella*[63], many species of which are known to produce sialidase. *Prevotella* was also found in other studies, which analysed the seminal microbiome using 16S rRNA sequencing, along with *Gardnerella*, another potential sialidase producer[62,64], both of which are associated with sialidase activity in bacterial vaginosis. Whilst the identification of bacteria capable of producing sialidase is not an absolute indication of sialidase activity, it does indicate the possibility that bacterial sialidases may be present within the male urogenital tract as well as the female urogenital tract.

Figure 5- Summary of previous Seminal Microbiome Studies

Study	Hou 2014	Weng 2014	Mandar 2015
Location	Shanghai, China	Hsinchu, Taiwan	Tartu, Estonia
Sequencing primer target	V1-V2 Region	V4 Region	V6 Region
Most abundant taxa	Ralstonia	Lactobacillus	Lactobacillus
	Lactobacillus	Pseudomonas	Flavobacterium
	Corynebacterium	Prevotella	Porphyromonas
	Streptococcus	Gardnerella	Garderella
	Staphylococcus	Rhodanobacter	Prevotella
	Prevotella	Streptococcus	Atobium
	Finegoldia	Finegoldia	Clostridiales
	Anaerococcus	Staphylococcus	Dialister
	Peptoniphilus	Corynebacterium	Finegoldia
	Incertae. Sedis. XI	Clostridium	Shuttleworthia

Figure 5. Summary of the results of three previous seminal microbiome studies. The location of the study is listed in addition to the region of the 16S rRNA gene amplified by the primers used. The bacterial taxa found in each study are listed in order of abundance, with the most commonly identified taxa being at the top of the table. Certain taxa have been highlighted, to demonstrate the similarity between their findings (for example Prevotella and Lactobacillus were found in high abundance in all three studies). Note that this table only shows the top 10 most abundant taxa from each study. Figure constructed using Microsoft Powerpoint.

4.3 Potential Impact of Sialidases in Semen

The presence of sialidases in the male urogenital tract presents a potential cause of infertility, that is thus far unstudied. It is possible that as the vaginal microbiome fluctuates, sometimes causing bacterial vaginosis, the male urogenital microbiome male may also fluctuate, but with less obvious symptoms. This could potentially lead to some men having an abundance of sialidase producing bacteria in their semen, prematurely removing the sperm sialome, and impeding fertility. The sialidase producing bacteria may spread from the male to the female partner potentially exacerbating the problem, and possibly causing bacterial vaginosis. It is also possible that the sialidase producing bacteria originate in the female partner, perhaps as the result of pre-existing bacterial vaginosis, and then spread to the male partner.

In addition to the effect of sialidases in semen on fertility, there may be an effect on urogenital pathogens. *Neisseria gonorrhoeae,* the causative agent of gonorrhoea, relies on a coating of sialic acid (like many pathogens) to survive within the host[67]. *N. gonorrhoeae* obtains cytidine

monophosphate N-acetyl neuraminic acid from within host cells[67,68], the N-acetyl neuraminic acid portion of which is then attached to the outer membrane LPS by a sialyltransferase[69]. Therefore, urogenital sialidases may potentially have a beneficial effect by removing sialic acid from *N. gonorrhoeae*, limiting the pathogens survivability in the host and preventing dissemination. Such a benefit of sialidases should certainly be considered if sialidase inhibitors are ever administered as a means of treating infertility. *N. gonorrhoeae* has recently become particularly problematic due to its increasing levels of antibiotic resistance[70,71], making infections harder to treat. It is not known to what extent sialic acid influences the antibiotic susceptibility of *N. gonorrhoeae,* but such a significant surface modification could have some impact. This presents another potential impact of urogenital sialidases to be considered.

5. Project Outline

Based on the afore mentioned observations in the literature, and previous studies by the group, it appeared likely that sialidases could have a clinically relevant impact on the male urogenital tract, worthy of further investigation. The aim of this project was to better understand this impact. Firstly, by helping to determine the sources of sialidase activity in the male urogenital tract. Secondly, by attempting to determine the impact of sialidase activity on fertility. And finally, by attempting to understand the effect of sialidases on the physiology of *N. gonorrhoeae,* the causative agent of a common urogenital infection.

This project intended to confirm the presence of sialidases in semen by optimising sialidase assays for the use in semen. Men both with and without apparent fertility problems were recruited to provide semen samples, allowing for the determination of the incidence of sialidase activity in semen, and the degree to which it is associated with fertility problems. 16S rRNA sequencing was used to analyse the bacteria present within the semen samples, allowing us to better understand the bacterial contribution to sialidase activity, and to identify organisms that are potential risk factors in sialidase mediated infertility. In addition to this, the effect of sialidases on *N. gonorrhoeae* was studied. The effect of sialidases on gonococcal serum resistance is well documented, but this project aimed to determine whether sialidases have any impact on gonococcal antibiotic susceptibility, with the aim of helping to provide a more complete understanding of the impact of sialidases on the urogenital tract.

Materials & Methods

1. Reagents

4-Methylumbelliferyl-n-acetyl-α-d-neuraminic acid (4MUSA) was purchased from Carbosynth Ltd. All other equipment and reagents were purchased from Sigma-Aldrich, unless otherwise stated.

2. Semen Sample Collection

Samples of semen were collected by two clinics. Andrology Solutions in London, and the Bristol Centre for Reproductive Medicine (BCRM). Patients were provided with a written patient information leaflet detailing the nature and purpose of the study and were given the opportunity to ask questions about the study, before being given a consent form to sign, confirming their agreement for the samples to be used in this study. Samples were produced by masturbation into a sterile container, and then frozen, before being transported to the University of Bristol on dry ice, using an approved courier. Initial freezing was done at -80^{o}C at Andrology Solutions, and -20^{o}C at the BCRM. Samples were anonymised prior to shipment. Men were excluded from the study if they had taken antibiotics in the last 4 weeks, were aged under 18 years, or were unable to provide informed consent. Ethics approval can be found under IRAS project ID- 168675 and REC ID- 15/SW/0093; 02.04.15.

3. Measurement of Semen Parameters

A portion of each sample was retained by the clinics and used to measure semen parameters. Both the Bristol Centre for Reproductive Medicine, and Andrology Solutions measured sample volume, sperm concentration, the percentage of sperm with normal morphology, the percentage of sperm with progressive motility, and the pH of the sample. In addition to these parameters, Andrology Solutions also measured the reactive oxygen species, the concentration of round cells, the concentration of leukocytes, the percentage of living cells, the concentration of IgA, the concentration of IgG, and the percentage of sperm with any form of motility in the samples. Andrology Solutions also provided a qualitative assessment of sample appearance, viscosity, and the amount of debris. All parameters were not measured in all samples, due to limited sample volumes. Data was identified using numerical sample ID's, and no identifying information such as patient name or birth date was included in the datasets.

4. Sample Processing and Storage

After arrival at the University of Bristol. Samples were thawed on ice for a minimum of 1 hour before being divided into aliquots for enzyme assays and sequencing. Samples were then quickly frozen using liquid nitrogen and stored at -80°C. Detailed records were kept listing the location of each sample in accordance with the Human Tissue Act (2004). If at any point details emerged indicating the sample was no longer eligible for use in the study, the sample was destroyed.

5. Sialidase Assay

100µl of each semen sample was thawed on ice for 1 hour. 50µl of each sample was mixed with 50µl of 2mM 4MUSA in assay buffer (400mM sodium acetate pH 4.2), and 50µl of each sample was mixed with assay buffer only (the autofluorescence control). A blank was prepared by mixing 50µl of 2mM 4MUSA in assay buffer, with 50µl of Dulbecco's phosphate buffered saline. All reactions were incubated at 37°C for 1 hour, and then stopped by addition of 100µl of 850mM glycine pH 10 (stop buffer). 100µl of each stopped reaction was added to separate wells in a black polystyrene 96-well plate, and the fluorescence of each reaction was measured at an excitation of 355nm and emission of 460nm, using a BMG Labtech plate reader.

6. Activity Calculation

Sialidase activity for each sample was calculated by subtracting the fluorescence of the blank reaction (4MUSA in assay buffer and PBS), and the autofluorescence (sample and assay buffer only), from the total fluorescence measured in the assayed sample (sample with 4MUSA in assay buffer). The value generated by this calculation provided an estimate of the fluorescence produced just by sialidase activity within the sample. The final activity for each sample was calculated as the average of three experiments.

7. Sequencing

Genomic DNA was extracted from the semen samples using a QIAamp DNA Blood Mini Kit, following the supplied spin protocol for DNA purification from blood or body fluids with no alterations. A barcoded amplicon library was prepared by PCR using the forwards primer 5'-XXXXGGATTAGATACCCBRGTAGTC-3' and the reverse primer 5'-XXXXTCACGRCACGAGCTGAC GAC-3' where the X's are replaced by a four codon long barcode used in a combination unique to each semen sample. Each 50µl PCR reaction contained 5µl purified sample DNA, 0.5µM of each primer, 1 unit of Phusion polymerase, 200µM dNTPs, 1x Phusion HF buffer, and 28.5µl of nuclease free water. The cycle conditions were as follows: 30 seconds initial denaturation at

98°C; followed by 35 cycles of 10 seconds at 98°C, 20 seconds at 50°C, and 30 seconds at 72°C; followed by a final extension of 10 minutes at 72°C. The size of the products was checked by gel electrophoresis and then sent to the Bristol Genomics Facility. Staff at the Bristol Genomics Facility carried out sample purification, normalisation and pooling. The pooled amplicon library was then sequenced using 2 Ion Torrent PGM 318v2 chips. The results were returned as FASTQ files.

8. Sequencing Data Analysis

The FASTQ files were sent to a computational biologist who deconvoluted and analysed the data using the QIIME bioinformatics pipeline. Data was returned to us as the relative percentage abundance of each 16S rRNA amplicon sequence found in each semen sample, taxonomically identified to the highest level possible.

9. Bacterial Strains and Growth Conditions

3 different strains of *N. gonorrhoeae* were used in these experiments: P9, FA1090, and SN1. Two variants of P9 were used, the nonpiliated P9-1 and the piliated P9-2. All strains were grown on GC agar (see below) and incubated at 37°C, 5% CO_2. Sialylated gonococci were grown by spreading a solution of CMP-NANA in sterile water, onto the surface of the agar to the specified final concentration and allowing the plate to dry before inoculating with bacteria.

10. GC Agar

400ml of agar contains 4g proteose peptone no.3, 0.4g starch, 4g Difco agar, 1.6g K_2PO_4, 0.4g KH_2PO_4, 2g NaCl, 0.4g glucose, 0.04g L-glutamine, 1mg B-NAD, 0.4mg cocarboxylase, 0.012mg thiamine HCl, 0.04mg cyanocobalbumin, 0.132mg $Fe(NO_3)_3$. $9H_20$, 0.052mg 4-aminobenzoic acid, 4mg adenine, 0.12mg guanine HCl, and 0.104mg L-cysteine HCl.

11. Immunodot Blot Test for Sialylation

Immunodot blotting was used to determine the sialylation status of the bacteria. Bacteria were suspended in sterile water and lysed by the freeze-thaw method. The resulting lysates were dotted onto nitrocellulose membrane by vacuum. The unoccupied nitrocellulose surface was then blocked by incubation at room temperature with 3% bovine serum albumin (BSA), in PBST (8mM Na_2HPO_4, 150mM NaCl, 2mM KH_2PO_4, 3mM KCl, 0.05% Tween 20, pH 7.4) for 1 hour. After blocking the nitrocellulose was incubated with the primary antibody SM82, which is specific to lacto-N-neotetraose, for 1 hour. The nitrocellulose was then incubated with the secondary antibody, AP conjugated anti-mouse IgM, for 1 hour. Finally, the nitrocellulose was

placed in AP buffer (100mM NaCl, 100mM Tris-HCl, 50mM $MgCl_2$, 1% Tween 20, 0.5mM BCIP, 0.4mM NBT) until purple colour developed. The nitrocellulose was washed three times between each antibody incubation using ELISA wash (0.15M NaCl, 0.55M Tween 20).

12. Effect of Sialylation on Growth Rate

Both sialylated and unsialylated P9 bacteria were grown on agar as described in section 9, and then suspended in GC broth to a concentration of approximately $5x10^6$ cfu ml^{-1}. CMP-NANA was added to the broth to give a final concentration of 25µg ml^{-1} in the sialylated bacteria. No CMP-NANA was added to the unsialylated bacteria. 200µl of each suspension was added to separate wells in a clear 96-well plate and placed in an incubator at 37°C 5% CO_2. The plate was removed from the incubator at hourly intervals and the OD600 was measured using a plate reader.

13. Antibiotic Susceptibility Assays

Antibiotic susceptibility assays were set up as per growth rate assays (see above) but with the following changes. The bacteria were resuspended to a concentration of approximately $1x10^7$ and then 100µl of bacterial suspension was added to 100µl of the appropriate concentration of antibiotic dissolved in GC broth. The resulting 200µl was incubated in a 96-well plate as per the growth rate assay, but rather than measuring the A600 at hourly intervals, the absorbance at 600nm was only measured at the end of a 20-hour incubation period.

14. Relative Growth Calculations

The relative growth was calculated using the following equation:

Relative Growth= Optical Density of tested condition (AU)/ Optical Density of control condition

Relative growth was used to normalise growth rate data to allow comparison of the wild type to the mutant strains.

