Association of ABO, Lewis and Secretor phenotypes and genotypes with *Neisseria gonorrhoeae*

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Abstract

BACKGROUND: Previous studies of association of ABO phenotypes with gonorrhoea have shown contradictory results. Despite the interdependencies, none have examined the combined effect of ABO, Lewis and Secretor phenotypes. Furthermore, none have used genotyping to confirm phenotyping. This study is ground-breaking in this regard, and illustrates how such an association study should be performed.

STUDY DESIGNS AND METHODS: The study examined 175 individuals who tested positive for gonorrhoea, and 211 individuals who tested negative for gonorrhoea. Strain typing was not performed. The following blood grouping methods were performed on the study participants:

ABO phenotyping

Lewis phenotyping, and genotyping of selected samples

Secretor genotyping

Chi-square and p values were used to examine whether or not there is an association of ABO, Lewis and Secretor blood group related molecules with gonorrhoea infection.

RESULTS: Neither random statistical analysis of data sets, nor statistical analysis of data sets arranged by blood group, yielded a statistically significant association of ABO, Lewis and Secretor phenotypes and genotypes with *Neisseria gonorrhoeae* that could not be refuted when the data was disaggregated for ethnicity. The study did show a statistically significant difference in the incidence of the partial secretor phenotype (26.7%) in the gonorrhoea positive population and the incidence of the partial secretor phenotype (15.4%) in the gonorrhoea negative population, when all ethnic groups were analysed together. However, when the data was disaggregated for ethnicity, the p values were no longer statistically significant.

CONCLUSION: There is no association of ABO, Lewis and Secretor phenotypes and genotypes with *Neisseria gonorrhoeae*. Nevertheless, this study still has merit, because, to the author's knowledge, it is the first time a study of these human blood groups with a disease has been performed correctly.

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Statement of Originality

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement is made in the acknowledgements.”

……(signed)

……(date)

Abbreviations

ASHS	Auckland Sexual Health Service
bp	Base pairs
Cer	Ceramide
CDC	Centers for Disease Control
CPD	Citrate phosphate dextrose
CSL	Commonwealth Serum Laboratories
DIC	Disseminated intravascular coagulation
DGI	Disseminated gonococcal infection
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
Fuc	Fucose
FUT	Fucosyltransferase
g	Unit of relative centrifugal force (rcf)
G+	Gonorrhoea positive
G-	Gonorrhoea negative
Gal	Galactose
GalNAc	N-acetyl galactosamine
Glc	Glucose
GlcNAc	N-acetyl glucosamine
GRC	Glycoscience Research Centre
HIV	Human immunodeficiency virus
^{125}I	Iodine isotope 125
kDa	Kilo dalton
lele	Lewis negative genotype
Lele	Lewis positive genotype (heterozygous for *Le* gene)
LeLe	Lewis positive genotype (homozygous for *Le* gene)
LOS	Lipo-oligosaccharide
NYC	New York City
Opa	Opacity proteins
Por	Porin
PID	Pelvic inflammatory disease
PCR	Polymerase chain reaction
R	Remainder of molecule
RCLB	Red cell lysis buffer
RFLP	Restriction fragment length polymorphism
sese	Non-secretor genotype
Se	Secretor genotype
Se^w	Partial secretor phenotype
SDS-PAGE	Sodium lauryl sulphate-polyacrylamide gel electrophoresis
STI	Sexually transmitted infection
TM	Thayer Martin
U	Unit
UV	Ultraviolet

Chapter One: Gonorrhoea

1.1 Introduction

This chapter outlines the following aspects of gonorrhoea:

- history
- symptoms
- incidence
- treatment and prevention
- laboratory diagnosis
- human defence mechanisms against gonorrhoea
- adherence properties of *Neisseria gonorrhoeae*

The chapter also introduces the hypothesis of this thesis; namely whether *Neisseria gonorrhoeae* prefers some profiles of carbohydrates, expressed on the mucous membranes of the reproductive tract as a result of ABH, Lewis and Secretor histo-blood groups, over others.

1.2 History

Gonorrhoea is a sexually transmitted disease caused by the bacterial organism *Neisseria gonorrhoeae*, commonly known as gonococcus. Gonorrhoea is one of the oldest human diseases. In the Bible, (Leviticus., 15; 2-17) there is reference to urethral discharge. In 130 A.D, Galen called the disease we know as gonorrhoea “flow of seed”, evidently believing the urethral discharge to be semen. Hippocrates in the 4th century wrote of a disease called “stangury” which is today ascribed to the organism *Neisseria gonorrhoeae* as reviewed by Sparling (Sparling, 1999). Today we recognise that the disease is transmitted by direct physical contact with mucosal surfaces of an infected person, most commonly during sexual intercourse. The disease was accurately ascribed to the bacterial genus *Neisseria* and species *gonorrhoeae* after Neisser’s demonstration of diplococci in stained vaginal, urethral and conjuctival smears, in 1879, as reviewed by Kampmeier (Kampmeier, 1978). Humans are the only known biological host for *Neisseria gonorrhoeae*, and the organism quickly dies outside the human host unless placed on suitable microbiological culture media.

1.3 Symptoms

Clinical manifestations of gonorrhoea vary widely. The disease can be symptomatic or asymptomatic, and uncomplicated or complicated. The symptoms of gonorrhoea also differ between men and women. In men, infection is often symptomatic within seven days of infection, with frank purulent discharge from the urethra, which may be accompanied by dysuria, and an increase in urinary frequency. These infections, if treated with antibiotics, are usually uncomplicated. Between 10% and 50% of men may have infections, which are asymptomatic (Knapp et al, 1999). In women however, acute infection is much more likely to be asymptomatic, and women may not present until they have complications or even disseminated infection (Holmes et al, 1971). Symptoms in women are similar to those seen in many lower genital tract infections; namely increased vaginal discharge, dysuria, bleeding between periods, and menorrhagia. There may be cervical abnormalities such as purulent discharge, or bleeding. Rectal infection is seen in 35% - 50% of infected women and is common in homosexual men (Hook et al, 1999). Orogenital contact (particularly fellatio) can lead to pharyngeal infection (Weisner et al, 1973). Over 90% of pharyngeal infection is asymptomatic, and can lead to disseminated gonococcal infection (Weisner et al, 1973). Gonococcal conjunctivitis is rare in adults, and when seen is presumably the result of poor hand washing practice. Gonococcal conjunctivitis is a small but recognised risk for laboratory workers working with positive cultures (Bruins et al, 1979). Conjunctivitis is also seen in neonates born to infected mothers, as a result of infection during passage through the birth canal. Opthalmia neonatorum can result in blindness in children. Gonorrhoea in pre-pubertal children may be an indicator of sexual abuse (White et al, 1983).

1.3.1 Complicated gonococcal infections

Pelvic inflammatory disease (PID) is the most common complication in women (Eschenbach et al, 1975). Between 9% and 15% of women with gonorrhoea develop PID (Eschenbach et al, 1975), and between 10% and 70% of PID cases have had *Neisseria gonorrhoeae* isolated (Knapp et al, 1999). Gonococcal PID can lead to chronic pelvic pain, infertility, or ectopic pregnancy.

Other complications in women include urethral vestibular abscesses, salpingitis and tubo-ovarian abscesses.

In men, complications are less likely, partly inherently, and partly because the disease is more likely to be symptomatic, and therefore more likely to be treated. In men, complications are acute epididymitis, prostatitis and urethral stricture. Penile lymphangitis is a rare complication (Hook et al, 1999).

Untreated patients may develop disseminated gonococcal infection (DGI). DGI tends to be more common in women than in men, because the acute urethritis seen in men is more likely to receive early treatment (Holmes et al, 1971). DGI results from gonococcal bacteraemia and leads to arthritic syndromes and/or dermatitis. Endocarditis (Holmes et al, 1971) and meningitis (Sayeed et al, 1972) following DGI are rare complications. For the purpose of this research, complicated infection was defined as patients having PID, epididymo-orchitis, or DGI.