15. Construction of *N. gonorrhoeae* Lst knockout

P9-2 was used as a starting strain used to produce a Δ*lst* knockout mutant, by insertion of a kanamycin resistance cassette into the *lst* gene. The insertion was created using SOEing PCR. The 5' *lst* flanking region was amplified using the forward primer 5'-GCAAATGCCGTC TGAACAGACGGCATCGCGCCG-3' and the reverse primer 5'-ATCCGGGGAATTCACTGGCTC GTGGCAGGATTTGCAGC-3'. The 3' *lst* flanking region was amplified using the forward primer 5'-CCCTGCAGGTCGACGGATATATCCCGAAGCAAAAAACAATCAG-3' and the reverse primer 5'-ACGTTTCAGACGGCATCGGGGAATGCCGCCAAAATG-3'. The kanamycin resistance cassette was amplified using the forward primer 5'-GCTGCAAATCCTGCCAC GAGCCAGTGAATTCCCCGGAT-3'

and the reverse primer 5'-CTGATTGTTTTTTGCTTCGGGATATATCCGTCGACCTGCAGGG-3'. These PCRs were carried out using *Taq* DNA polymerase and standard PCR protocol. These three fragments were then spliced together by PCR in a reaction containing 550ng of template DNA comprised of equimolar concentrations of each fragment in nuclease free water, 1x Standard *Taq* reaction buffer, 200µM dNTPs, 1.25 units of *Taq* polymerase. The conditions for the splicing PCR were as follows: An initial denaturation at 95°C for 30 seconds, followed by 15 cycles of 95°C for 25 seconds, 58°C for 50 seconds, and 68°C for for 2 minutes 45 seconds. After 15 cycles 0.2µM each of the forward primer 5'-GCAAATGCCGTCTGAACAGACGG-3' and the reverse primer 5'-ACGTTTCAGACGGCATCGGGG-3' were added to the reactions. The reactions were then subjected to 20 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 68°C for 2 minutes 35 seconds. This was followed by a final extension of 68°C for 5 minutes. The splicing reaction produced an insert containing regions homologous to the gonococcal *lst* gene, flanking a kanamycin resistance cassette, extended with the gonococcal DNA uptake sequence. The PCR mixture was then pipetted directly onto a fresh streak of P9-2 on GC agar, which was then incubated overnight at 37°C 5% CO_2. Bacteria were then scraped from the region of the streak onto which the DNA had been pipetted, and re-streaked onto GC agar containing 50µg ml^{-1} Kanamycin and incubated overnight again at 37°C. Colonies were picked from the kanamycin plate and checked for successful transformation by PCR using the forward primer 5'-GCAAATGCCGTCTGAACAGACGGCATCGCGCCG-3' and the reverse primer 5'-CTGATTGTTT TTTGCTTCGGGATATATCCGTCGACCTGCAGGG-3' with *Taq* DNA polymerase. Further confirmation of the mutant strain was provided using immunodotblotting as described in section 10**.**

16. Statistical Analysis

The relationship between sialidase activity and fertility status was first analysed using a student's t-test. Mean sialidase activity was grouped into three categories: Fertile (samples from BCRM), Infertile (samples from Andrology Solutions confirmed to be unsuccessful after >1 year of attempting to conceive), and Fertility clinic (all samples from Andrology Solutions). The infertile group, and the fertility clinic group were each independently checked for significant difference to the fertile group. First a two sample F-test was used to check for equal variance. It was found that the variances of the fertile samples and the infertile samples were unequal, whilst the variances of the fertile samples and the fertility clinic samples were equal. For comparing the fertile to the infertile samples, a two-sample t-test assuming unequal variances was used. For comparing the fertile to the fertility clinic samples, a two-sample t-test assuming equal variances was used. In both tests it was found that the t-stat was between the two-tail t-

critical value, and the -1(two-tail t-critical value), therefore indicating that there was no significant difference between these groups.

To check for significant relationships between sialidase activity and numerical semen parameters the samples were separated into three groups: High activity (sialidase activity >1000AU), Low activity (sialidase activity 1-1000AU), and No activity (sialidase activity ≤0). The values for each parameter were separated into these three groups and checked for significant difference using a single factor ANOVA test. The F statistic was found to be higher than F-critical in only two parameters: sperm concentration, and sample volume. For these samples the three groups were compared using first an F-test to check whether variance was equal or unequal, and then a two-sample t-test to confirm which groups were significantly different to one another. Excel Analysis ToolPak was used for all statistical test calculations.

Results & Discussion

1 Development and optimisation of sialidase assay for use in semen.

Assessment of the impact of sialidases on semen first required a means of measuring sialidase activity in semen. 4-Methylumbelliferyl-n-acetyl-α-d-neuraminic acid (4MUSA) acts as a fluorogenic substrate for sialidases[72] and is often used to assay sialidase activity. Before using the substrate to measure activity in semen samples, attempts were made to maximise the sensitivity of the assay, by testing the use of lower volumes of more concentrated stop buffer (Figure 1).

Figure 1- The Effect of Stop Buffer Concentration on 4MU Fluorescence

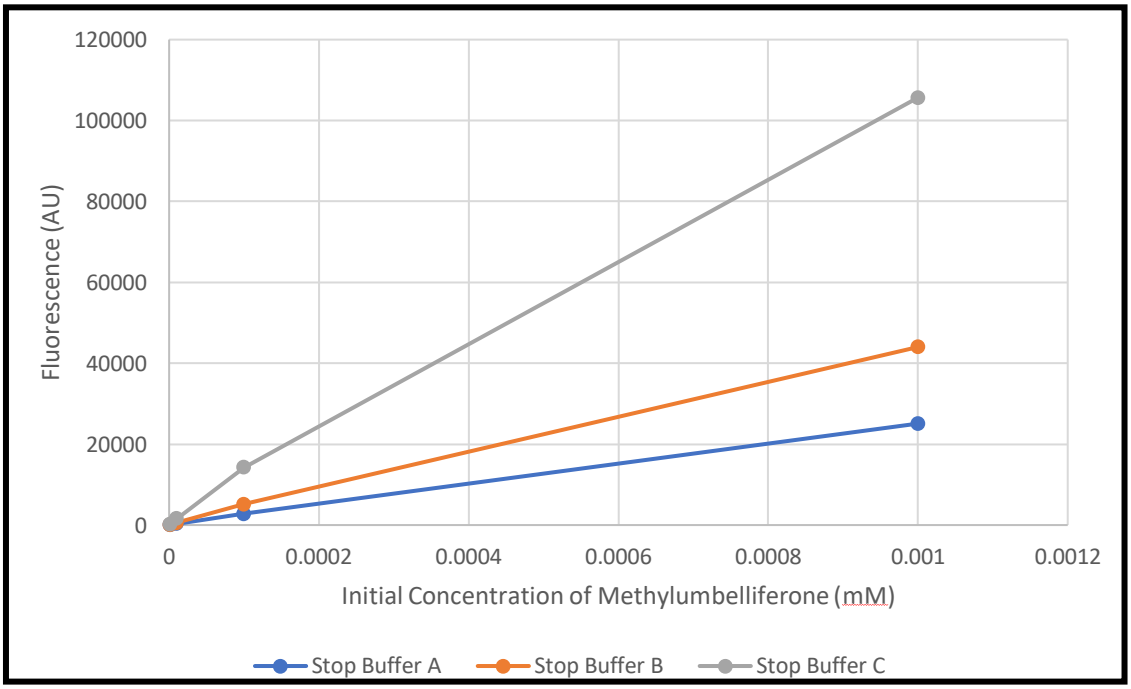

Figure 1: The Effect of Stop Buffer Concentration on 4MU Fluorescence- Three different stop buffer variations were tested using a standard curve of 4MU. For stop buffer A, 1ml of 85mM glycine (pH 10) was added to 0.1 ml of 4MU. For B, 0.5 ml of 170mM glycine was added. For C, 0.1ml of 850mM glycine was added. N=1.

The original assay protocol recommended the addition of 1 ml of 85mM glycine buffer to stop the reaction. It was found that the addition of 0.1 ml of 850mM glycine (pH 10) to 4-methylumbelliferone (4MU) produced a much larger fluorescence signal. This was likely due to maintaining a higher concentration of 4MU, whilst still successfully raising the reaction pH to 10 thereby stopping the reaction and allowing the 4MU to fluoresce. Using lower volumes of stop buffer could also likely increase the signal of any background fluorescence in the samples,

but this would be unlikely to be a problem should the signal-noise ratio remain constant at different volumes.

Two different pH values of the reaction buffer were tested in order to determine which should be used in the wider screen of clinical samples. Different sialidases have a different optimum pH, but due to restraints on sample volume, it would only be possible to test the samples at one pH. A selection of samples was assayed at both pH 4.2 (the average vaginal pH[73] and close to the optimum pH for many bacterial sialidases[74–77]), and pH 7.7 (the average pH of semen[78]).

Figure 2- The Effect of Reaction pH on Seminal Sialidase Activity

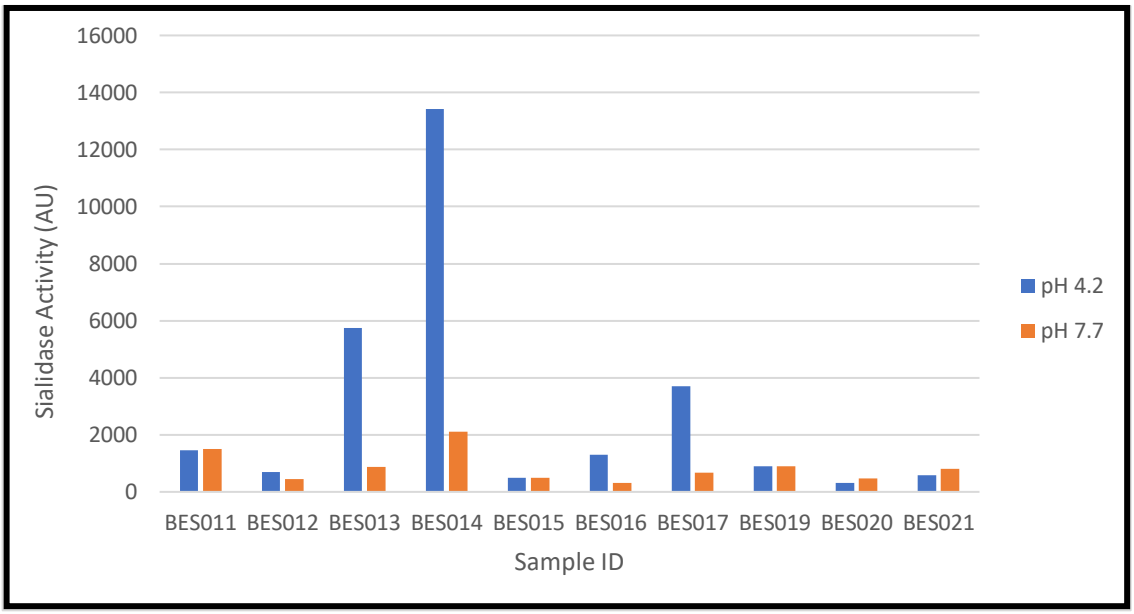

Figure 2- A comparison of sialidase activity in semen samples assayed at both pH 4.2 and pH 7.7. Whilst some samples remained mostly unaffected the pH change, the fluorescence for several samples was dramatically higher at pH 4.2. N=1.

It was found that in four of the ten samples tested, the sialidase activity was much higher at pH 4.2 (Figure 2). The other six samples were mostly unaffected by the pH change, although in two low activity samples samples (BES020 and BES21) the activity was slightly higher at pH 7.7. Whilst carrying out the reactions at pH 7.7 would have been more representative of *in vivo* conditions, it was decided that pH 4.2 should be used to maximise signal, thus maximising the sensitivity of the assay.

To determine the effect that sample storage might have on sialidase activity, one sample was split into three portions immediately after collection at the BCRM, and each portion was stored under different conditions before both the sialidase activity and the sample autofluorescence were measured. Storage conditions were designed to mimic variations in storage procedure at different clinics collecting the samples. One part of the sample was immediately stored at -80°C

whilst another was stored at -20°C. A third portion was stored at -80°C after being left at room temperature for 1 hour.

Figure 3- The Effect of Sample Storage Conditions on the Sialidase Assay

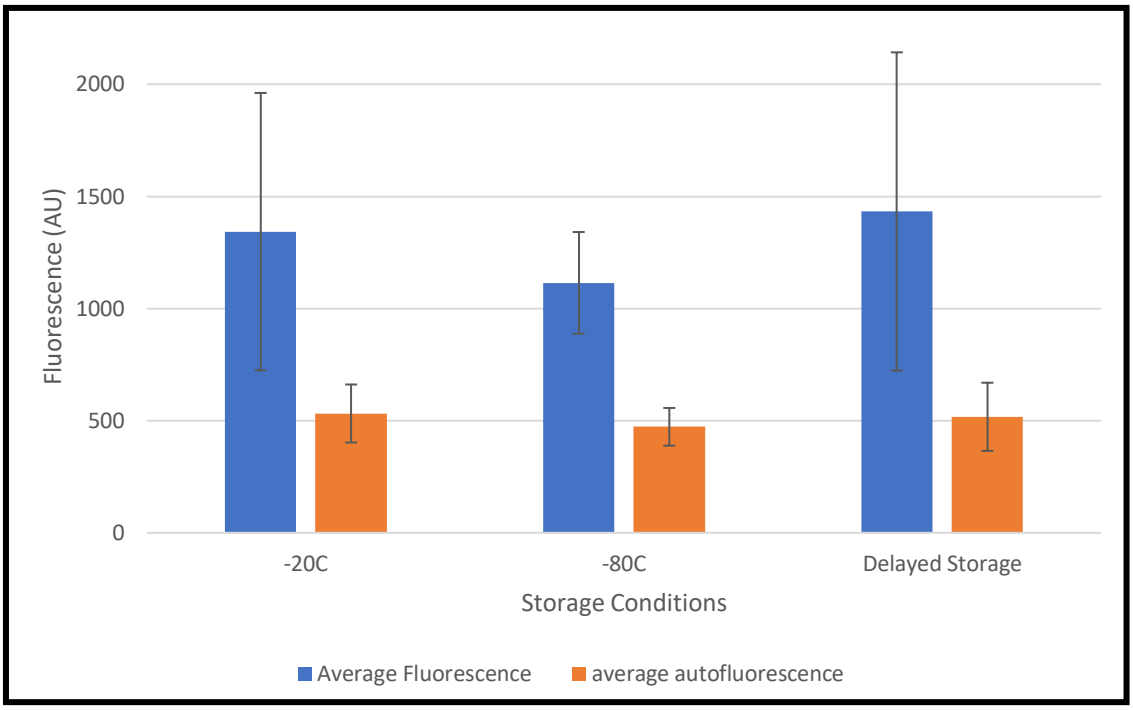

Figure 3- A single semen sample was split into three and stored under three different conditions. One sample was stored immediately at -20°C (-20C), one at -80°C (-80C) and one was left at room temperature for 1 hour before being stored at -80°C (Delayed Storage). The blue bars show the total fluorescence of the sample (sialidase activity + autofluorescence) whilst the orange bars show just the sample autofluorescence. Error bars show one standard deviation to either side of the mean. N=3.

Immediately storing the sample at -80°C provided the most consistent results across three repeats (Figure 3). However, altering the storage conditions did not appear to have a statistically significant effect on the mean sialidase activity and autofluorescence. This experiment whilst limited, demonstrated that samples from clinics without access to -80°C freezers should still provide useful data.

The effect of long term sample storage was tested by measuring the sialidase activity and sample autofluorescence of a random selection of semen samples, both before and after the samples had been stored at -80°C for 1 year (see figure 4). It was found that both the sialidase activity and autofluorescence were reduced after a year of storage, with many samples that previously tested positive for sialidase activity now testing negative. For example, BES024 was recorded as having an activity of 1860AU (after autofluorescence was subtracted), but after one

year of storage this was reduced to 0AU. BES034 had the highest recorded activity of 11930AU, but this was reduced to less than 3000AU after storage.