1.4 Incidence

Amongst sexually transmitted infection (STI), gonorrhoea is the second most common infection worldwide (second only to *Chlamydia trachomatis*) (Knapp et al, 1999). The Centers for Disease Control and Prevention in Atlanta, USA (CDC) estimate only half of the total gonorrhoeal infections are reported (CDC, 1997). CDC report a steadily increasing number of infections from 1964 to 1977, fluctuation through the 1980's, and then an annual decline from 1987 (CDC, 1997). Britian, however, reported an increase in gonorrhoea during the mid-1990's (Hughes et al, 2000). Internationally, there is an increasing incidence of gonorrhoea amongst men who have sex with men (Donovan et al, 2001; Knapp et al, 1999).

In New Zealand, gonorrhoea is not a notifiable disease, and so incidence figures are not annually reported. However, in 2000 and 2001, a study was published in the New Zealand Public Health reports, reporting incidence figures for 1999 and 2000 respectively (ESR, 2001; Turley et al, 2000). These figures report only on New Zealand Sexual Health Clinic attendees, and do not include gonorrhoea infection diagnosed at patients' general practitioners, or other health providers. Contacts of gonorrhoea patients are not routinely traced in New Zealand (Brokenshire, 2002, personal communication).

In 1999, the national average of incidence of gonorrhoea in attendees at New Zealand Sexual Health Clinics was 0.6% (Turley et al, 2000). Rates were higher than the national average in some geographical locations, as shown in Table 1. Nationally, 69% of gonorrhoea cases were in persons aged under 25, and rates of gonorrhoea were also disproportionately high in Maori and Pacific Islands people. The high positivity rate in persons aged under 25 is in keeping with international findings. Knapp reports gonorrhoea to be most common in high-density populations, and particularly amongst people under 24 years of age having unprotected sex (Knapp et al, 1999).

Table 1. *Gonorrhoea rates in New Zealand sexual health clinic attendees*. The table illustrates the incidence of gonorrhoea as a percentage of sexual health clinic attendees in regions of New Zealand, in 1999, as compared to 2000 (Turley, 2000).

Location	Incidence of gonorrhoea %	
	1999	2000
Auckland	0.9	0.9
Whakatane	2.5	2.2
Napier	2.0	2.5
Gisborne	1.2	1.1
Rotorua	1.1	0.9
Porirua	1.0	0.7
Hastings	3.3	3.3
New Plymouth	0.3	1.0
Timaru	0.2	2.7

The decline of gonorrhoea in the United States of America (CDC, 1997) is not the experience in New Zealand. Rather, the rate of gonorrhoea is increasing. Turley reported a 40% increase in gonorrhoea (and *Chlamydia trachomatis*) in sexual health clinic attendees between 1996 and 1999 (Turley et al, 2000). In 2000, gonorrhoea infections had once more increased in the population tested (namely those attending at New Zealand Sexual Health Clinics). The national average rate of confirmed gonorrhoea in 2000 was 0.7%, which was significantly higher ($p<0.01$) than the rate of 0.6% in 1999 (ESR, 2001). The fact that gonorrhoea continues to increase in New Zealand, despite education campaigns, is of great concern, particularly as it is predominately a disease of young people. The fact that contacts of those diagnosed with gonorrhoea are no longer routinely traced in New Zealand may be contributing to the increasing

rate of gonorrhoea. Turley et al suggest national increases may reflect failures in safe sex campaigns, and are a worrying trend, because high gonorrhoea rates can precede increases in infection with human immunodeficiency virus (HIV) (Donovan et al, 2001). It is well accepted internationally that good control of STI, including gonorrhoea, is a good control strategy for HIV. Treatment of urethral gonorrhoea in men has been shown to reduce the level of HIV in semen (Donovan et al, 2001).

Turley (Turley et al, 2000) suggests that increased incidence in Maori is probably multi-factorial, including change in attendance patterns at sexual health clinics, increase in number of people self-identifying as Maori, and high pregnancy rates, which suggests low contraceptive use, including condoms. Internationally, high gonorrhoea rates have been linked to low socio-economic conditions, and limited access to health care (CDC, 1999).

1.5 Treatment

If left untreated, gonococcal infection in men is usually self-limiting, with clearance of the organism within 6 months (Pelouze, 1941). However, treatment is highly desirable, since it prevents the spread of infection, and prevents complications in women. *Neisseria gonorrhoeae* is also quite sensitive to antibiotics, making an early-diagnosed infection relatively easy to treat. The first effective antimicrobial treatment was developed in the 1930's, and consisted of sulphonamides (Kampmeier, 1983).

In modern medicine, gonorrhoea has been successfully treated for many decades with the antibiotic penicillin (Cohen et al, 1999). However, with the prolonged usage of antibiotics, there has been a gradual increase in antibiotic resistant mutant strains. Strains are now commonly encountered which are resistant to penicillin (Faruki et al, 1985), and tetracycline (Morse et al, 1986). Resistance to penicillin in 1999 was reported at 10.4% (Brett et al, 2001). In New Zealand, resistance to penicillin has fallen over the past 10 years, due to the success of treating gonorrhoea with quinolines. However, there is now an increasing resistance to fluoroquinoline (1.8% in 1999) (Brett et al, 2001). When this study started, the choice of antibiotic treatment for gonorrhoea was ciprofloxacin (500mg orally); a quinoline. During the study, Auckland Sexual Health Service (ASHS) began prescribing ceftriaxone (250 mg intramuscularly), when the gram stain was suggestive of *Neisseria gonorrhoeae,* and antibiotic

sensitivity results were pending. This change was due to emerging resistance to the quinolines. Ceftriaxone is a third generation cephalosporin.

1.6 Prevention

Proper use of condoms provides effective protection against gonorrhoea, and other genital infections (Donovan et al, 2001). Silver nitrate can be used in eyedrops in infants born to infected mothers to prevent gonococcal conjunctivitis (Forbes, 1971). There has been some work performed to find a suitable vaccine against gonorrhoea. Gulati has produced a monoclonal antibody which mimics the natural immune response to the gonococcal lipooligosaccharide 2C7 (Gulati et al, 1996a; Gulati et al, 1996b; Gulati et al, 2001).

1.7 Laboratory Diagnosis

A swab sample is taken at the affected site, and is transported to the laboratory, where gram stain and culture of the swab are performed. Presence of intracellular gram negative diplococci in polymorphonuclear white blood cells in the gram stain is suggestive of *Neisseria gonorrhoeae*. However, it does not rule out other species of the *Neisseria* genus.

Stained smear identification has a high sensitivity for symptomatic men (90% - 95%), but a low sensitivity for women, with only 50% - 70% of cases of uncomplicated gonorrhoea diagnosed after gram stain of endocervical smear (Rothenberg et al, 1976). A positive culture of *Neisseria gonorrhoeae* is diagnostic. It is also important to grow the organism in order to determine its susceptibility to a range of antibiotics. Culture media for *Neisseria gonorrhoeae* consist of New York City (NYC) or modified Thayer-Martin (TM). Both these media are selective; providing nutrients that encourage growth of *Neisseria* species, and inhibit growth of normal vaginal, cervical, or rectal flora, by addition of antibiotics. Single cultures on selective media have high sensitivity detection rates. Gram stain and culture of swabs constitute routine screening techniques.

For the purposes of this study, all samples were confirmed either positive or negative for *Neisseria gonorrhoeae* by culture on selective media. Routine practice at ASHS is to culture cervical and urethral swabs on NYC media, and oral and rectal swabs on TM media. No growth on these media constitutes a

negative result. Characteristic colony growth, gram stain showing gram negative diplococci, positive oxidase test and a range of tests for sugar utilization, constitute a positive result for *Neisseria gonorrhoeae*. LabPlus at Auckland Healthcare, who perform the testing of laboratory samples from ASHS, use the RapID NH commercial system, a qualitative micromethod employing conventional and chromogenic substrates for identification of medically important species of *Neisseria*, and other pathogens.

Advances have also been made in genotyping *Neisseria gonorrhoeae*. The polymerase chain reaction (PCR) has been used to amplify genes coding for Opacity proteins (Opa). Deoxyribonucleic acid (DNA) amplification techniques are now available for the diagnosis of *Neisseria gonorrhoeae.* Commercial identification systems available in New Zealand include the Becton Dickinson strand displacement amplification (SDA), the Abbott ligase chain reaction (LCA), and the Roche polymerase chain reaction (PCR). Testing can be performed on both swabs and urine specimens, and is highly sensitive and specific. Culture confirmation of *Neisseria gonorrhoeae* is recommended in cases of positive DNA amplification tests.