Figure 4- The Effect of Long-Term Storage on the Sialidase Assay

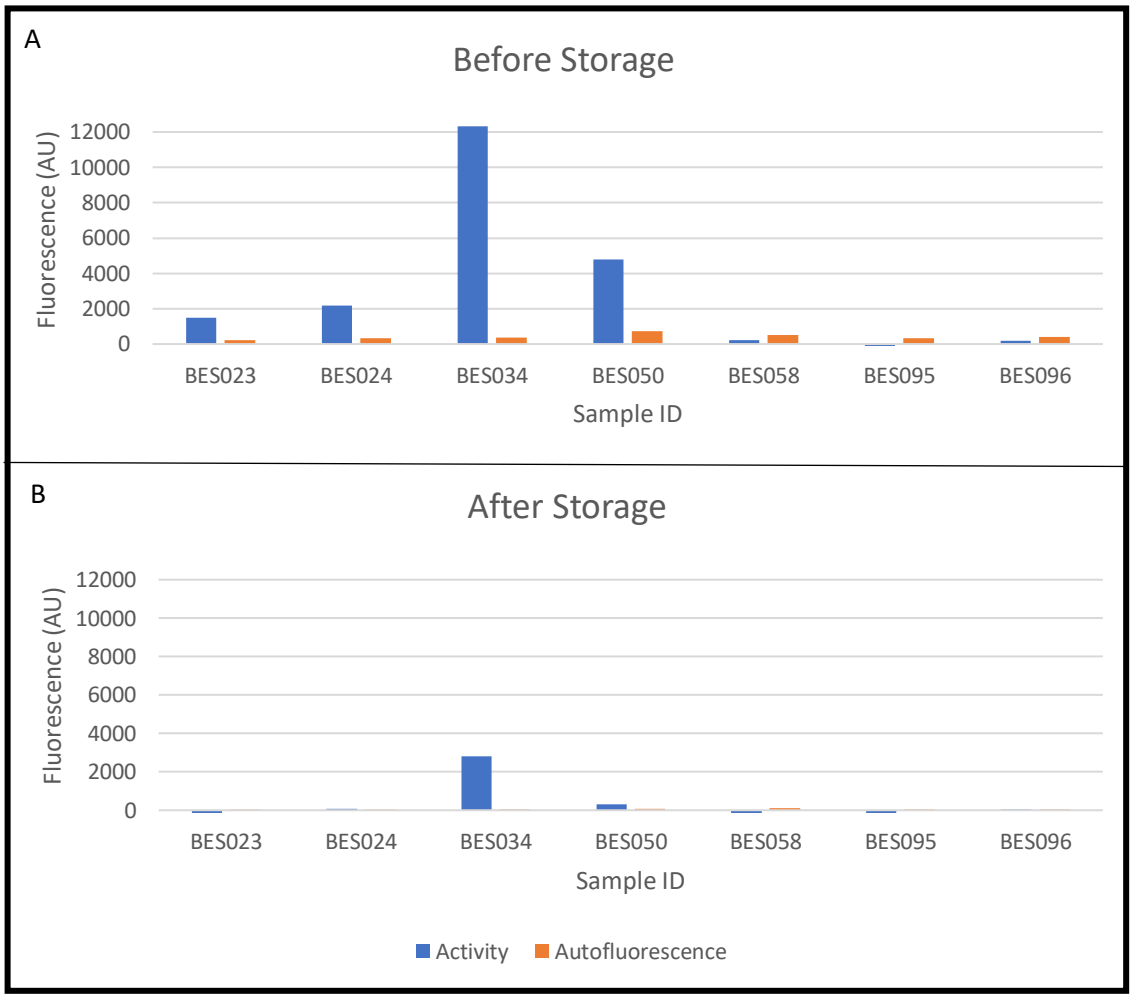

Figure 4- Panel A shows the total fluorescence of the sample after an hour of incubation with 4MUSA (activity) and without 4MUSA (autofluorescence) before the samples were stored for a year at -80°C. Panel B shows the results of the same assay being conducted on the same samples after the year of storage. Both the activity and autofluorescence were severely reduced by the long-term storage. N=1.

These results provide important considerations for future studies, as they indicated that testing for sialidase activity after long term storage of semen samples at -80°C is not viable. Most enzymes are stable at -80°C but living cells can lose some viability after long term cold storage. It is possible that these results indicate enzyme production by living cells (bacterial or human) could be the primary source of sialidase activity during the assay.

2. Sialidase Activity in Semen

Sialidase activity was measured in 142 semen samples. In addition to sialidase activity, other semen parameters were measured such as sperm motility and pH. The samples were also categorised based on the fertility status of the patient. 30 of the samples were grouped as 'fertile' as the donor had successfully conceived within the last 6 months. The other 112 samples were provided by a fertility clinic. Most of the donors for these samples were likely to be suffering from fertility problems, hence their visiting the fertility clinic. However, only 61 samples could be defined as 'infertile' as such a classification requires the patient to have being attempting to conceive for longer than 1 year, without success. For a large proportion of the samples from the infertility clinic, detailed information regarding the fertility status of the patient was not available, and therefore many of these samples could not be defined as infertile despite a high likelihood of fertility problems.

Initial analysis of the relationship between sialidase activity and fertility status shows no significant difference between any of the groups (*see table 1)*. This was not unexpected, as most of the infertile patients were likely to be suffering from conditions unrelated to sialidases. The results demonstrate that a certain level of sialidase activity can be present even in healthy semen samples, as a subset of sialidase positive samples was present within both the fertile and infertile samples.

Table 1- Patient Fertility Status and Sialidase Activity

Sample Group	Mean Sialidase Activity (AU)	P-value (*t-test against fertile*)
Fertile (n=30)	169.6 ± 113.4	N/A
Infertile (n=61)	219.7 ± 121.6	0.76
All Fertility Clinic (n=112)	470.0 ± 168.8	0.37

Table 1- The average sialidase activity for each group is shown (± standard error). Data was analysed using a student's t-test to check for significant difference between the infertile and fertile patients, and between all samples from the infertility clinic and the fertile samples, the p-values from these tests are shown in the table. No significant differences were found.

Despite there being no overall significant difference between the average sialidase activity of each group. It was still possible to identify differences in the distribution of sialidase activity

within the groups. A frequency distribution was generated by grouping the samples from the fertile, infertile and fertility clinic samples, into bins of 500 based on sialidase activity.

It should be noted here that due to the methods used to calculate sialidase activity in this assay (see Materials and Methods section 6), many samples appeared to have negative activity. This could be due to limitations of the assay discussed in section 2.1 of the general discussion (page 56). However, until more is learnt about the causes of the negativity of these samples, it was decided that samples with negative activity should be recorded as such, and the range of the frequency distribution should be inclusive to such samples.

Figure 5- Sample Distribution by Sialidase Activity

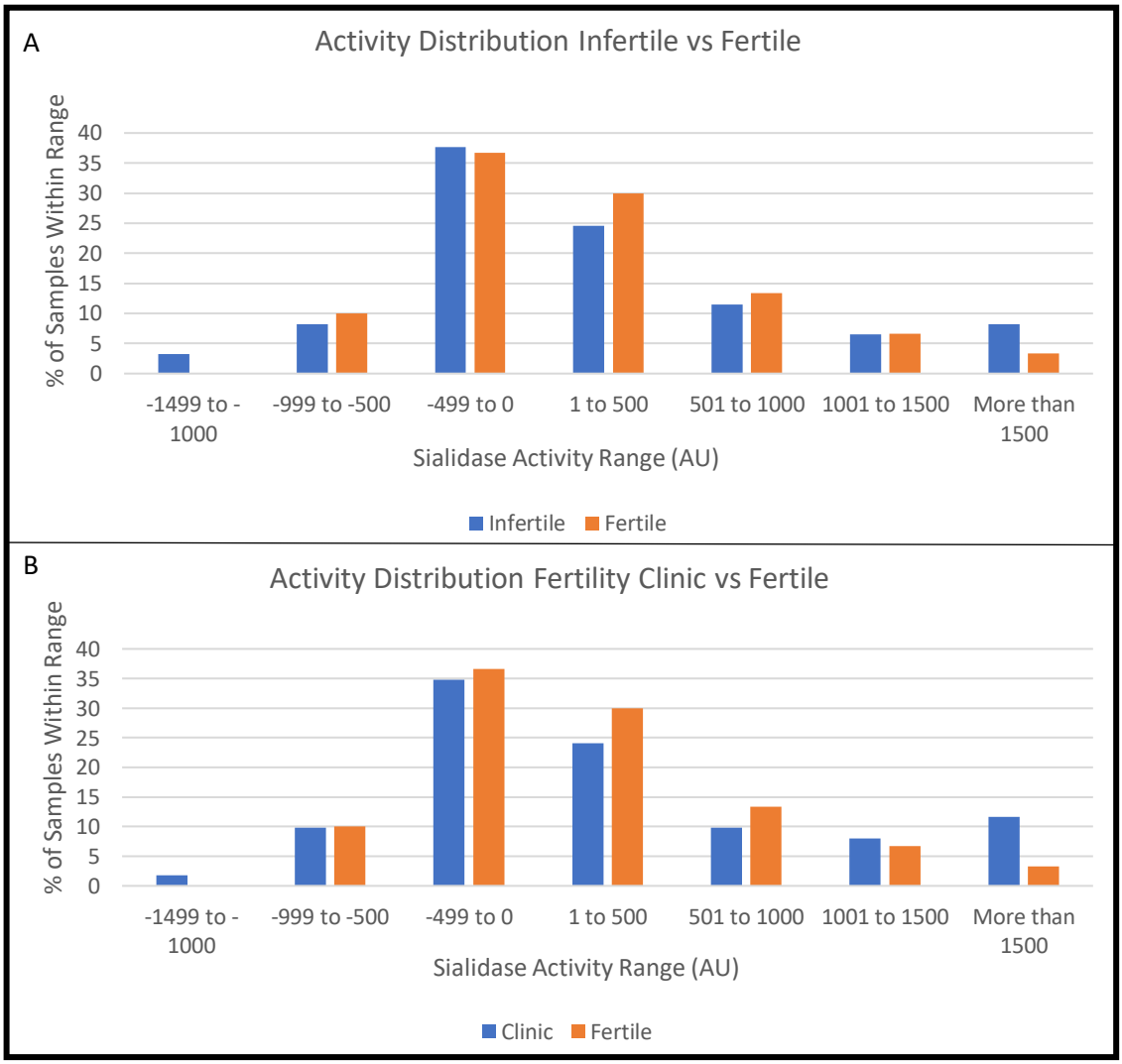

Figure 5- The distribution of sialidase activity is shown. Panel A compares the fertile samples to all samples defined as infertile. Panel B compares the fertile samples to all samples from the fertility clinic regardless of whether detailed fertility information was available for the sample. The bars show the % of samples within that group that fall within the specified range.

Studying the distribution of sialidase activity across the samples, highlights features not observable when only comparing the mean activity of fertility status groups (see Figure 5). For all 3 sample sets the majority of samples have activity between -500 and 500 AU. This range can be considered the 'normal' range of sialidase activity in semen. The difference between the sample sets can be observed at the tails of the distribution. Particularly noteworthy is the higher proportion of infertile samples, and fertility clinic samples with abnormally high levels of activity. 10% of the fertile samples have sialidase activity greater than 1000AU, whilst 15% of the infertile samples, and 20% of the samples from the fertility clinic have activity greater than 1000AU.

The sensitivity of the fluorogenic assay was useful in identifying the presence of sialidase activity but did not provide a means of determining the level of activity needed to influence the sample. It is possible that activity that low levels of activity, whilst detectable by this assay, have no effect on the sample, and that a certain threshold is required to compromise the fertility status of the patient. These results could indicate that this threshold lies above 1000AU (as calculated by our assay).

3. Association of Sialidase Activity with Semen Parameters

The distribution of sialidase activity did not confirm whether or not sialidase activity is associated with infertility. However, the 5% increase in infertile and 10% increase in infertility clinic samples, with activity greater than 1000AU, indicates the potential for a small subset of samples in which high levels of sialidase activity could be influencing fertility.

To further investigate this, the samples were split into three groups based on sialidase activity: negative samples (sialidase activity is equal to or less than 0), low activity samples (sialidase activity is between 1 and 1000), and high activity samples (activity is greater than 1000). Semen parameters were compared between these groups, to identify any possible relationships between semen parameters and sialidase activity. In the majority of measured parameters there was no significant difference between the three groups (Table 2).

Table 2- Semen Parameters not Significantly Associated with Sialidase Activity

Parameter	Activity Group	Mean Value	P (ANOVA)
pH	Negative	8.15	0.06
	Low Activity	8.07	
	High Activity	7.97	
Total Motile Sperm (%)	Negative	58	0.94
	Low Activity	59	
	High Activity	58	
Progressively Motile Sperm (%)	Negative	51	0.75
	Low Activity	49	
	High Activity	49	
Sperm with Normal Morphology (%)	Negative	4.2	0.55
	Low Activity	4.9	
	High Activity	4.7	
Sperm Vitality (%)	Negative	64	0.36
	Low Activity	68	
	High Activity	67	
Leukocytes (million/ml)	Negative	0.90	0.38
	Low Activity	0.49	
	High Activity	0.44	
Age of Patient (Years)	Negative	38	0.64
	Low Activity	36	
	High Activity	38	

Table 2- Table shows the mean values for several semen parameters, and the P-value from an ANOVA comparing the three sialidase activity groups. Negative samples have activity equal to or less than 0AU, low activity samples have activity between 1 and 1000AU, high activity samples have activity greater than 1000AU.

Of the parameters listed in Table 2, pH has the most significant relationship with sialidase activity. Whilst the data is not significant enough to give a p-value lower than 0.05, it appears possible that slightly lower pH could be linked to higher sialidase activity. This could be due to the acidic optimal pH of many bacterial sialidases. There appeared to be no association between sialidase activity and morphology, motility, or vitality. This indicates that raised sialidase activity has no effect on sperm cells observable microscopically. This was as expected, as there was no reason to suspect that the removal of sialic acid would have an immediately obvious impact on

sperm function. Furthermore the average values for each group was above lower reference limits for all measured parameters, as described by the 2010 WHO laboratory manual for the examination and processing of human semen[79], demonstrating that these samples were suitably representative of normal human semen.

However, some parameters did appear to differ between the 3 sample groups. A larger proportion of the high activity samples were described to have 'normal' appearance, whilst more of both the negative and low activity samples were described to be 'streaky' upon macroscopic evaluation (see Figure 6).

Figure 6- Sample Appearance and Sialidase Activity

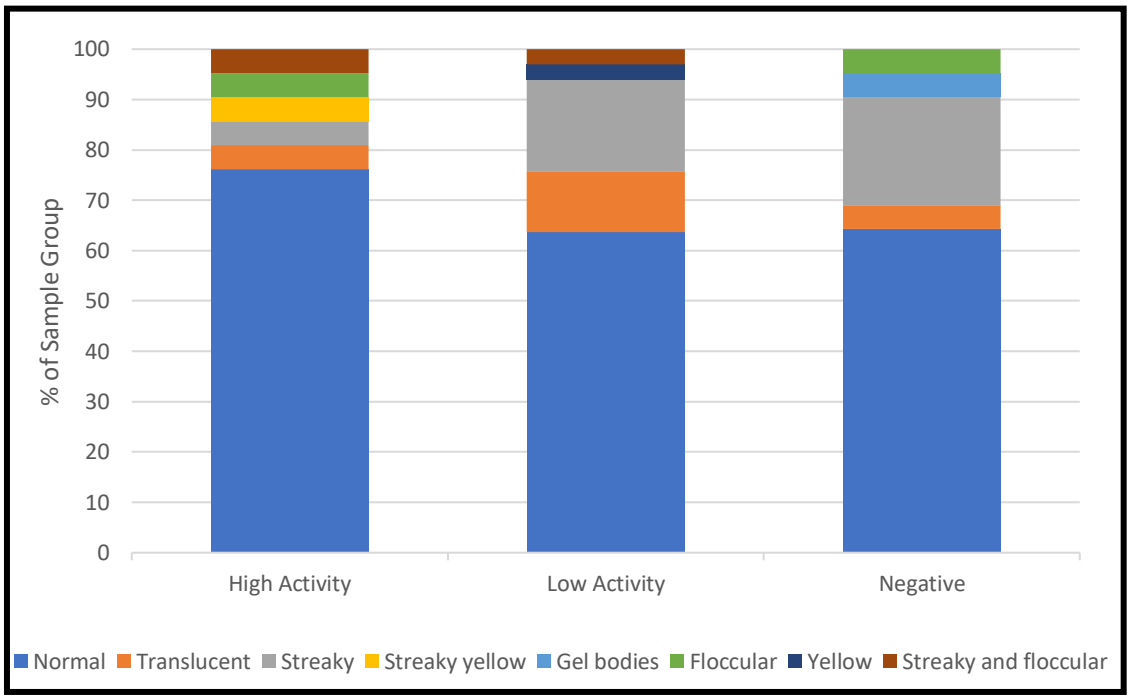

Figure 6- The bars show the % distribution of samples within the activity groups with various descriptions of macroscopic appearance. 'Streaky' samples are represented by the grey bar which is larger for the low activity and the negative groups. 'Normal' samples are shown in blue and make up a lower proportion of the negative and low activity groups.

Streaks in semen are often caused by mucus, and it is possible that the raised sialidase activity broke up the mucus, by cleaving free the terminal sialic acid residues holding mucins together. It is also possible that sialidase activity is indicative of bacteria producing other enzymes with mucus degrading activity. For example many *Prevotella* species produce sialidases which will likely be detected by the sialidase assays, but also mucin degrading sufatases[80] which may be partly responsible for the reduction in mucus streaks.

Further evidence of sialidase mediated degradation of seminal mucus can be seen in the viscosity of the samples (see Figure 7). The negative and low activity groups both contain more samples with raised viscosity than the high activity group, with the negative group containing the most. More than 30% of the negative samples are considered more viscous than normal, whilst less than 20% of the high activity samples are. However, the high activity group does also contain the largest proportion of samples within the highest viscosity group: 'hyperviscous'.

Figure 7- Sialidase Activity and Sample Viscosity

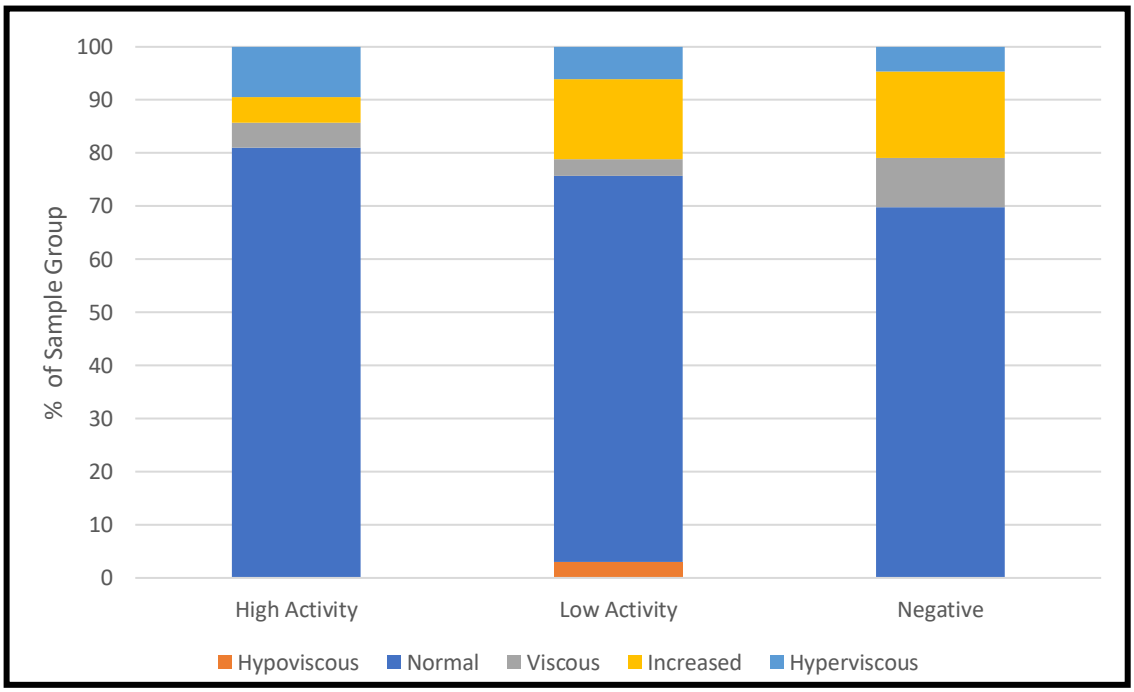

Figure 7- The bars show the distribution of viscosity classification for each of the sialidase activity groups. 'Hypoviscous' is the lowest viscosity classification, followed by 'normal', 'viscous', 'increased', and finally 'hyperviscous' which is the highest viscosity classification.

Sialidase mediated degradation of seminal mucus could result in lower viscosity, accounting for the increased proportion of samples with 'normal' viscosity or lower within the high activity group. It is difficult to determine what caused the increase in 'hyperviscous' samples within the high activity group. Seminal hyperviscosity can be indicative of infection of the prostate glands or seminal vesicles[81], and it could be that there were more patients suffering from such infections amongst the high activity patients.

Whilst both the effect of sialidase activity on appearance and viscosity seem to indicate possible sialidase mediated mucus degradation, it appears to only affect abnormal samples. High sialidase activity has an impact on the presence of observable 'streaks' but it does not appear to be associated with any abnormalities in appearance. The activity appears to reduce increased

viscosity in the samples but is not associated with hypoviscosity. It is possible that this is because sialidase activity in semen is a normal enough occurrence that its observable effects are considered to be 'normal'. Alternatively, it is possible the effect of sialidase activity on mucus is only obvious in samples that would otherwise contain abnormally large amounts of mucus. Mucus is thought to be an important component of seminal plasma, and sialidase mediated degradation of it, could present another potential means for sialidases to influence fertility.

Debris within the samples was also assessed, and there appeared to be some difference between the proportions of samples with significant amounts of debris across the activity groups (see Figure 8). The negative group contained the highest proportion of samples without significant debris, whilst the high activity samples contained the least. Additionally, the high activity group contained the most samples described as having "plus plus" debris, indicating particularly large concentrations of debris.

Figure 8- Sialidase Activity and Sample Debris

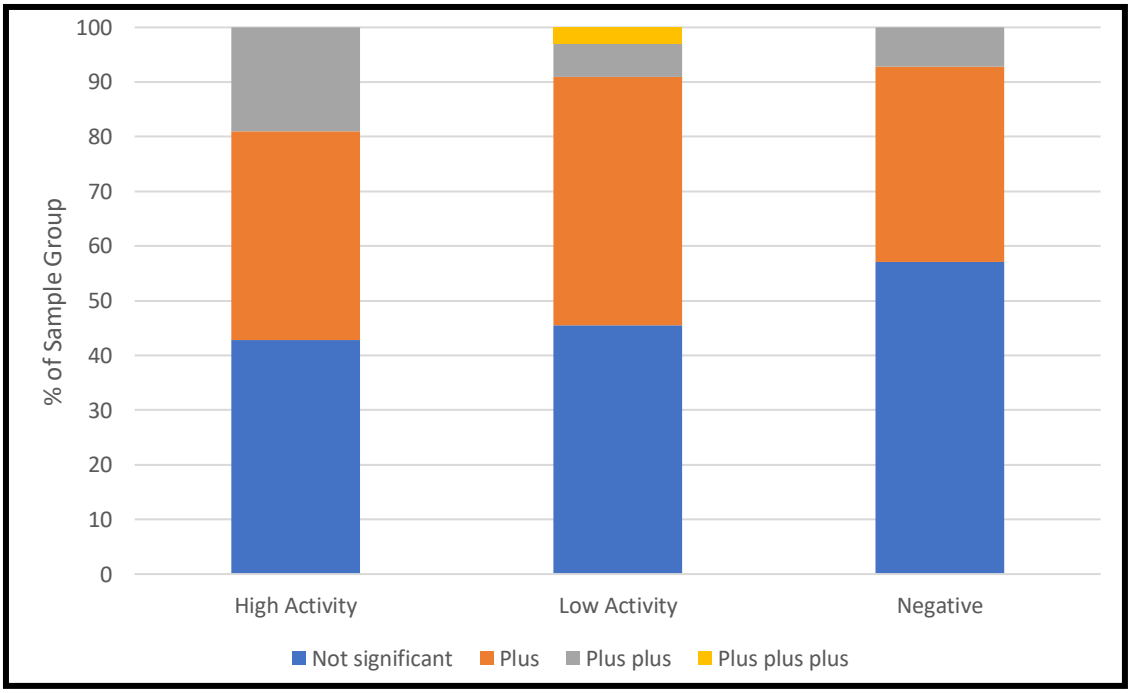

Figure 8- The bars show the % distribution within the activity groups of various debris classifications. Classifications are based on qualitative microscopic assessment of the sample by the clinician, with 'not significant' being the lowest amount of debris, and 'plus plus plus' being the highest.

Abnormal amounts of debris in a semen sample is often associated with microbial activity and can be indicative of urogenital infection[82,83]. This perhaps provides evidence that the sialidase activity in these samples is bacterial in origin.

There also appeared to be a significant relationship between the sialidase activity and the sample volume and sperm concentration (see Table 3). The samples with high activity tended towards lower volume and higher sperm concentration, although it is likely that these two variables are linked, with lower volume resulting in higher sperm concentration. Despite this relationship, the average sperm concentration for all three groups was well above the lower reference limit of 15 million cells per ml, and the average sample volume was above the lower reference limit of 1.5 ml[79], showing that there was no association between sialidase activity and abnormality in these parameters.

Table 3- Sialidase Activity and Sperm Concentration

A- Sperm Concentration

Group	**Mean Sperm Concentration (million/ml)**	**P (*t-test against negative group)***
High Activity (n=24)	70.3 ± 11.3	0.03
Low Activity (n=45)	50.7 ± 6.3	0.09
Negative (n=57)	42.2 ± 4.9	N/A

B- Sample Volume

Group	**Mean Sample Volume (ml)**	**P (*t-test against negative group*)**
High Activity (n=24)	3.19 ± 0.28	0.01
Low Activity (n=46)	3.50 ± 0.21	0.02
Negative (n=56)	4.23 ± 0.21	N/A

Table 3- Table A shows the average sperm concentration (± standard error) for each sample group, and the P-value generated by a student's t-test against the negative group. Table B shows the same as table A but for sample volume.

This association could indicate a contribution to the sialidase activity by the sperm associated human sialidases. It was thought that these would be contained within sperm cells and not act

extracellularly, but perhaps small amounts are secreted, or released by lysed sperm cells. It is possible that the 4MUSA enters sperm cells present within the samples and is degraded there. It is also possible that sialidase producing bacteria are associated with the sperm cells, as bacterial adherence to sperm cells has previously been observed[84,85]. Alternatively, the results could be explained just by the volume difference, with lower seminal volume resulting in both higher concentrations of sialidases and sperm cells, causing a co-association.

Dividing the samples into these 3 activity groups has proven to be an effective tool for identifying potential effects of sialidase activity. It indicates that the effect of lower levels of activity (<1000AU) is limited, and that perhaps these samples should not be considered to be truly sialidase positive. It is however, possible that the observed association of high activity with fertility problems is coincidental. Perhaps the lower volume of the samples is having an impact on fertility, whilst also increasing the observed sialidase activity, leading to more highly positive samples being present amongst those visiting a fertility clinic. Although, the lack of streaky or more viscous high activity samples indicates that the sialidase activity is having some effect on the semen samples.

4. Distribution of Bacterial Species in Semen

To provide more insight into the potential sources of sialidase activity in semen, the microbial content of a selection of the samples from the infertility clinic was analysed using 16S rRNA sequencing. This allowed a microbial profile to be produced for each of these samples, detailing the bacterial taxa present within the sample. An equal number of samples were selected from the 'high activity', 'low activity', and 'no activity' groups.

Two unusual taxa appeared to be present within the majority of the sequenced samples (see Figure 9): the genus *Shewanella* and the family *Halomonadaceae*. Neither of these taxa have previously been isolated from the urogenital tract and are both more commonly associated with oceanic environments. Approximately 73% of the sequenced samples contain *Halomonadaceae* and *Shewanella* (HaShe) and in most of these samples they are the dominant taxa, along with smaller amounts of *Corynebacterium* and *Enterbacteriaceae*. However, the other 27% of samples instead contain a mixture of other taxa including *Prevotella, Porphyromonas, Lactobacillus* and various taxa within the order of *Clostridiales*. A small number of samples (around 12%) contained a more even mixture of these two groups of taxa.