Serological assays, which use monoclonal antibodies to *Neisseria gonorrhoeae* epitopes, are useful in identifying strains of the organism. Monoclonal antibody typing schemes have been developed based on 2 chemically and immunologically distinct classes of Por; the Porin protein of *Neisseria gonorrhoeae*. Por can be isolated from the outer membrane of *Neisseria gonorrhoeae*. It is a 34-36 kDa protein, as visualised on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Por is located on the membrane surface, and its function may be to carry nutrients through the organism's lipid-rich outer membrane (Sparling, 1999). It exists in 2 forms; Por A or Por B, but never both. Many distinct strains of Por A and of Por B can be distinguished by reaction patterns with monoclonal antibodies to unique epitopes of the protein. The Opa family of proteins is important in cellular adhesion. Opacity proteins assist in adherence of gonococci to epithelial cells. These proteins vary in size from 24 to about 28 kDa when separated by SDS-PAGE, and all share the property of heat modifiability. That is they take on a different shape and higher molecular weight when heated to 100°C (Swanson, 1982). A single strain of *Neisseria gonorrhoeae* may exhibit no Opa proteins, or up to 5 proteins of the family simultaneously. Studies have shown that

expression of Opa proteins increases adhesion of *Neisseria gonorrhoeae* to epithelial cells (Fischer et al, 1988; Sugasawara et al, 1983). The cellular protein CD66 has been identified as a host cell receptor for Opa (Virji et al, 1996a; Virji et al, 1996b).

Neisseria gonorrhoeae can be split into strain types depending on the genetic polymorphisms of these Opa genes (O'Rourke, 1995). Strain typing, based on expression of Opa, and Por proteins, is important when undertaking epidemiological studies. Although strain typing would have been of interest to this study, the cost of serotyping prevented its inclusion.

1.8 Innate genito-urinary defence mechanisms to pathogens, including *Neisseria gonorrhoeae.*

The mucosa, and the epidermal surfaces of the genito-urinary tract, provide innate protection from invading pathogens. The mucosa of the urethra in the male and female, and the endocervix of the female, is comprised of simple columnar epithelium, and the vagina and the ectocervix are comprised of stratified squamous epithelium. The area where the endocervix meets the ectocervix is called the squamocolumnar junction, and this is increased in size in pregnancy, adolescence, and in women using oral contraception. Increased numbers of cells of squamous epithelium in this area may favour the growth of some pathogens (Cohen et al, 1999). Epithelial cells and bacterial cells both have a negative net charge, and this acts as a physical repellant to invading bacteria. This negative charge on epithelial cells results from carboxylic groups of N-acetyl-neuraminic acid, and also other anionic groups, including alkaline phosphatase, and the beta and gamma-carboxy groups of aspartic and glutamic acid. Mucociliary activity, and flow of mucus, act as primary defence mechanisms, since organisms will simply be swept away, unless they can adhere. Huge variations can be seen in the vaginal mucus of women throughout their menstrual cycle, which may make them more vulnerable to pathogens at specific times in the cycle (Holmes et al, 1971). It is of note that the mucosa bears the blood group antigens (see later).

Lactobacilli, which are present in the vaginal tract as normal flora, can inhibit the growth of some bacterial pathogens, since they produce an acid environment by virtue of production of lactic acid. In addition, they produce several anti-bacterial

compounds, notably lactocidin, acidolin, lactacin B, acidophilin, and hydrogen peroxide. Hydrogen peroxide has been shown to inhibit growth of *Neisseria gonorrhoeae in vitro* (Zheng et al, 1994). However, studies have not found any significant differences in gonorrhoea infection rates between exposed males and females, so the protective effect of lactobacilli *in vivo* is not well established (Cohen et al, 1999).

1.9 Adherence properties of *Neisseria gonorrhoeae*

Whether pathogens can adhere to the mucosa of the genito-urinary tract or not, is critical to their establishment in a given human host. In order to penetrate the mucus, *Neisseria gonorrhoeae* uses pili and outer membrane proteins (Opa). Pili and Opa are the main proteins involved in cellular adhesion. The other membrane structures are not discussed in any detail, except where they are implicated in resisting host defence mechanisms. Lipo-oligosaccharide (LOS) is mentioned because it plays a role in resisting antibody and complement mediated host defence mechanisms. LOS is made up of a lipid, and a core polysaccharide. It may be “short” or “long”. Long LOS is designated because it can accept host neuraminic acid by means of a gonococcal sialyltransferase (Smith et al, 1995) (van Putten et al, 1995). If LOS is sialylated, antibody mediated complement lysis is not effective, because antibody access to LOS is sterically blocked.

Antigenic variation is seen amongst both pili and Opa (and is used as the basis for antigenic strain typing as previously described). Some variants may be more effective in adhering to human epithelial cells than others, by virtue of attaching to different ligands on the cell. Pili are tentacle-like projections, which cover virtually the entire surface of the gonococcal cell. They are arranged either as individual fimbrils, or in bundles. They are made up of the protein fimbrillin. Their function is thought to be exchange of genetic information between organisms, as reviewed by Mallinson (Mallinson, 2000). They are also strongly associated with the ability of *Neisseria gonorrhoeae* to adhere to epithelial cells of the host. Pili have been classified into 5 types, and *Neisseria gonorrhoeae* possess type 4, characterised by N-methylphenylalanine in the amino terminus region of the major subunit (Mallinson, 2000). These type 4 pili express 2 distinct sub-units; PilE, expressed on the pilus fibre, and PilC, associated with the tip. PilC has been shown to adhere to human epithelial cells, and PilE to red

blood cells (Rudel et al, 1992). In culture, *Neisseria gonorrhoeae* can be piliated or non-piliated, but fresh isolates from patients are usually piliated, suggesting that piliated strains are more effective in penetrating the host's innate immune mechanisms (Sparling, 1999). Piliated *Neisseria gonorrhoeae* adhere better to human columnar epithelium than to squamous cells. The mucosa of the urethra in the male and female, and the endocervix of the female, comprises simple columnar epithelium. Antibodies against pili have been shown to decrease adherence to red blood cells and to epithelial cells (McChesney et al, 1982; Schoolnik et al, 1983; Virji et al, 1984). This phenomenon has been used as a basis for developing vaccines, with, as yet, limited success against the wide variety of strains seen in clinical practice.

Pili in different bacteria have been shown to bind to specific carbohydrate receptors. A piliated *Escherichia coli* has been shown to use mannose as a receptor in the human buccal epithelium cells (Ofek et al, 1978).

> "The precise nature of the mucosal receptor(s) for binding of gonococcal pili is still under investigation, but carbohydrates may be involved" (Cohen et al, 1999).

This observation is part of the basis of this research, since carbohydrates, which are determined by the genes of the blood group systems ABO, Secretor, and Lewis, are expressed on the mucosal surfaces of the genito-urinary tract. Our hypothesis is that particular carbohydrate molecules (dependent on one's blood group genes) act as receptors for *Neisseria gonorrhoeae,* and this may convey either a pre-disposing, or conversely, a protective, effect.

> "Since mucus is rich in carbohydrates, it has been suggested that one or more sugar moieties may be offered to the invading pathogen and occupy a critical bacterial ligand, thereby interfering with the adherence of the pathogen to the epithelial layer" (Cohen et al, 1999).

Earlier studies that have examined whether or not there is a relationship between blood groups and gonorrhoea are contradictory. Three separate studies in the 1970s and 1980s reported gonorrhoea to be more common in blood group B individuals (Foster et al, 1976; Kinane et al, 1983a; Kinane et al, 1983b; Miler, 1977). However, three further workers conducted similar studies, and found no relationship between ABO blood group and gonorrhoea (Johnson et al, 1983; Matzkin, 1987; Schofield, 1966). Understanding of interaction of

gene products of ABO, Lewis and Secretor blood groups has developed since the 1980's (Henry, 1996a). We now know that the genes of the ABO, Lewis and Secretor blood group systems produce transferases, which compete for common substrates in the body's tissues, and affect blood group expression on the mucosa of the reproductive tract. Since transferases controlled by three blood group systems are involved, and their action is integrated, it is essential to determine the influence of each of the blood group systems ABO, Lewis and Secretor.