Figure 9 – Distribution of Bacteria in Semen Samples

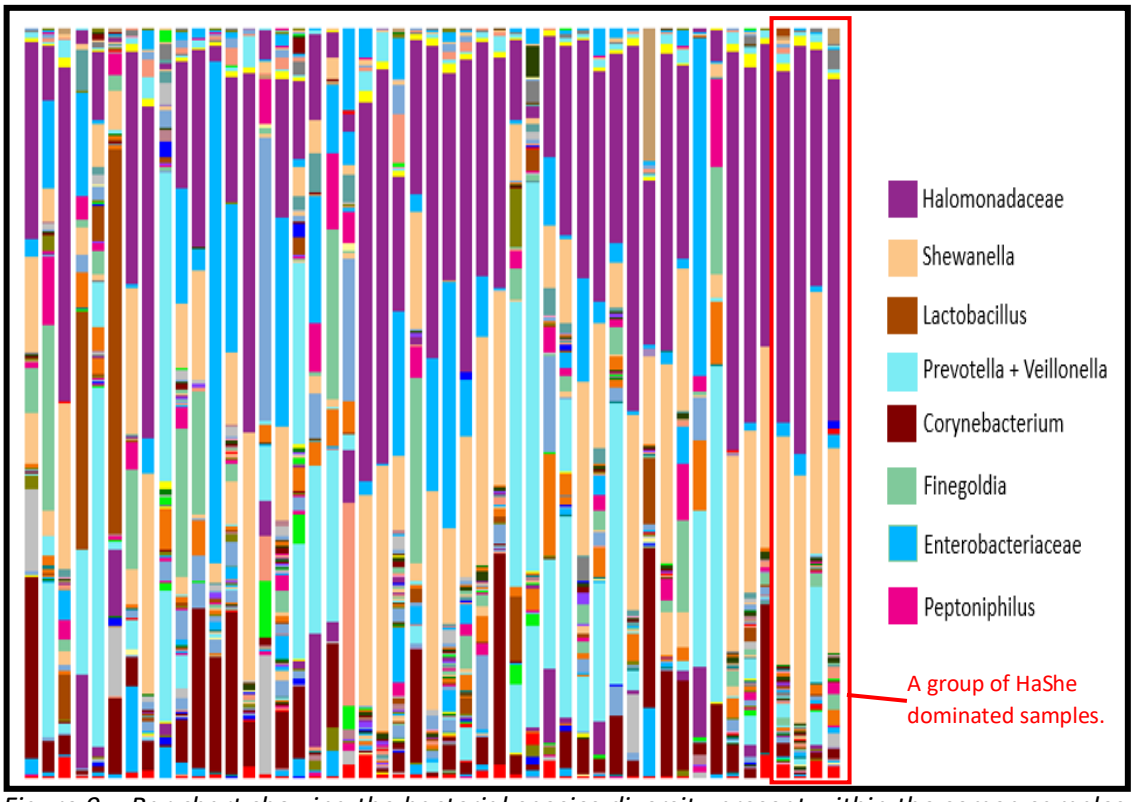

Figure 9 – Bar chart showing the bacterial species diversity present within the semen samples. Each column corresponds to a single sample, and the different colours each show the relative abundance of 16S rRNA sequences corresponding to different bacterial taxa. The colour key to the side of the chart shows which colours correspond to the more common taxa. A red box has been placed around a group of HaShe dominated samples to highlight their prevalence.

There is a degree of concurrency to some of the identified taxa (as may be seen in Figure 9). The already mentioned *Shewanalla* and *Halomonadaceae* are almost always present together, and *Veillonella* is rarely seen in samples that do not also contain *Prevotella*, although *Prevotella* can occasionally be seen without *Veillonella.* This could indicate a degree of co-dependency to some of the bacteria found within the male urogenital tract and provide evidence for symbiotic relationships.

The HaShe presence in the samples is difficult to explain. In some cases, parts of the 16S rRNA gene can be similar enough in two species that analysis software can inappropriately label one as another, and it is possible that the HaShe are actually other taxa more likely to be found in the urogenital tract. There is a possibility that the HaShe taxa did not originate within the samples and are the result of contamination of the samples, despite every care being taken to avoid such an occurrence. However, as all samples were treated the same at every stage any contamination would be expected to be present in all samples, and this is not the case. The

presence of HaShe could also possibly be explained by the methods used to analyse and present the sequencing data. To make the samples comparable the species abundance is given as a percentage of total reads within the sample. This means that if all the samples contain a small amount of a certain taxon, the samples containing the least amount of anything else will over represent this taxon. It could be that a higher abundance of HaShe is linked to a lower overall bacterial load in the sample.

The taxa identified, other than HaShe, are similar to those previously found in other seminal microbiome sequencing studies, and the methods used appear to have provided a good overall view of the microbial content of these samples.

5. Sialidase Activity and Bacteria

Certain bacterial taxa appear to be differentially distributed between sialidase positive and negative samples (see Figure 10). HaShe positive samples can be found across the entire range of sialidase activity but make up a greater proportion of the samples with activity less than 500. Around 60% of the samples with activity greater than 500 are HaShe positive, whilst 100% of samples with sialidase activity less than 500 are HaShe positive. *Prevotella* (along with the often concurrent *Veillonella*) is more commonly associated with samples with higher sialidase activity. 57% of samples with sialidase activity greater than 500 were positive for *Prevotella*, whilst only 16% of samples with activity less than 500 were *Prevotella* positive. In addition to *Prevotella* other taxa such as *Poryphyromonas* and *Bifidobacteriaceae* could be found at higher levels of sialidase activity whilst they were absent from samples with lower levels of activity. There was a greater degree of taxa diversity in samples with higher levels of sialidase activity.

Figure 10- Distribution of Bacterial Taxa by Sialidase Activity

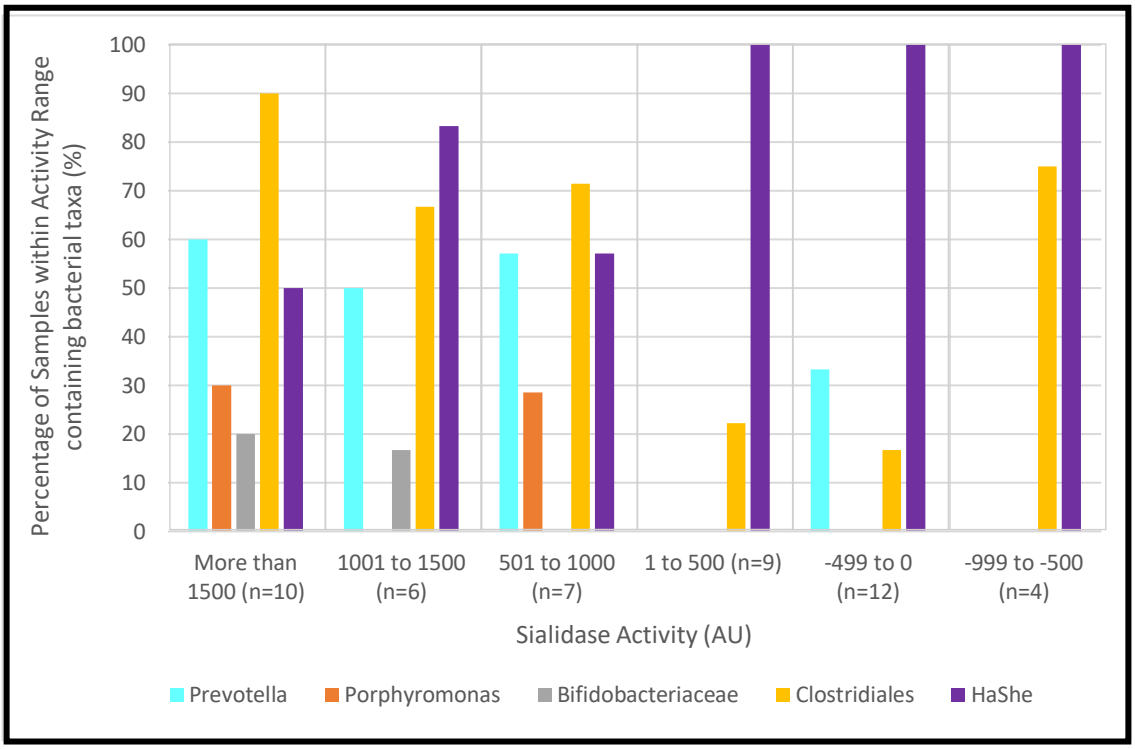

Figure 10- In this figure the samples have been grouped into sialidase activity bins of 500, and the bacterial taxa present within these samples has been presented as the percentage of samples within that activity range positive for that particular taxon. 'Positive' in this case is defined as having more than 5% of total 16S rRNA sequence reads within the sample, correspond to that taxon. Certain taxa have been grouped in a manner which better demonstrates the trends present within the samples, such as the various genera within the Clostridiales order, and HaShe.

It appears that sialidase activity could be an indication of a disruption of the normal microbial presence of the urogenital tract, with various species contributing towards the raised activity. Several species of *Prevotella* are known to be sialidase producers, so it is not unexpected that this species demonstrates one of the most notable associations with sialidase activity in this study. However, not all sialidase positive samples contain an abundance of *Prevotella*. In most of these cases there are other taxa present which could possibly be providing a source of sialidase activity, although it is difficult to fully assess this without species specific data. The various potential sources of sialidase activity in these samples means that there does not appear to be a single taxon strongly associated with raised sialidase activity, but rather a strong negative association with the taxa they are replacing, in this case appearing to be *Shewanella* and *Halomonadaceae*.

Shown in Figure 11 are examples which appear to break the trends held by other samples. Two samples demonstrated particularly high sialidase activity and both of these samples also appear to carry an unusual set of bacterial taxa. BES034 was the only sample to contain such a large proportion of *Enterobacteriaceae*. Many other samples appear to contain organisms from this family, including several sialidase negative samples, but none are so completely dominated by the taxon. It seems likely that this high proportion of *Enterobacteriaceae* is linked to the markedly high sialidase activity. Some species within the *Enterobacteriaceae* are capable of producing sialidases, whilst some are not, and it is possible that the *Enterobacteriaceae* present within sample BES034 produces sialidases, whilst those present within the sialidase negative samples do not.

BES014 was consistent with many of the sialidase positive samples, containing no HaShe but instead containing *Prevotella* and *Veillonella.* However, BES014 was unusual in that it contained a particularly large percentage abundance of *Veillonella* when compared with other sialidase positive samples and was also the only sample to contain the taxon *Treponema*. It is difficult to determine which of these factors is contributing to the raised sialidase activity, as there is no evidence that any species of *Veillonella* is capable of producing sialidases, and the *Treponema*, though unusual and a potential sialidase producer, was in relatively low abundance, making up only 3.9% of the total sequence reads for that sample.

Another group of unusual samples are BES097, BES005, BES100, and BES072. These were all samples negative for sialidase activity which contained *Prevotella*. This was unusual as most of the *Prevotella* positive samples were sialidase positive, as expected due to many species of *Prevotella* being sialidase producers. It is possible that the *Prevotella* species detected in these samples are some of the few that do not produce sialidase, such as *Prevotella intermedia*[86]. Alternatively, there may have been some other factor within those samples preventing either sialidase expression, or detection in our assay, although there was no other notable abnormality in the other measured parameters for these samples.

Figure 11- Bacterial Taxa Distribution in Unusual Samples

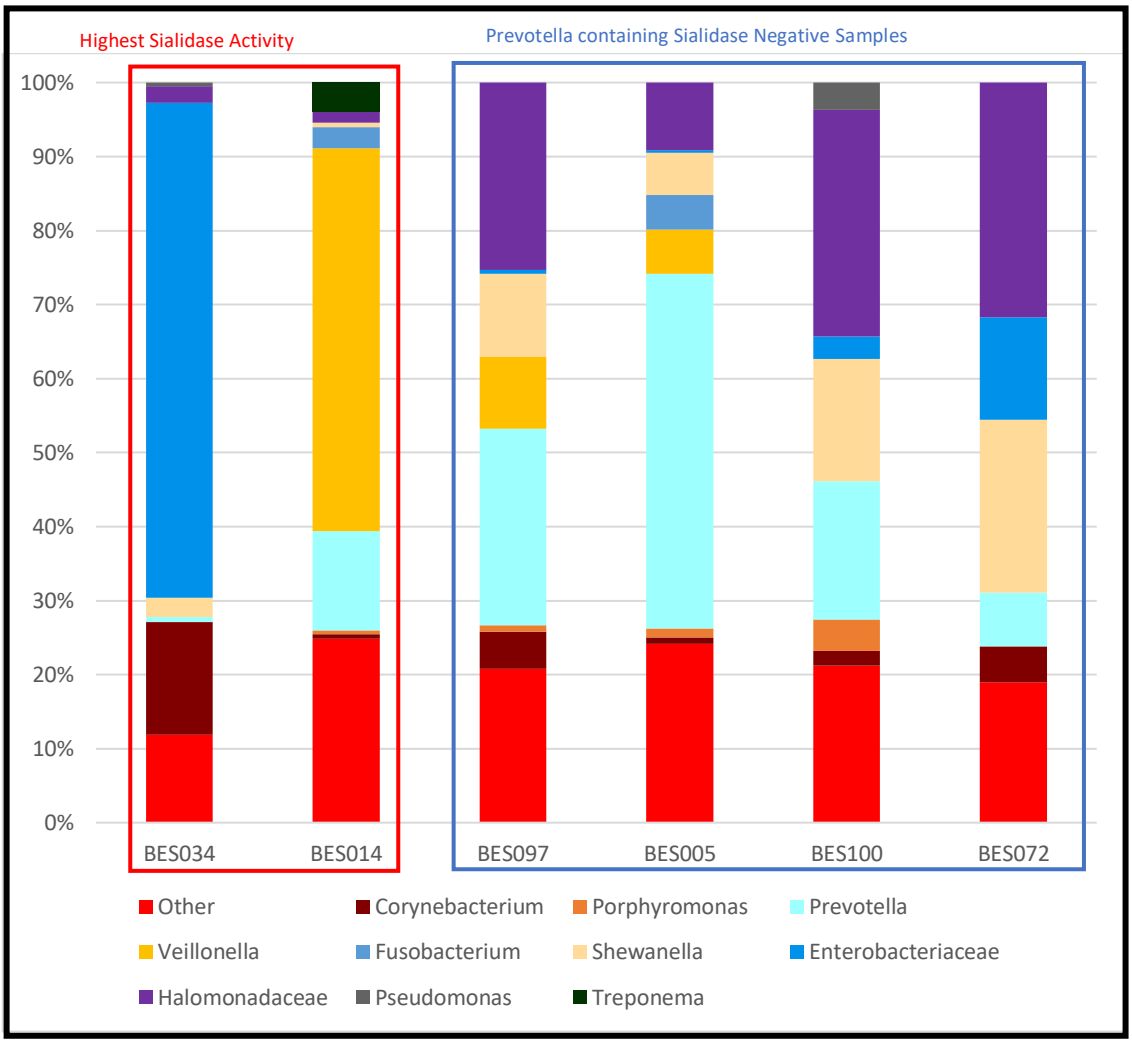

Figure 11- This figure shows a selection of six 'unusual' samples. Two of these were samples with substantially higher activity than most (in the red box). Four of these are samples which contained an abundance of Prevotella, despite having no sialidase activity (in the blue box). For clarity, only taxa with an abundance of at least 4% in at least one of the samples are shown. All other taxa are grouped into 'other'.