1.10 Cellular and antibody mediated defense mechanisms to *Neisseria gonorrhoeae*

Once *Neisseria gonorrhoeae* has successfully adhered to the genitourinary tract, cellular defense mechanisms are mounted. Neutrophils are attracted to the adhesion site by the release of chemokines from the damaged epithelial cells, and from the gonococcal cells, as reviewed by Sparling (Sparling, 1999). Neutrophils are able to engulf and lyse bacterial pathogens, by virtue of toxic oxygen-reduction products produced by oxygen and glucose metabolism. These products include hydrogen peroxide and superoxide. *Neisseria gonorrhoeae* is sensitive to oxidative attack. Lysosomal proteins such as cathepsin G also contribute to the killing of *Neisseria gonorrhoeae* (Shafer et al, 1986). Most gonococcal cells taken up by neutrophils are killed, but studies suggest that up to two % of ingested *Neisseria gonorrhoeae* survive the bactericidal attack by neutrophils (Casey et al, 1986). T lymphocytes also recognise and present gonococcal cells to B lymphocytes, which begin production of IgM, IgG and IgA. Recently, infection with *Neisseria gonorrhoeae* has been shown to produce an antibody response directed against the gonococcal LOS epitope 2C7 (Gulati et al, 1996a). IgG and IgM directed against Porin (Por) and LOS in the outer membrane are able to activate complement to begin complement- mediated lysis. However, if LOS is sialyated, complement mediated lysis is not effective. Once inside the epithelial cell, *Neisseria gonorrhoeae* is immune to the actions of specific antibody, and of complement.

1.11 Chapter review

Neisseria gonorrhoeae is a significant pathogen both internationally, and in New Zealand. The adherence properties of the organism, in particular to carbohydrates, are not well understood, and therefore make *Neisseria gonorrhoeae* an ideal candidate for this study.

Chapter Two: Human Blood Groups

2.1 Introduction

ABO and Lewis are two of the blood group systems expressed in humans. They differ from some other blood group systems, in that the antigenic molecules are carbohydrate, and they are expressed throughout the body. Individuals express a profile of these carbohydrates on their mucous membranes as well as on their red cells. This profile differs, depending on whether or not the individual possesses the Secretor gene, and on the genes inherited in the ABO, and Lewis systems. Furthermore, the expression varies depending on the type of tissue involved. There are at least 18 major and distinctively different carbohydrate profiles which can be expressed on the mucosal tissues (Henry, 2001a).

2.2 ABO

The ABO blood group system was the first major blood group system to be recognised, and remains the most important in human blood transfusion (Landsteiner, 1901). There are four major groups in the system; A, B, AB and O. Expression of the groups is controlled by enzymes known as glycosyltransferases, produced under control of inherited genes. On red cells, sugars are sequentially added to type 2 precursor chains under control of the glycosyltransferases. Type 2 chains are characterised by a β1-4 linkage between the terminal N-acetylglucosamine (GlcNAc) and galactose (Gal) in the saccharide chain.

In the body fluids (saliva, tears, semen, urine and breast milk), and on the mucosal surfaces, type 1 chains are also found. Type 1 chains are characterised by a β1-3 linkage between the terminal N-acetylglucosamine (GlcNAc) and galactose (Gal) in the saccharide chain.

The steric differences between type 1 and type 2 chains resulting from the β1-3 or β1-4 linkages respectively can be appreciated much more easily when the molecules are viewed in 3-dimensional models. The concept is illustrated in Figure 1, which is reproduced with permission (Henry et al, 1995).

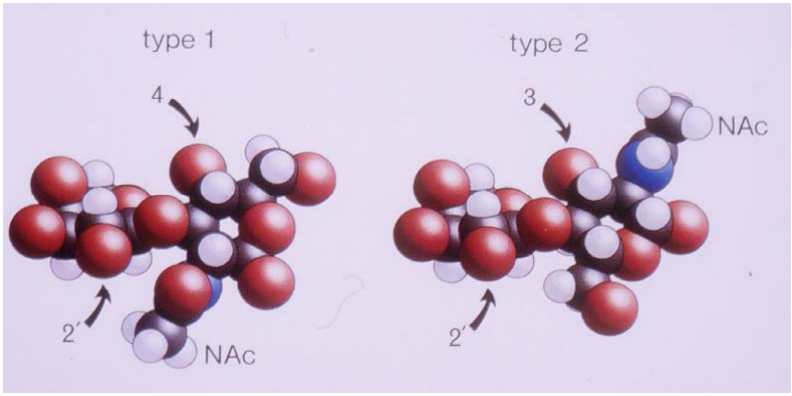

Figure 1. Galβ1-3GlcNAc vs Galβ1-4GlcNAc. The model illustrates the steric difference between type 1 and type 2 chains, especially at carbon 2 of Gal.

Precursor chains types 3, 4 and 6 also exist, but to date, type 5 has not been found in humans (Daniels, 1995; Henry et al, 2000). Table 2 shows the molecular structure of types 1, 2, 3, 4, 5 and 6 (Oriol et al, 1986).

Table 2. *Carbohydrate precursor determinants*.

Type 1	Galβ1→3GlcNAcβ1→R
Type 2	Galβ1→4GlcNAcβ1→R
Type 3	Galβ1→3GalNAcα1→R
Type 4	Galβ1→3GalNAcβ1→R
Type 5	Galβ1→3Galβ1→R
Type 6	Galβ1→4Glcβ1→R

The fucosyltransferase enzyme produced by the H gene, adds the sugar fucose to type 2 precursor to produce an antigen known as H type 2.

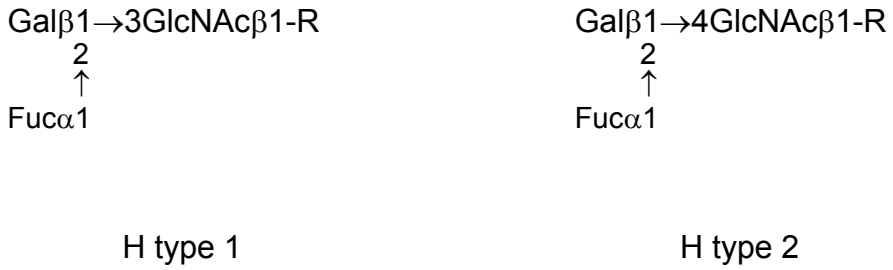

Figure 2. *H type 1 and H type 2.* These molecules are formed by the addition of fucose to type 1 and type 2 precursors.

Once the precursor is fucosylated to form H, A and B specific sugars can be added. In A individuals, the A gene produces an α1-3 N-acetylgalactosaminyl transferase, which adds N-acetylgalactosamine to H, thus forming A.

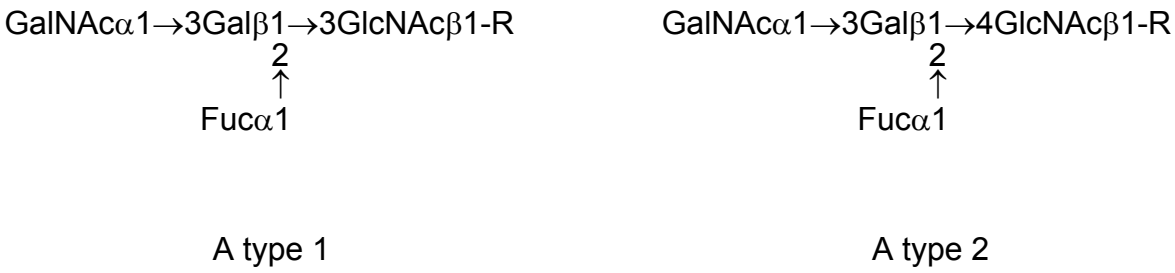

Figure 3. *A type 1 and A type 2.*These molecules are formed by the addition of N- acetylgalactosamine in α1-3 linkage to H type 1 and H type 2 respectively.

In blood group B individuals, the B gene codes for production of galactosyltransferase, which adds a galactose to H, forming B.

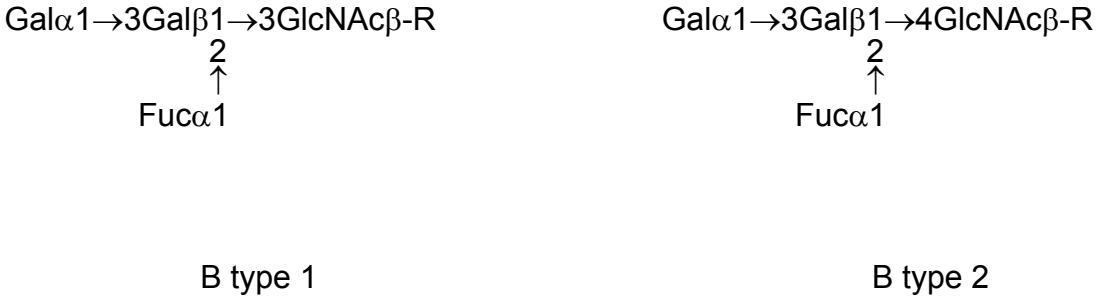

Figure 4. *B type 1 and B type 2.* These molecules are formed by the addition of galactose in α1-3 linkage to H type 1 and H type 2 respectively.

In blood group AB individuals, the A and B transferases compete for the precursor substrates with sufficient success that both A, and B, are expressed.

In blood group O individuals, the O gene encodes an inactive protein so H remains unaltered; thus large amounts of fucose are expressed as the terminal sugar. The A, B, and O genes are located on chromosome 9, as reviewed by Daniels (Daniels, 1995).

These blood groups, with their different steric structures and different terminal sugars, are of major importance for safe blood transfusion, because antibodies are common. Blood group A individuals express anti-B in their plasma. Blood group B individuals express anti-A in their plasma. Blood group O individuals express anti-A and anti-B in their plasma, predominately as an inseparable form of anti-A + anti-B (anti-A,B). Blood group AB individuals do not express either anti-A or anti-B. Allo-anti-H is not expressed because all common ABO blood groups still express some H antigen. These ABO antibodies are of major significance, since they have the ability to activate complement. Transfusion of incompatible blood has potentially fatal consequences.

2.2.1 Biological significance of the ABO system

In addition to clinical significance for transfusion and transplantation, it is becoming increasingly apparent that ABO and H antigens are of biological significance and may be associated with predisposition to, or protection from, many diseases. The reader is referred to the chapter entitled “ABH polymorphisms and their putative biological relationship with disease” in the book *Human Blood Cells; Consequences of Genetic Polymorphisms and Variations*, for a full discussion (Henry et al, 2000). Of course, ABO is only one factor, and environmental triggers, health of the individual, level of medical intervention and other genes may all contribute to whether or not an individual succumbs to a particular disease. Bacterial diseases are of particular interest, since many bacteria have been shown in vitro to react with specific ABH-related structures, and may use them *in vivo* as receptors when invading the host.
Helicobacter pylori, for instance, has been shown to bind to H type 1 in vitro, reviewed in (Henry et al, 2000) and H type 1 in the stomach may be the ligand which *Helicobacter pylori* uses to establish itself in the human host.

The adhesion properties *of Neisseria gonorrhoeae* are not completely understood, as discussed in the previous chapter. As mentioned, those carbohydrates, which are probably used as ligands, are not yet elucidated. It is

the postulate of this thesis that *Neisseria gonorrhoeae* may use specific blood group related ABH, Lewis, or their precursors, as antigens to bind to the human host. Deal and Krivan (Deal et al, 1990) demonstrated that *Neisseria gonorrhoeae* bound preferentially to some glycolipids isolated from endocervical epithelium. They labelled *Neisseria gonorrhoeae* with iodine isotope ^{125}I and bound these to a profile of glycolipids separated by thin layer chromatography. *Neisseria gonorrhoeae* showed preference for asialo-GM1 and GM2, lactotriaosylceramide (the precursor to Le^c) and paragloboside (type 1 precursor, also known as Le^c). These are epithelial glycolipids, which are present in all individuals, but in varying amounts; because they are precursors which are modified depending on expression of the *Le*, *Se* and *ABH* genes. Our hypothesis is that *Neisseria gonorrhoeae* may prefer some presentations of carbohydrates found in different blood group phenotypes.

The ABO and H antigens are widely distributed throughout the human tissues. In relation to this thesis, the expression of ABO and H antigens on the epithelium of the genito-urinary tract, and in the mucous secretions of that tract, are of interest. Expression of blood group ABH and some Lewis antigens in the tissues is also under control of the Secretor (*Se*) gene, and hence we must consider the action of the fucosyltransferases of this family next.

2.3 Secretors and non-secretors

The *H* gene and the *Se* gene, are controlled from different loci on chromosome 19 (Ball et al, 1991). *H* and *Se* loci are closely linked, and exhibit linkage disequilibrium, as reviewed by Daniels (Daniels, 1995). Both code for fucosyltransferases. The α1-2 fucosyltransferase (FUT 1) produced by the *H* gene is able to fucosylate type 2 precursor, found on erythrocytes, and also types 3 and 4 (Oriol et al, 1986).

The *Se* gene also produces an α1-2 fucosyltransferase, (FUT 2) but a different enzyme nevertheless, because it can fucosylate both type 1 and type 2 precursors (Oriol et al, 1986). However, FUT 2 shows a definite preference for type 1 (Le Pendu et al, 1985). In addition to expression of the H antigen on erythrocytes, and in the secretions, H antigen can be found on human tissues. Type 2 structures are found on tissues of endodermal and mesodermal origin (e.g skin, vascular epithelium and bone marrow). Tissues of endodermal origin (e.g digestive, and respiratory, mucosa) express type 1 and type 2 structures, where both the H antigens are produced by FUT 2 (Oriol et al, 1986).

In individuals who possess the *Se* gene in homozygous or heterozygous form, FUT 2 adds a fucose to type 1 and type 2 precursors, to produce the structures known as H type 1 and H type 2 respectively. These are shown in Figure 2.

H types 1 and 2 are expressed in the secretions, and on the mucosal surfaces, in individuals who possess the *Se* gene.

Once the precursor is fucosylated into H, A and B specific sugars can be added to produce A type 1 and B type 1 respectively, as shown in Figure 3 and Figure 4. Therefore, in the secretions, and on the mucosal surfaces, individuals who are Secretors express their ABH substances.

In concordance with their red cell blood group, determination of secretor status was previously performed by an indirect method, which involved testing the saliva against diluted human anti-A and anti-B. If the individual was secreting A substance, the anti-A was neutralised, and subsequently failed to agglutinate A positive red cells. If the individual was secreting B substance, the anti-B was neutralised, and subsequently failed to agglutinate B positive red cells. This was a crude method, which failed to take account of the varied mutations we now know to exist amongst the Secretor genes, and often resulted in incorrect results (Henry et al, 1995).

In this study, Secretor genes were determined using a molecular genotyping method, which takes into account the wild type and mutated genes (Svensson et al, 2000). About 20% of Caucasians do not secrete ABH substances in their body fluids, as reviewed by Harmening (Harmening, 1999).

At a genetic level, this is due to one of:

- A series of point mutations which result in a stop codon in the Secretor gene, and a resulting non-transcription of enzyme protein (G428A, C571T, C628T, and G849A)

- A three nucleotide deletion at nucleotide 685, which results in an inactive protein
- The pseudogene “fusion” which results in an inactive protein
- The deletion gene (del) which results in an absence of the *Se* gene (Svensson et al, 2000)

The genetic basis of these non-secretor phenotypes is summarised in Table 3.

Table 3. *Secretor gene mutations which produce non-secretor phenotypes*. The nucleotide change from the wild type gene is shown in the first column. The amino acid change that this nucleotide change produces is shown in the second column. The effect this has on the emzyme is shown in the third column. The populations in which each mutation has been observed are referenced in the last column.