These unusual samples indicate that the findings of this study do not yet present a complete picture of the link between bacteria in semen, and sialidase activity in semen. Although there were certain clear trends, not all samples followed these trends, and the many outliers indicate that unknown factors may have influenced the results. It is possible that something other than the bacteria in the samples is contributing to the sialidase activity, such as the human neuraminidases. In addition, it is possible that under certain conditions, either sialidase production or sialidase activity may be somehow inhibited. It does still seem apparent that

there is a link between bacteria in semen, and sialidases in semen. The positive association between potential sialidase producers such as *Prevotella* and raised sialidase activity, provides a strong indication of bacterial contribution to sialidase activity in semen. However, further experiments will be necessary to confirm these findings, and to better understand the unknown factors affecting samples which do not appear to follow the observed trends.

6. Effect of Sialidase Activity on Gonococcal Physiology

The urogenital tract provides a dynamic ecological niche, inhabited by a complex microbial community. It is also the frequent site of infection, with sexual intercourse providing a mechanism by which pathogenic organisms transfer from one urogenital tract to another. Any major change to the urogenital tract, such as raised sialidase activity, would be likely to have an effect on these pathogens, potentially influencing their virulence, or interfering with treatments. Part of the study sought to determine what these effects could be. *Neisseria gonorrhoeae* (known as gonococci) the causative agent of gonorrhoea was selected for this part of the study due to gonorrhoea being a particularly common sexually transmitted infection, and because of the known importance of sialic acid to the gonococcal lifecycle (see Introduction section 4.3).

Gonococci become sialylated when grown in the presence of CMP-NANA, and so growing bacteria with and without CMP-NANA produced both sialylated and unsialylated gonococci. This allowed simulation of bacteria that had become sialylated during infection, as is normal for gonococci, and bacteria that had been desialylated by high concentrations of sialidase, as would potentially happen in a 'high activity' patient.

Assessment of the sialylation status of the bacteria was made possible using the SM82 antibody. SM82 binds specifically to lacto-N-neotetraose (LnT), a moiety found at the end of LPS chains (see Figure 12A). Gonococcal sialyltransferase attaches sialic acid to LnT during sialylation preventing SM82 from binding to the bacteria (see Figure 12B). The impact of CMP-NANA on measurable sialylation was tested in three different laboratory strains of gonococci: MS11, P9, and FA1090. It was found that CMP-NANA had the greatest effect on P9, and so P9 was selected for further studies. A range of concentrations of CMP-NANA was tested on P9, and it was found that at least 20 μg ml^{-1} was required for complete sialylation (see Figure 12C).

Figure 12- Blocking of SM82 Antibody Binding by Sialylation

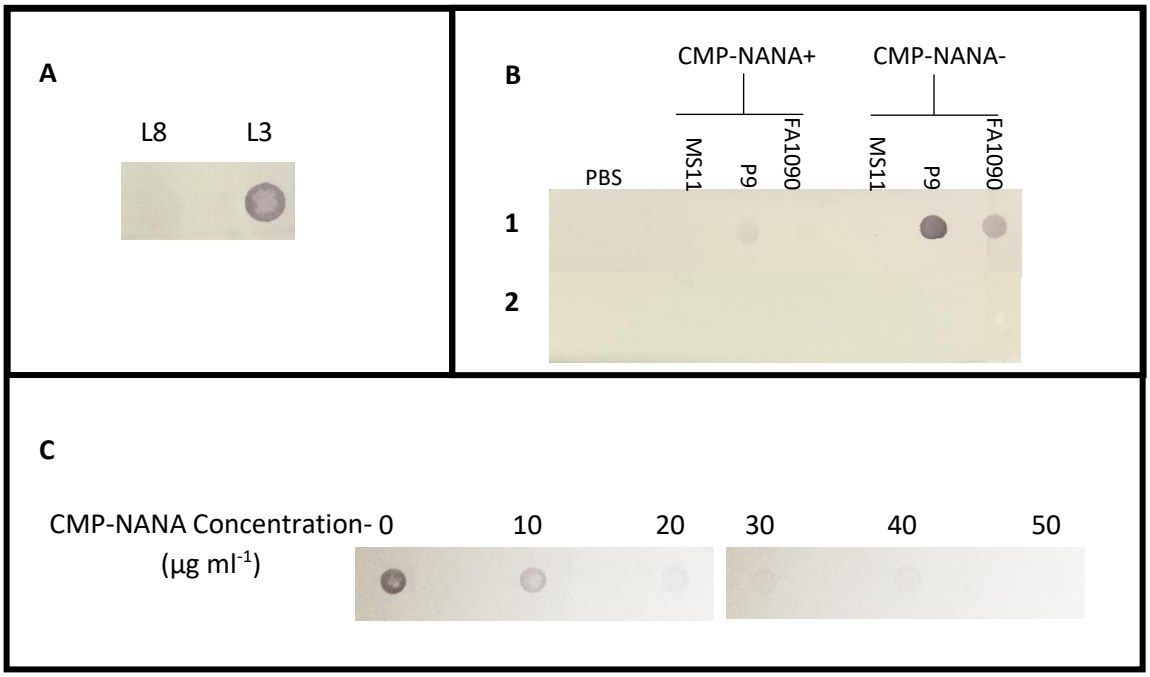

Figure 12- The results of three different dot blots using SM82 with AP conjugated anti-mouse IgM as a secondary antibody. Panel A shows a blot using two strains of Neisseria meningitidis, one with L8 LPS (without LnT) and one with L3 LPS (with LnT). Panel B shows the blot against 3 strains of gonococci grown both with and without CMP-NANA. Row 1 shows SM82 with the secondary antibody, row 2 shows the secondary only. Panel C shows a blot with P9 grown in varying concentrations of CMP-NANA.

It was found that CMP-NANA supplementation of nutrient broth increased the growth rate of gonococci (see Figure 13A). The growth rate during the log phase was increased to 160% of that of bacteria grown without CMP-NANA, when 25µg ml^{-1} CMP-NANA was added to growth media. This was unexpected, and if caused by sialylation could have important implications for sialidase positive patients. However, it was possible that CMP-NANA was having an as yet unknown effect on gonococcal metabolism, completely unrelated to sialylation, in which case sialidases would have no effect on the change in growth rate.

To determine whether the effect on growth was due to sialylation, sialyltransferase knockout mutants were created by disrupting the *lst* gene. These mutants were incapable of sialylation even in the presence of CMP-NANA (see Figure 13B), and therefore any effect of CMP-NANA on these bacteria would be unrelated to sialylation and would be unaffected by the presence of sialidases. It was found that the growth rate of the mutants was completely unaffected by the presence of CMP-NANA (see Figure 13C) indicating that sialylation was required for the effect to occur, meaning that the presence of sialidases would likely reverse this effect.

Figure 13- Sialylation Increases Gonococcal Growth Rate

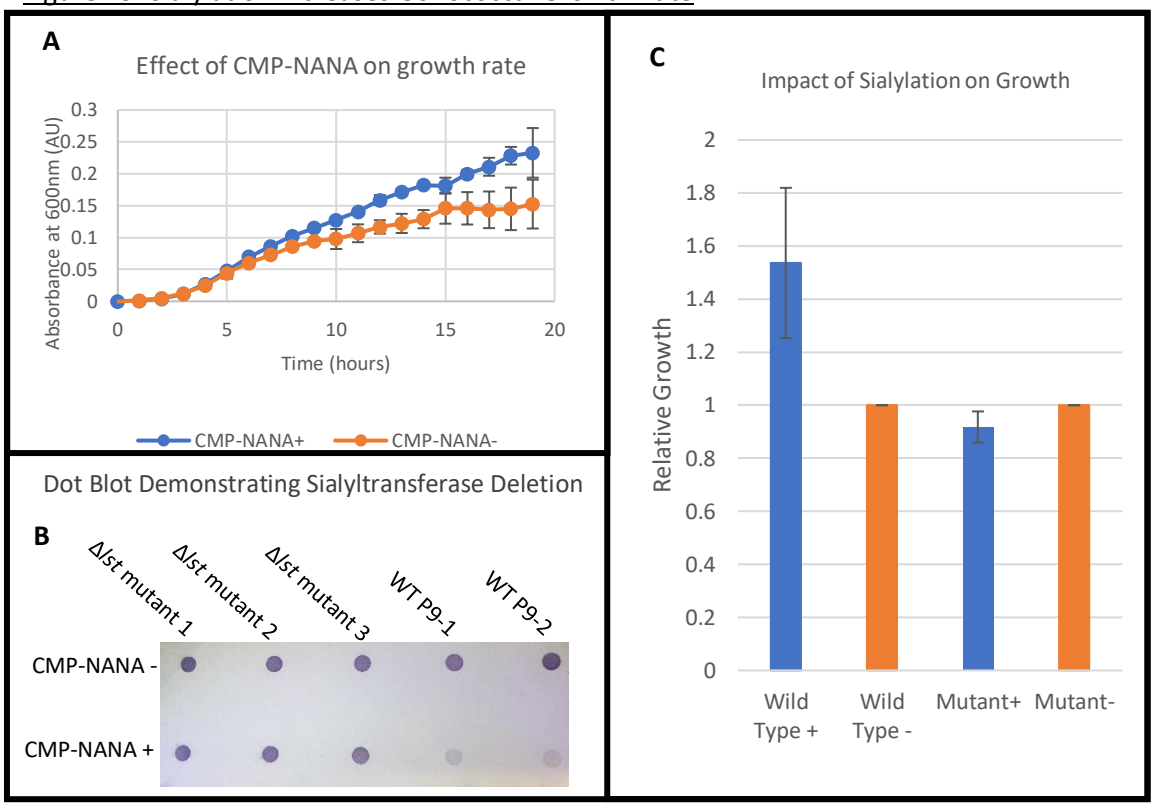

Figure 13- Panel A shows a growth over time curve for gonococci both with and without CMP-NANA, each point is the average of three experiments. Panel B shows the result of an SM82 dot blot against 3 sialyltransferase knockout mutants, and two wild type strains all grown both with and without CMP-NANA. Panel C shows the effect of CMP-NANA on the endpoint growth of the wild type and sialyltransferase mutant. Values given are the endpoint growth (absorbance at 600nm) normalised to the growth endpoints without CMP-NANA. Values are the average of three experiments.

There are several potential explanations for the effect of sialylation on growth rate. The negative charge of sialic acid[87,88] could potentially prevent bacteria from aggregating, allowing them to more evenly disperse throughout the growth media preventing competition for nutrients. It is also possible that sialylation triggers a change in gene expression in the bacteria, resulting in a faster growing phenotype. The bacteria normally become sialylated upon contact and invasion of host cells, where they are exposed to intracellular CMP-NANA. It is therefore possible that sialylation acts as a signal to the gonococci to switch to a phenotype more suitable to intracellular life, perhaps leading to an increase in growth rate.

N. gonorrhoeae was one of the first organisms to demonstrate resistance to antibiotics[89,90], and over the decades since the introduction of antibiotics, gonococci have become significantly harder to treat[91,92], with highly resistant isolates becoming more prevalent[93]. The particular

importance of antibiotic efficacy in gonococci prompted the study of the impact of sialylation on antibiotic susceptibility. Initial results indicated that there was no significant effect of sialylation on antibiotic susceptibility, as there was no change in the minimal inhibitory concentration (MIC) of azithromycin, ceftriaxone, chloramphenicol, or tetracycline.

However, bacterial interactions with antibiotics can be too complex to be defined only by an MIC. Often several factors with small impacts on antibiotic susceptibility can compound together to produce higher levels of antibiotic resistance. To determine whether sialylation could have a smaller effect on antibiotic resistance, not detectable in an MIC assay, the bacteria were grown in the presence of sub-MIC concentrations of the antibiotic, both with and without CMP-NANA (see Figure 14). It was found that after 20 hours of incubation the growth was sufficient to be able to measure the impact of sialylation on antibiotic susceptibility by comparing the change in growth reduction with and without CMP-NANA. For further experiments a single OD600 measurement was taken at 20 hours, rather than taking measurements every hour.

Figure 14- Impact of Azithromycin and Sialylation on Gonococcal Growth Kinetics

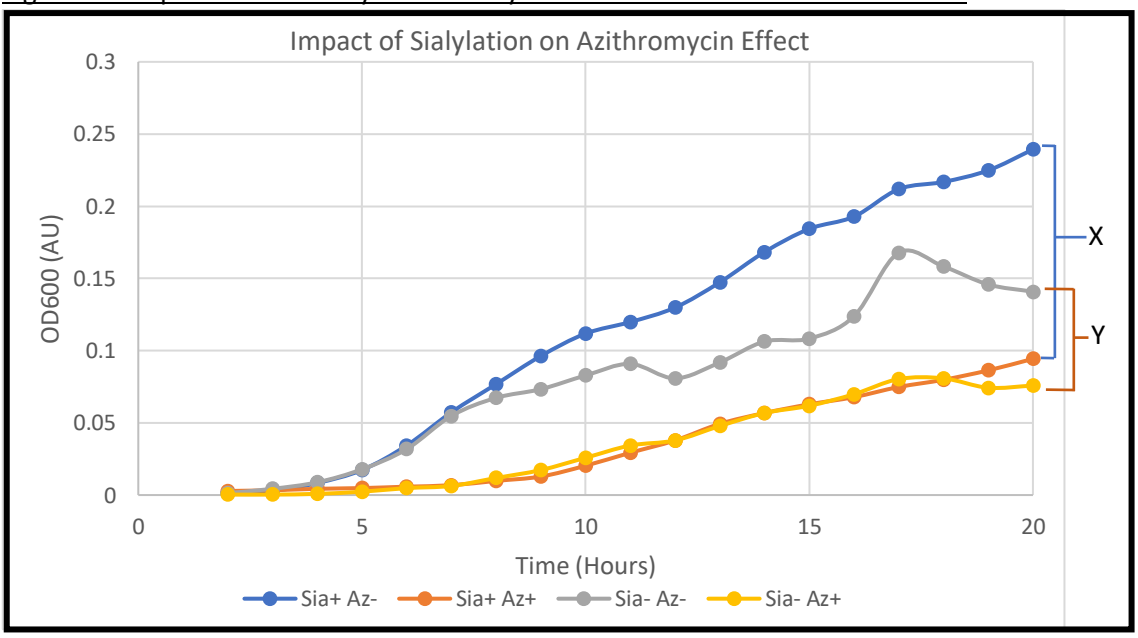

Figure 14- Shows growth curves of sialylated and unsialylated gonococci with and without 0.09 µg ml^{-1} of azithromycin. Sia+ Az- is grown with CMP-NANA but no azithromycin, Sia+ Az+ is grown with CMP-NANA and azithromycin, Sia- Az- is grown without CMP-NANA and without azithromycin, and Sia- Az+ is grown without CMP-NANA and with azithromycin. It was found that after 20 hours of growth, the difference between the OD600 of the bacteria with and without antibiotic could be used to compare the impact of antibiotics on bacteria grown under different conditions. X shows the impact of azithromycin on bacteria grown with CMP-NANA, Y shows the impact on bacteria grown without CMP-NANA. When Y is normalised to 1 (see Materials and Methods 14) X equals 0.73.