Nucleotides	Amino Acids	Effect on enzyme	Population
428 G→A	143 Try →STOP*	Inactivate	Caucasian (Kelly et al, 1995)
571 C→T	191 Arg →STOP*	Inactivate	Polynesian (Henry et al, 1996d) Chinese (Yu et al, 1996) Japanese (Koda et al, 1996)
628 C→T	210 Arg →STOP*	Inactivate	Japanese (Koda et al, 1996)
685 del TGG	230 Val excluded	Inactivate	Taiwanese (Yu et al, 1999)
849 G→A	283 Trp →STOP*	Inactivate	Chinese (Yu et al, 1996)
Deletion		Absent	Indian (Fernandez-Mateos, et al 1998)
211-272	Fusion with a pseudogene	Inactivate	Japanese (Koda et al 1996)
385 A →T	129 Ile → Phe	Unstable **	Asian (Koda et al, 1996; Kudo et al, 1996; Yu et al, 1995) Indonesian (Henry et al, 1996c) Polynesian (Henry et al, 1996b)

* STOP= Stop codon
**(Henry et al, 1996b)

2.4 Lewis

A third set of blood group genes is also involved in producing the varied carbohydrate profiles, which the human host presents to bacteria. The Lewis (LE) locus is also located on chromosome 19 (Reguine-Arnould et al, 1995). Two major alleles are expressed: *Le* and *le*. In fact the term “*le*” refers to a group of genes which produce no active protein i.e. no fucosyltransferase activity. These almost certainly represent mutations of the wild-type gene (Oriol et al, 1986). Elmgren et al have described five point mutations that render the *Le* gene non-functional (Elmgren, 1996). Individuals can be homozygous for the *Le* gene (*LeLe*), heterozygous (*Lele*) or homozygous for the *le* gene (*lele*), with

94% of Whites, and 75% of Blacks having the *Le* gene (Harmening, 1999). Six percent of Whites are *lele* and 25% of Blacks are *lele* (Harmening, 1999). *lele* individuals do not modify type 1 precursor (also known as the Le^c antigen) or H type 1 (also known as the Le^d antigen).

Genetic interactions between Le, ABO and Se loci produce our Lewis phenotype. The *Le* gene codes for an α-3/4-L fucosyltransferase (FUT-3), which fucosylates type 1 chains (Oriol et al, 1986). FUT 3 has been detected in breast milk, saliva, gastric mucosa and cyst fluids, but not in plasma, or in red cell stroma (Salmon, 1984). FUT 3 pins carbon 1 of L- fucose to carbon 4 of GlcNAc on type 1 precursor. In type 2 chains, the carbon 4 of GlcNAc is already bound to galactose, so carbon 3 is used. FUT-3 catalyses the addition of fucose to H type 1 and H type 2 in Secretors to produce Le^b antigen and Le^Y (or Y) antigen, respectively (Figure 5).

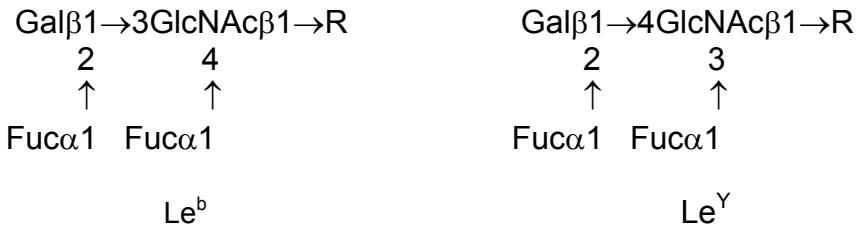

Figure 5. *Le^b and Le^Y antigens.* FUT-3 joins carbon 1 of L- fucose to carbon 4 of GlcNAc on H type 1 to form the Le^b antigen. FUT 3 joins carbon 1 of L- fucose to carbon 3 of GlcNAc on H type 2 to form the Le^Y antigen.

In non-secretors, FUT-3 catalyses the addition of fucose to type 1 precursor to produce Le^a antigen, or to type 2 precursor to produce Le^X (or X) antigen (Figure 6).

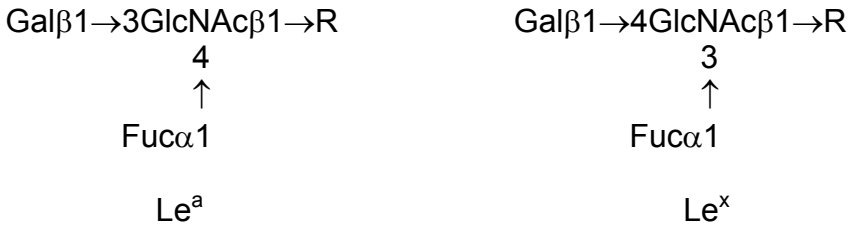

Figure 6. *Le^a and Le^x antigens*. FUT-3 catalyses the addition of fucose to type 1 precursor to produce Le^a antigen, and to type 2 precursor to produce Le^X. Other enzymes can also produce Le^X.

The Lewis antigens are not synthesised by the red cell, but instead by exocrine epithelial cells (Henry et al, 1995). Glycolipids bearing Lewis antigens are absorbed from the plasma onto red cells, thus labelling them with these antigens. At the simplest level, red cells of secretors with the *Le* gene may be considered to have the phenotype Le(a-b+), and red cells of non-secretors with the *Le* gene may be considered to have the phenotype Le(a+b-). Secretors do in fact produce a small amount of Le^a (but in much less quantity than Le^b). The Lewis transferase (FUT-3) and the Secretor transferase (FUT-2) compete for type 1 precursor. Because the Secretor transferase (FUT-2) is generally the more efficient, more H type 1 is made than Le^a in people who possess both *Le* and *Se* genes. H type 1 is then converted to Le^b by the Lewis transferase. Le^a cannot subsequently be converted to Le^b. This gives rise to the red cell phenotype Le(a-b+), as previously discussed. Manufacturers of Lewis antisera deliberately adjust the titre and avidity of these sera, so that the small amount of Le^a produced by secretors, is not detected on red cells (Henry, 2002, personal communication). However, if sensitive techniques are used, for example indirect antiglobulin test with enzyme treated cells, Le^a can be detected with selected anti-Le^a on group O Le(a-b+) cells (Mollison et al, 1997).

The Le(a-b-) red cell phenotype is produced in individuals who are homozygous for the *le* gene. These individuals may be secretors, or non-secretors, of ABH substances, depending on whether or not they possess the *Se* gene.

2.5 The partial secretor phenotype

The 385 A $\rightarrow$T mutation, at nucleotide 385 in the Secretor gene, produces an amino acid change from Ile to Phe. This codes for a FUT-2 transferase which produces the phenotype known as Se^w (Secretor weak; also known as the partial secretor phenotype) (Henry et al, 1989). The enzyme is unstable and thus has a lower activity ouput (Henry, 1996a). The red cells of these individuals give positive reactions with anti-Le^a and anti-Le^b, and so have the phenotype Le(a+b+). Because the fucosyltransferase (FUT-2) is less efficient, the Le transferase (FUT-3) competes more effectively, and forms more Le^a, whilst less Le^b is formed. The Le(a+b+) phenotype is virtually absent in Caucasian adult populations (Mourant et al, 1976). It is however frequent in Polynesians, (Henry et al, 1990), (Henry et al, 1989), Australian Aboriginals, (Vos et al, 1967)

Japanese, (Sturgeon et al, 1970) Chinese, (Lin-Chu et al, 1988) and Blacks (Greenwell et al, 1986). The frequency of the phenotype is often not truly recognised in a population, because its detection when typing red cells with Lewis antisera depends on the potency and brand of antisera used, and the technique by which it is used. Henry estimates that using solely serological methods to phenotype red cells results in at least a 15% anomaly rate; because of the inadequacy of all typing reagents (Henry et al, 1995).
Lin-Chu confirms Henry's findings. In an International workshop study of Chinese people, the author found that the frequency of the Le (a+b+) phenotype varied from 13% to 25%, depending on antisera used (Lin-Chu et al, 1990).

A third set of enzymes also competes in this "soup" for type 1 precursor; a set of branching and elongation enzymes. In secretors, between 1% and 5% of type 1 chains are not fucosylated by FUT-2 or FUT-3, but instead become elongated type 1 molecules, with repeating units of Gal-GlcNac units (Henry, 2003, personal communication). These are subsequently fucosylated (by other fucosyltransferases beyond the scope of this thesis). In non-secretors, up to 40% of chains go into the elongation and branching cycle. In the partial secretor, 10% - 20% of chains go into the elongation and branching cycle (Henry, 2003, personal communication).

At a biological level, the Le(a+b+) phenotype associated with the partial secretor phenotype tends to produce more elongated glycolipids than other Lewis positive phenotypes (Henry et al, 1995). Failure to efficiently fucosylate type 1 precursor allows more molecules into the alternate pathway, where chains elongate and branch. Henry demonstrated a 2.5 fold increase in elongated glycolipids taken from the small intestine of an Le(a+b+) individual, as compared to an Le(a-b+) individual (Henry, 1994). The Lewis negative non-secretor also makes elongated chains of Le^c (type 1). The Lewis positive non-secretor makes elongated chains of Le^a. The Lewis positive partial secretor makes elongated chains of Le^a and Le^b.

2.6 Interactions between ABO, Secretor and Lewis

As can be seen, the transferases of these 3 blood group loci compete for many common precursors. These concepts are illustrated in Figure 7; reproduced with permission (Henry, 1996a).

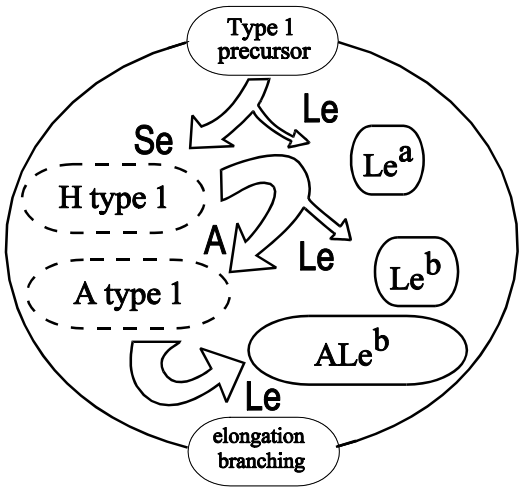

Figure 7. *Lewis biosynthetic pathway***.** This figure demonstrates the interaction of Le, Se, and ABO genes in the secretions of an individual with the genes A, Le and Se. The thickness of the arrows on this figure represents the relative efficiency of the transferase. The individual expresses type 1, H type 1, A type 1, Le^b, ALe^b, and trace amounts of Le^a in her secretions, and on her mucosal surfaces. Note that Le^b is not converted to by ALe^b by the action of GalNAc transferase, as FUT-3 effects chain termination. Therefore ALe^b is formed by the action of FUT-3 on A type 1. Minimal branching occurs in a normal A Le(a-b+) individual. In an A Le (a+b+) individual, the same enzymes are present, but the FUT-2 transferase produced by the Se^w gene is less efficient, and so less H type 1, and in turn, A type 1, and less Le^b, are formed. More branching occurs in the Le(a+b+) individual.

In addition to the structures already discussed, there are 2 further structures to be considered. In group A secretors who possess a functional Lewis gene, A type 1 can be converted to ALe^b. The type 2 equivalent also exists and is known as AY. ALe^b and AY are shown in Figure 8.

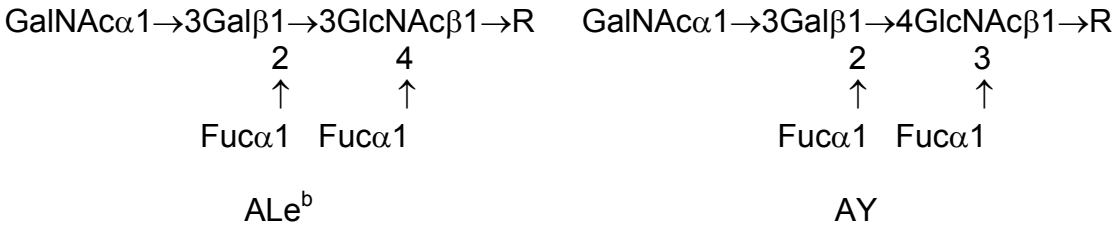

Figure 8. *ALe^b and AY*. The ALe^b molecule is formed in group A secretors who possess a functional Lewis gene. The type 2 equivalent moleucule is known as AY.

In group B secretors who possess a functional Lewis gene, B substance can be converted to BLe^b. The type 2 equivalent also exists and is known as BY. BLe^b and BY are shown in Figure 9.

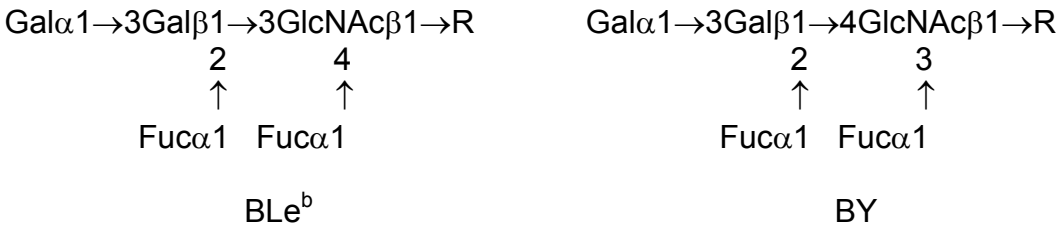

Figure 9. *BLe^b and BY*. The molecule BLe^b is formed in group B secretors who possess a functional Lewis gene. The type 2 equivalent moleucule is known as BY.

Before the A and B transferases can effect the addition of N-acetyl galactosamine (GalNAc) or galactose (Gal) respectively, fucose must be added to terminal Galactose (Gal) by FUT-2, to produce H type 1. This does not occur in individuals who lack the *Se* gene. The addition of the AB immunodominant sugar cannot occur after the molecule is converted into Le^b, i.e. A type 1 is the precursor, not Le^b.

False negative results are often seen in red cell reactions of group A individuals with anti-Le^b sera. This is because the N-acetylgalactosaminyl transferase, which is under control of the A gene, and which adds N-acetylgalactosamine to H type 1 in the secretions, and the Lewis transferase; FUT-3, compete for the same substrate (namely H type 1). The A transferase is more efficient, and produces more A type 1, than the Lewis transferase produces Le^b. The A type 1, once formed, is mostly converted into ALe^b by the Lewis transferase. The smaller amount of Le^b is often insufficient to be detected by some anti-Le^b reagents (Henry, 2002, personal communication). These phenotyping discrepancies are not of great concern in routine blood banks where the main concern is provision of compatible blood, because Lewis antibodies are almost never implicated in haemolytic transfusion reactions (Harmening, 1999). However, the 15% error rate in Lewis typing is of great significance in anthropological work, where blood groups are a valuable tool, and in disease association studies, such as this one.

Table 4 shows the possible interactions of Le, Se and ABH genes, and the resulting antigens expressed in the secretions, and on the mucosal surfaces (Henry, 2003, personal communication). It is important to note that this table does not consider any of the major subgroups of ABO, for example A_1 and A_2,

where the profiles would be quantitatively different. The red cell phenotypes are also listed in each case.

Table 4. *Interactions of ABO, Le and Se genes, and quantitative antigens.* The table was constructed using available and tacit knowledge to compare and contrast relative expression of molecules on the tissues, and in the secretions, of individuals with different ABO, Le and Se genetic profiles.

Genes present	Antigens Secretions and mucosal surfaces	Relative quantity	Red cell phenotype
A, Le, Se	Type 1precursor	(+)	A
	H type 1	(+)	Le(a-b+)
	A type 1	++	
	ALe^{b}	++++	
	Le^{b}	++	
	Le^{a}	+	
B, Le, Se	Type 1	(+)	B
	H type 1	(+)	Le(a-b+)
	B type 1	++	
	BLe^{b}	++++	
	Le^{b}	++	
	Le^{a}	+	
O, Le, Se	Type 1	(+)	O
	H type 1	+	Le(a-b+)
	Le^{b}	++++++	
	Le^{a}	+	
ABO, Le, sese	Type 1	+	ABO Le(a+b-)
	Le^{a}	++++++	
A or *B, lele, Se*	Type 1	(+)	A or B
	H type 1	++	Le(a-b-)
	A or B type 1	+++++	
O, lele, Se	Type 1	(+)	O
	H type 1	+++++++	Le(a-b-)
ABO, lele,sese	Type 1	++++++++	ABO Le(a-b-)

(+) Low or almost negative

As stated in the introduction to this chapter, there are at least 18 different possible carbohydrate profiles that may be expressed. An individual expresses one of these profiles in the mucous tissues, depending on the combination of ABO, Secretor and Lewis genes inherited. These 18 profiles are summarised in Table 5 (Henry, 2001b).