Endpoint measurements were then used to measure the effect of sialylation on the action of both azithromycin and ceftriaxone (Figure 15). It was found that at sub-MIC concentrations, growth with CMP-NANA made the bacteria more susceptible to the antibiotics. CMP-NANA caused the effect of azithromycin and ceftriaxone on gonococcal growth to increase by almost 20%. The experiment was repeated with the sialyltransferase knockout mutants, and it was found that the CMP-NANA did not have the same impact on the effect of antibiotics on these organisms, confirming that the observed effect was caused by sialylation.

Figure 15 - The Effect of Sialylation on Antibiotics

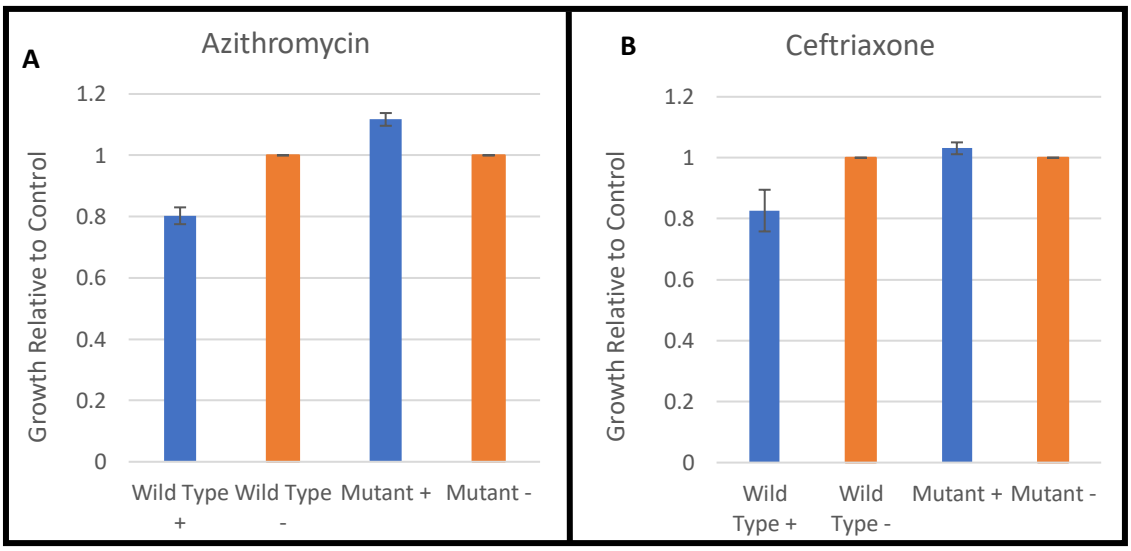

Figure 15- The figures show the effect of sialylation on response to sub MIC concentrations of antibiotic. Panel A shows the effect on 0.09 µg ml^{-1} of azithromycin, Panel B shows the effect on 0.002 µg ml^{-1} of ceftriaxone. The experiment was carried out on both wild type gonococci (P9) and the lst negative mutant (labelled mutant here). '+' indicates growth with CMP-NANA, '-' indicates growth without CMP-NANA. The values are given as relative growth values, with the growth (relative to growth without antibiotics) of the CMP-NANA negative control equalling 1. Values in both panels are the average of three experiments.

There are several potential explanations for this observed increase in the susceptibility to antibiotics of sialylated organisms. Antibiotics have a greater observable effect on growing organisms, either because growth is required for their action (as is the case for ceftriaxone) or because they act to slow bacterial growth, and their effect is more noticeable in growing organisms (as is the case for azithromycin). It is likely that the increased growth rate of sialylated gonococci is at least in part, responsible for the observed increased effect of the antibiotics on the bacteria.

However, there may also be a more direct influence of the sialic acid on antibiotic susceptibility. Glycans have been demonstrated to influence the structure and spatial arrangement of surface proteins in eukaryotic cells,[94,95] and it is possible that a similar effect in gonococci could render the membrane more permeable to antibiotics. Alternatively, if sialylation does induce a change in gene expression to produce a phenotype better suited to the intracellular stage of its lifecycle, (as proposed as a mechanism influencing the growth rate) then this intracellular phenotype could be more susceptible to antibiotics. Host contact mediated changes in phenotype have been demonstrated in other bacteria such as *Staphylococcus aureus*[96] and *Legionella pneumophila,*[97] and gonococci have been shown to possess a transcriptional regulator (CrgA) which responds to host cell contact[98] indicating a need for phenotypic changes to take place upon infection of host cells. It is possible that in the relative safety of the host cell interior, efflux pumps become less active, and the membrane becomes more permeable to outside substances.

Although the effect of sialylation status on antibiotic susceptibility is small, it could still have clinical relevance in some circumstances. Combined with other resistance mechanisms, lack of sialylation could tip the balance towards treatment failure, meaning high sialidase activity in the urogenital tract could make gonorrhoea harder to treat. Additionally, small numbers of unsialylated gonococci surviving treatments could play a role in developing higher levels of antibiotic resistance. Lack of sialylation may allow bacteria with only slightly reduced susceptibility to survive treatment, giving them the opportunity to acquire additional resistance, through mutation or horizontal gene transfer.

However, it could be argued that the advantages of sialylation to gonococci far outweigh the disadvantages. The increase in growth rate, coupled with the serum resistance granted by sialylation, will likely make it easier for bacteria to survive within the host, and could facilitate the development of a disseminated infection. It is possible, based on these results, that patients with higher levels of sialidase activity in their urogenital tract, could suffer from harder to treat, but milder less virulent gonorrhoea infections. This could prevent the use of sialidase inhibitors to increase antibiotic treatment success, as an increase in sialylated bacteria could increase the severity of the infection. The multitude of potential effects sialidase activity could have on gonorrhoea infections, provides an example of the complex dynamics influencing infections in the context of host microflora.

General Discussion

1. Outcomes of Study

The data produced by the experiments carried out during this study has provided a more detailed understanding of several linked areas of biomedical research and will hopefully provide the groundwork for further research into areas that have yet to be studied in detail.

1.1 Outcomes of Sialidase Assays

Previous studies have examined the presence of sialidases in the mouth[99,100], gut[37], and female genital tract[20,101]. This project has expanded upon sialidase research by confirming the presence of sialidase activity within human semen samples, and therefore demonstrating their presence in the male genital tract. The data also shows that not all semen samples contain measurable levels of sialidase, indicating the influence of a yet to be identified variable. The demonstration of such significant variation in the activity of an enzyme present within human tissue is important, as it is possible that this variation could have an effect on health. The data will hopefully prompt further research into this variation and its underlying mechanisms, potentially improving our understanding of factors influencing sexual health.

The optimisation of the 4MU based sialidase assay for use in semen has provided a starting point for being able to differentiate between sialidase positive and sialidase negative men, which could prove valuable should the health impacts of raised sialidase activity in semen be confirmed. The study also identified two samples with activity several fold higher than most other sialidase positive samples. Such samples provide data with which varying levels of 'normal' can be determined. We now know that sialidase activity in semen is relatively common, with roughly half of the men in this study testing positive. However, only a relatively small number of these samples (around 20%) presented activity greater than 1000AU, and only in rare cases (2 samples of the 142 tested) were samples be found with activity greater than 10000AU. Understanding how these varying levels of activity can affect the semen, and the health of the patient, will be crucial for developing effective treatments and diagnostic procedures.

Recent research has begun to highlight the importance of better understanding male factor infertility[102], and the potential explanations for idiopathic cases. Researchers have investigated potential genetic causes[103,104], as well as the possible link between the presence of reactive oxygen species and infertility[105,106]. This data has provided some insight into the potential impact of sialidase activity on male factor infertility and indicates sialidases are another

potential avenue of research which may help to explain the large numbers of cases of idiopathic male factor infertility. The experiments did not identify a strong link between seminal sialidase activity and the fertility status of the patient, but there was an increase in the number of samples with activity greater than 1000AU within the infertile samples, and the samples of those visiting a fertility clinic. The study did not produce enough evidence to confirm that the higher levels of sialidase activity in these samples caused the fertility issues of the patients who provided them, but this increase in samples above a certain threshold of activity is worthy of further investigation. Various limitations to the techniques used in these experiments (see section 2.1) may have prevented the confirmation of a strong link between sialidases and infertility. However, should the link be confirmed by further experiments, it could help to diagnose many cases of male factor infertility which are currently left unexplained, and to develop treatments for those men affected by sialidase mediated fertility problems. For couples suffering from unexplained infertility, such developments in the field are very important.

Links were also discovered between sialidase activity and various other semen parameters. When the threshold of 1000AU of sialidase activity was applied to looking at parameters such as sample viscosity and appearance it was found that there were some significant relationships. These relationships provide more evidence of a biological effect of sialidase activity on semen, with potential clinical implications. Although the initial hypothesis that sialidases could interfere with the sperm glycocalyx is yet to be confirmed, we now know that certain levels of sialidase activity appear to reduce the amounts of mucus in some semen samples. Mucus makes up less than 1% of the composition of seminal fluid, so it is unsure how significant an effect its degradation by sialidases will have on fertility, if indeed that is the cause of the observed trends. However, evidence that the sialidase activity is having a detectable effect on the semen will be important to take into consideration when evaluating the potential clinical significance of sialidases in semen.

1.2 Outcomes of 16S rRNA Sequencing

16S rRNA analysis of the bacteria present within the semen samples provided an insight into the possible sources of sialidases in semen. Understanding the biological mechanisms behind raised sialidase levels in semen will be important for the development of diagnostic tools and treatments, should the morbidity associated with raised sialidase activity be deemed significant enough to warrant such measures. The data indicates that the sialidase activity could be bacterial in origin, meaning antibiotics could likely be used to reduce the activity if necessary. It also appears that, similar to bacterial vaginosis, sialidase activity in semen seems to be linked

to a disruption of the normal urogenital microbiota, perhaps indicating that the two phenomena are closely linked. It is possible that an asymptomatic condition similar to bacterial vaginosis also occurs in men. Should this be the case, seminal sialidase assays could prove to be the easiest way to diagnose the condition, just as sialidase assays can be used to diagnose bacterial vaginosis[107].

The bacterial involvement in seminal sialidase activity also potentially provides explanation for the difficulty confirming a link between sialidase activity and infertility. Microbiomes can be dynamic and can often shift between different dominant organisms over time. This is particularly true with the vaginal microbiome which changes in response to different stages of the menstrual cycle[108]. As there is a degree of sharing between the urogenital microbiomes of men and women who frequently engage in sexual intercourse, it is likely that there will be a degree of temporal variation to the male urogenital tract too. This means that there may be fluctuations in the abundance of sialidase producing bacteria in the male urogenital tract, meaning the sialidase activity threshold required to impede fertility may not be constantly maintained. This could explain why we found cases of men who were fertile, who also displayed relatively high levels of sialidase activity. It could also help to explain the weak overall association between sialidase activity and fertility problems. However, it does seem plausible that in some cases sialidase producing bacteria could become more stably established within the urogenital microbiomes of both the male and female partner, leading to potential long-term fertility problems.

1.3 Outcomes of Gonococcal Experiments

It was found that there is more to gonococcal sialylation than just serum resistance. Sialylated *N. gonorrhoeae* appear to be faster growing, but more susceptible to both azithromycin and ceftriaxone. This coupled with their serum resistance presents a complex view of the effect of sialylation on gonococci. In some ways sialylation is clearly beneficial to gonococci, allowing them to better survive in the host and reproduce more quickly, but it carries the disadvantage of making them more susceptible to antimicrobial drugs. It is important that bacterial phenotypic changes that occur in response to host contact, such as gonococcal sialylation, are understood in their entirety. Such changes are likely to impact infection, and better understanding them could lead to the development of novel clinical tools. Studies of gonococcal sialylation have mostly been restricted to its effect on serum resistance, but it is possible that the data produced by these experiments could prompt research into its other potential effects on gonococcal physiology.

Coupled with the results of the sialidase assays, the findings from the gonococcal experiments are given additional context, with potentially important clinical ramifications. It is likely, although yet to be experimentally confirmed, that more gonococci infecting a highly sialidase positive urogenital tract will be in their unsialylated state. In some ways this could be to the benefit of the patient, as the bacteria will not be serum resistant, and will grow more slowly. This could limit the spread of the infection, and perhaps even make it harder for the gonococci to establish an infection at all. However, it is possible that the lack of sialylation will also make it slightly harder to treat the patient with antibiotics. These details will be important to consider should efforts ever be made to reduce sialidase activity to aid fertility, and patients should be thoroughly screened for sexually transmitted infections before such treatments are administered. It is possible that treatment with sialidase inhibitors could potentially exacerbate an underlying gonorrhoea infection.

Furthermore, when treating gonorrhoea, it may be important to take into consideration variations in urogenital sialidase activity between patients. Doses of antibiotic sufficient to treat a sialidase negative patient, may not be enough to treat a sialidase positive patient, due to the presence of greater numbers of more resilient unsialylated gonococci. It is also possible that this could drive the development of higher levels of antimicrobial resistance. Bacteria with only slightly reduced levels of antimicrobial susceptibility, normally not sufficient to allow survival of antibiotic treatment, may be able to survive treatment if they are unsialylated, giving them opportunity to develop other resistance mechanisms and become fully resistant to the antibiotics.

The potentially varied antibiotic response of gonococci infecting a sialidase positive urogenital tract versus a sialidase negative urogenital tract presents an example of the limitations of current laboratory antibiotic susceptibility assays. Bacteria are often taken out of context during antibiotic testing and placed on media designed to optimise growth rather than to mimic host conditions. Such conditions allow direct comparison between bacteria, but do not take into consideration variation they will be exposed to within the host. It is possible that whilst one bacterial strain may appear less susceptible to an antibiotic than another strain on agar, that strain may undergo different phenotypic changes in response to various host stimuli, making it more susceptible in the host. Bacteria are living organisms able to respond to and adapt to their surroundings and it is likely they will undergo phenotypic changes whilst in the host, which may also possibly vary from patient to patient. These experiments provide an example of how introducing a single host factor (CMP-NANA) to antibiotic susceptibility assays can alter the results. Coupled with the evidence that factors affecting this change (sialidase activity) will vary

from patient to patient, it becomes clear that traditional disc diffusion assays on agar do not provide a complete picture of gonococcal antibiotic responses. It is hoped that results such as these could prompt efforts to produce antibiotic susceptibility assays that more accurately recreate host conditions, and that an understanding of how these conditions may vary between patients is considered when interpreting the results of such assays.

2. Limitations of the Study and Possible Future Experiments

This study has provided a unique insight into the potential effects of sialidases on the urogenital tract. However, there were many factors which limited these experiments and the level of detail of the data that they produced. With hindsight, it is possible to suggest various improvements to the experiments carried out during this study, and to suggest further experiments that could be completed to provide a more robust dataset.

2.1 Limitations of the Sialidase Assay

The 4MUSA based sialidase assay provided a useful tool with which large numbers of semen samples could quickly be assessed for their sialidase activity. However, both the nature of the samples and of the fluorogenic substrate limited the level of detail that could be garnered from the assay. The semen fluoresced to some degree at the same wavelengths used to measure 4MU abundance, producing a high background signal. The 4MUSA substrate also degraded to some extent even if no sialidase was present. This meant that samples produced a relatively high fluorescence signal without any sialidase being present, and there was some difficulty differentiating between the background signal and signal produced by sialidase, as is evident by the numerous 'negative' activity values. These negative values are perhaps caused either by samples absorbing some of the fluorescence, or perhaps reducing the rate of natural substrate degradation. This meant it was impossible to convert the fluorescence measurements from these experiments into substrate turnover, as there was no way to generate a functional standard curve.

Another issue frequently encountered whilst carrying out sialidase assays was the heterogeneity of the samples. The aim of the assays was to detect extracellular sialidases. This necessitated the avoidance of over processing the samples in a manner which risked damaging cells and releasing internal sialidases, which made homogenising the samples more difficult. Some samples contained debris and mucus, and some samples would separate into two layers. Every effort was taken to gently resuspend samples to ensure that such components were evenly distributed between assay repeats, however due to the viscosity of the samples and the

size of some of the pieces of debris, it is likely that the samples will have maintained a degree of heterogeneity, increasing the error rate of the assay. The high viscosity of some samples also likely led to some pipetting inaccuracy, further increasing the error rate of the assay.

As previously mentioned, there was some debate as to the appropriate pH to use during the sialidase assay. Whether to use pH 7.7 to more closely replicate normal semen pH, or pH 4.2 to produce higher levels of sialidase activity, increasing the sensitivity of the assay. After testing a selection of samples (see section 1 of the Results and Discussion) it was found that many samples displayed much higher activity at the lower pH and it would be much easier to distinguish between sialidase positive and negative samples using pH 4.2. However, it is possible that this was a mistake, as many of the samples presenting lower levels of activity show no correlation with any other measured parameter. This is perhaps because at biological pH there is no sialidase activity present within these samples. In addition, optimum pH varies between sialidases, and there may have been rare samples with higher activity at biological pH which appeared negative in this assay. No such samples were detected in the selection tested at both pH's, but this does not eliminate the possibility of their existence. However, by determining a threshold of fluorescence representative of high enough levels of activity to affect the samples, useful data could still be produced.

Future experiments analysing sialidase activity within semen samples could further optimise this assay to produce more consistent data. It is not yet known how sialidases in semen are localised and whether they are associated with cells, or free within the seminal fluid. If this can be determined, it may be possible to process the samples in a manner which will reduce interference with fluorescence-based assays. It may be possible to remove cells via centrifugation, and then use protein purification to produce a 'cleaner' sample, containing only extracellular proteins, allowing for more consistent sialidase assays. The risk of processing the samples in this way is that significantly altering the conditions surrounding the sialidases may change their activity, allowing inactive enzymes to become active, or reducing the activity of active samples. However, these risks may be worth the increase in reproducibility, and reduction in background noise. Another option is to use an assay which does not rely on methylumbelliferone fluorescence. An alternative assay uses a coupled enzyme system in which the desialylation of the galactose residues is detected first by a galactose oxidase which produces hydrogen peroxide after binding to unsialylated galactose, and then by a peroxidase which uses the hydrogen peroxide to oxidise a chromogenic substrate[109]. Though this assay is more complex than the 4MUSA based assay it is possible that sample autofluorescence will not be as high, when detecting an alternative product. However, it is possible that unsialylated

galactose within the semen sample may present another source of background noise, possibly causing problems with this assay. Future experiments could also take samples from the same patient at different time points, to test how stable sialidase activity is over time, and determine whether problems caused by sialidase activity are likely to be acute or chronic conditions.

2.2 Limitations of the 16S rRNA Sequencing

One of the chief limitations of the 16S rRNA sequencing carried out as part of this study was sample availability. At the time the sequencing was completed none of the samples from fertile patients were available, and a selection of 50 had to be picked from the first 100 samples from the fertility clinic. This meant no comparisons could be made between fertile and infertile samples. Therefore, we could not determine whether there were any links between bacterial taxa and fertility, and if this corresponded to differences in sialidase activity in both sample sets. Such as the data is, the conclusions that can be drawn on the effect of bacteria on fertility are limited, and any speculation to that end requires the assumption that correlations between bacteria and sialidase activity can be extrapolated to the fertile samples. In addition, the number of samples in each activity group was relatively small, with only 16 samples in each (17 in the high activity group) limiting the statistical significance of the data.

There are also general limitations to 16S rRNA sequencing, which may have affected the results. The level of identification possible using the 16S rRNA gene is limited, rarely allowing identification at any phylogenetic level above genus, and sometimes being limited to the family or even order level. This made identifying potential sialidase producers difficult as although there are some genera containing large numbers of sialidase producing organisms (such as *Prevotella*) it is rare that sialidase production is ubiquitous throughout a genus. This creates a high risk of confirmation bias when interpreting the results with assumptions being made that sialidase negative members of a taxon are present within a sialidase negative sample whilst sialidase positive members of a taxon are present within a sialidase positive sample. It was also difficult to determine the abundance of taxa identified within the samples. 16S rRNA sequencing relies on PCR to amplify the targeted part of the 16S rRNA gene. This means that sequences more efficiently bound by the primers or amplified by the polymerase will be overrepresented, whilst sequences less efficiently amplified will be underrepresented. This means the percentage abundance of sequence reads produced by the analysis is unlikely to accurately correspond to the actual number of organisms within the sample. The processing of the sample may also have impacted the results, with some organisms potentially being lost when the samples were frozen, and some being lost during the DNA extraction process.

Another issue with the microbiome analysis was that of contamination. The patients producing the samples are unlikely to be trained in aseptic technique and, due to the private nature of the act of sample production, cannot be supervised by trained staff whilst they produce the samples. The nature of sample production also makes it difficult to place any restrictions on the patients to reduce the risk of contamination. The patients were encouraged to wash their hands before producing the sample, and the rooms in which the samples were produced were maintained to a high level of cleanliness, but despite these measures it is likely that there was a degree of contamination within the samples, likely originating from the patient's skin flora. Certain taxa often isolated from the skin flora such as *Streptococcus* and *Staphylococcus* were present within some samples, although a large proportion of the identified taxa are not usually found on the skin, and so were unlikely to be the result of contamination.

Now that more samples have been collected by the study, it is possible to carry out the sequencing on a larger scale, and to include samples from healthy patients. A larger number of samples will allow higher powered statistical analysis to be done and will potentially allow for hypotheses formed, based on the sequencing completed during this study, to be more rigorously tested. Future experiments will also allow for alternative regions of the 16S rRNA gene to be sequenced. A study in 2012[110] indicates that the V4 region provides the best coverage, whilst in this experiment we used primers spanning the V5 to V6 regions. It is possible that sequencing an alternative region of the 16S rRNA gene may provide a more detailed analysis of the seminal microbiome. Though difficult, it may be possible to reduce the risk of skin flora contamination during sample production. The use of sterilised polyurethane collection condoms may be one method to help reduce the risk of samples contamination. Alternatively, the use of sample collection machines, such as the Sanwe SW-3701 which allows for 'hands free' sample collection, may help both to reduce sample contamination, and to standardise the sample collection process. As with future sialidase assays, it will be useful to take several samples from each patient, at different time points, to monitor the temporal dynamicity of the seminal microbiome.

2.3 Limitations of the Gonococcal Experiments

The aim of this project was to assess the potential impact of sialidases on the male urogenital tract. Whilst the gonococcal experiments did produce data on the impact of sialic acid on gonococci, linking this to the project aim required the assumption that sialidases within the urogenital tract will alter the sialylation status of gonococci. It is highly likely that sialidases will desialylate gonococcal cell surface components, but experimental data confirming this would

improve the relevance of this data to the rest of the study. It is possible that the level of sialidase activity present within sialidase positive men will be insufficient to fully desialylate gonococci. The gonococci often become sialylated after entering host cells, as it is within cells that they often encounter CMP-NANA. It is not yet known whether bacterial sialidases can enter human cells, and it is possible that many sialylated gonococci within host cells will be protected from desialylation. Furthermore, the experiments were only completed with a single strain of *N. gonorrhoeae*. Until more experiments are done it is possible that the observed results are the product of a phenomenon which only occurs in P9, and no other strains of gonococci.

Future experiments should compare several different strains of gonococci, to verify the results and understand how varied gonococcal responses to sialylation are. The experiments studying the impact of sialylation on gonococcal antibiotic susceptibility should be repeated using bacteria with varying degrees of resistance to the antibiotics being tested to better understand how desialylation induced reduction in susceptibility will couple with other mechanisms of resistance. Species other than *N. gonorrhoeae* are known to undergo surface sialylation (such as *Haemophilus influenzae*) and it will be important to determine whether these species also experience changes in growth rate and antibiotic susceptibility. Therefore, experiments should be designed to test the effect of sialylation on these species, and perhaps the experiments could be expanded to include other surface modifications. In addition, the effect of sialidases on sialylated bacteria should be tested. An experiment could be designed to determine the level of sialidase activity required to desialylate the bacteria sufficiently to restore the serum sensitivity, slower growth rate, and grant the reduction in antibiotic susceptibility. This will allow better understanding of the effects of bacterial sialylation within the context of host levels of sialidase activity, and confirm whether host sialidase activity is sufficient to induce a level of phenotypic variation, with the potential to affect treatment outcomes or change the course of an infection.

Additional future experiments could examine the effects of sialylation on gonococci in more detail. The data from the gonococcal experiments prompted speculation as to how sialylation could increase growth rate, and how it could affect antibiotic susceptibility. Three theories regarding the potential effects of sialylation on gonococci were formed based on the observations. Firstly, the negative charge of the sialic acid could be significant enough to repel other sialylated bacteria potentially allowing the bacteria to more quickly disperse through growth media, reducing competition for nutrients and space. Secondly, sialylation could have an impact on membrane structure, possibly rendering it more permeable to antibiotics. Finally, sialylation could trigger changes in gonococcal gene expression, potentially acting as a signal to

adopt a phenotype more suitable to intracellular life. Future experiments could explore these hypotheses and help to gain an even deeper understanding of the impact of sialylation (and possibly sialidases) on gonococci. Microscopic observation of growing cultures could confirm whether sialic acid causes bacteria to be spaced further apart. Atomic force microscopy could potentially examine the surface of the bacterial membrane, to determine whether sialylation alters its structure. Transcriptomics of sialylated versus unsialylated bacteria could determine whether sialylation alters the expression of other gonococcal genes.

2.4 Further Research into Sialidases in Semen

This study should be repeated on a larger scale if the link between sialidase activity and infertility is to be more confidently established. Should the link between sialidase activity and infertility be confirmed, it will be crucial for future studies to confirm whether the link is causal. Clinical trials should be conducted to determine whether treating infertile men for raised sialidase activity is able to restore their fertility. Sialidase inhibitors could be used for this, or antibiotic treatments targeting potential sialidase producers. Trials should be conducted testing the efficacy of treating the male partner only, the female partner only, and both the male and the female partner. Should lowering the urogenital sialidase activity of infertile couples increase their reproductive success, it will confirm that the sialidases are directly causing infertility and are not merely a symptom of another unidentified fertility problem.

3. Concluding Remarks

This study has been successful in its aim to improve understanding of the impact of sialidases on the urogenital tract. Thanks to these results we now know that raised sialidase activity in semen is prevalent in men, and in some cases could be impacting their reproductive health. Links have been established between raised sialidase activity and several other semen parameters, showing that it is possible that these enzymes have an observable effect on the semen. In addition to this, the experiments revealed an increase in high activity samples in men with fertility problems, suggesting that the effects of sialidases on semen may be detrimental to male fertility. Furthermore, it appears that raised urogenital sialidase activity has the potential to impact gonorrhoea infections, possibly making the infection harder to treat, and creating an environment which may allow for the development of more resistant strains of gonococci.

Data from the study has also improved our understanding of various experimental techniques. Because of these experiments it may be possible to develop a sialidase assay better suited for

use in semen samples, which will be a key tool in expanding our understanding of these enzymes. The gonococcal experiments have increased our understanding of the limitations of conventional antibiotic susceptibility tests and will hopefully allow for the development of the more effective assays needed to tackle the rising problem of antibiotic resistance. It is hoped that the results of the experiments in this study will be useful in providing the groundwork for future experiments and allow for more sophisticated tools to be developed for these experiments.

Finally, this project has provided a unique insight into the delicate balance of the host-microbiome relationship. Human mucosal surfaces provide an environment in which a vast multitude of organisms can thrive, often becoming an important part of our biology and benefiting our health. However, should the conditions within this ecological niche begin to favour the wrong kinds of organism, the effects on our health can be detrimental. Looking at the impact of a single bacterial enzyme it is possible to see the range of effects such microbiome shifts could have on our health, in this case potentially rendering people infertile. It is hoped that such research will expand our understanding of bacterial disease beyond that of infection, and demonstrate how non-invasive, apparently asymptomatic changes to our microbiota, can also have serious consequences for human health.

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