Table 5. *Possible carbohydrate profiles depending on ABH, Lewis and Secretor type.* The numbers 1-18 in the table simply count the number of different glycoconjugate profiles, expected in a random mixed race population.

	Le positive			Le negative		
	Se	Se^w	se	Se	Se^w	se
A	1	5	9	10	14	18
B	2	6	9	11	15	18
O	3	7	9	12	16	18
AB	4	8	9	13	17	18
RBC phenotype	Le(a-b+)	Le(a+b+)	Le(a+b-)	Le(a-b-)	Le(a-b-)	Le(a-b-)

Se= *SeSe* or *Sese* or *SeSe^w*
Se^w= *Se^wSe^w or Se^wse*
Se= *sese*
Le positive = *LeLe* or *Lele*
Le negative = *lele*

2.7 Chapter review

The genes we inherit at the ABO, Lewis and Secretor loci control the glycoconjugate profile of antigens we express in our secretions, and on our mucous membranes. We know that some bacteria are able to use carbohydrates for attachment, thus enabling them to enter the human host cell. The adherence properties of *Neisseria gonorrhoeae* to blood group molecules such as ABO, H, Lewis and Secretor are not well understood, thus they provide a suitable subject for further investigation.

Chapter 3: Materials and Methods

3.1 Introduction

This chapter details all methods used in the research, including ethical aspects, design of the study, technical methods, and statistical methods used to analyse the data.

3.2 Study Design

The study was designed in collaboration with staff of Auckland Sexual Health Service (ASHS), which is part of Auckland Healthcare. It was planned to recruit 200 individuals with gonorrhoea, (*Neisseria gonorrhoeae* confirmed in microbiological culture and biochemical tests) and 200 individuals without gonorrhoea, (absence of *Neisseria gonorrhoeae* confirmed in microbiological culture and biochemical tests) to participate in the study. The 200 individuals without gonorrhoea were attending ASHS clinics. These individuals were used rather than published data for blood-groups (blood donor studies) because it was believed the control group from the clinic would be a better control of socio-economic and ethnic factors. Low socio-economic status is recognised as a risk factor for infection with gonorrhoea (Matzkin, 1987), and ethnic factors for blood group distribution (Mourant et al, 1976). The gonorrhoea negative cohort self-selected on the basis of exclusion for gonorrhoea positivity, and were therefore not a true randomly selected control group.

Table 6. *The study cohort*. A summary, showing characteristics of the test group, and the control group.

	G+ Group	G- Group
Gonorrhoea status	Positive	Negative
Number	175	211
Age in years	14-60	15-64
Gender; Male	128	151
Gender; Female	44	58
Sexual partners	0 - >10	0 - >10

G+ Gonorrhoea positive
G- Gonorrhoea negative

The study had the ethical approval of the Auckland Ethics Committee; Study 2001/113. Individuals were invited to participate in the study when visiting ASHS at one of the four clinics (Central Auckland, West Auckland, North Auckland and South Auckland). Medical and nursing staff received an initial information session, and then more detailed training from two clinic registrars, who were familiar with all aspects of the study, and who were the key clinical liaison point for the researcher.

Medical and nursing staff invited ASHS clinic attendees to participate by showing them the study information sheet. Participation in the study was completely voluntary, and anonymity was guaranteed by encoding the data. The researcher knew the participants only by a study code number. Participants were also offered a language interpretation service at this time.

If participants agreed to participate, a study pack was opened.
Each study pack contained:

- A coded blood collection tube.
- A coded consent form. Once the participant had read and signed the consent form, it was filed in the participant's notes, at ASHS.
- A coded questionnaire, which collected demographic information about the participant. Once completed, the questionnaire stayed with the blood sample. The questionnaire collected the following information:
    - Age
    - Gender
    - Ethnicity
    - Number of sexual partners in the last 3 months. Where this was more than 10, the number was recorded as "many".

- A coded checklist, which prompted clinic staff through participation procedures. The checklist was retained at ASHS, and provided the only link between the study code and the participant's unique identifying number. ASHS staff used this link to update the database, containing information as to whether or not gonorrhoea was diagnosed.

Condom use was not controlled in this study. If this study were repeated in other populations, it would be helpful to collect information on whether or not condoms are regularly used, as this is a factor that may affect incidence of gonorrhoea.

At this time, participants were shown a master code sheet and asked to self-identify their ethnic group on the questionnaire. Choices were limited to five groups; Maori, Pacific Island, Asian, NZ European and other, including Indian and Melanesian. Before the study began ASHS staff allocated a number between one and five to each ethnic group. Therefore, the researcher knew ethnicity only by a number. It was necessary to control for ethnicity in the study, as different ethnic groups have different blood group gene frequencies, and different rates of infection with gonorrhoea. However, the ethics committee did not wish to negatively label any one ethnic group as a result of the study.

The four clinics also recorded the number of participants who declined to participate. No demographic information was collected on this group (since they had not consented to participate) but merely a count was kept, so that the participation rate in the study might be known. Once the consent form and questionnaire were completed, the participants were sent to the laboratory staff in the clinic to have a blood sample taken. Wherever possible, this was taken at the same venepuncture as for other diagnostic tests, for which the participant was visiting the clinic. One 10 ml tube of blood, anticoagulated with citrate phosphate dextrose (CPD), was required from each participant. The participant was given the information sheet to take home. On the sheet appeared the participant's study code number. The participants had the opportunity to withdraw from the study at any time, by phoning the researcher or clinic and quoting only their study code number.

The samples were sent from the clinic to Microbiology at LabPlus, Auckland Healthcare. Each week, the researcher collected the samples from Microbiology, and took them to the Glycoscience Research Centre (GRC) at Auckland University of Technology for processing.

3.3 Technical Methods

Blood samples were centrifuged at 1000 g for 20 minutes to separate plasma, white blood cells, and red cells. ABO and Lewis phenotyping, and DNA extraction was performed on the centrifuged sample.

3.3.1 ABO typing

Cell and plasma blood grouping was performed in tubes with commercial antisera and red cells, by a standard accepted saline agglutination technique. Five percent suspensions of participant red cells were prepared in 0.9% saline, and tested against monoclonal anti-A (CSL 25202), and anti-B (CSL 25302). Plasma from each participant was tested against 5% suspensions of group A and B red cells from New Zealand blood donors. Reactions were left to sediment for 1 hour at room temperature, and then macroscopically examined for agglutination through a 10 x magnification eyepiece. Blood groups were assigned on the basis of known agglutination patterns, as shown in Table 7.

Table 7. *Interpretation of ABO phenotypes*. Both forward (reactions of study red cells with commercial antisera) and reverse (reactions of study plasma with donor red cells) typings were performed to assign ABO blood groups.

Red cells		Plasma		ABO Group
Anti-A	Anti-B	A cells	B cells	
+	-	-	+	A
-	+	+	-	B
+	+	-	-	AB
-	-	+	+	O

3.3.2 Lewis typing

Serological blood grouping was performed in tubes with commercial antisera and red cells, by a standard accepted saline agglutination technique. Five percent suspensions of participant red cells were prepared in 0.9% saline, and tested against monoclonal anti-Le^a (Ortho LAB104A), and anti-Le^b (Ortho LAB204A). The package inserts for these antisera recommend that a washed suspension of red cells is used. However, due to the relatively high-risk of blood borne infection transmission with the population of this study, it was decided to

use unwashed cell suspensions. This was considered to be justified, because of the recognized error rate of Lewis phenotyping, and the fact that this study includes Lewis genotyping. It is recognized there may be some false negative results in the phenotyping, due to neutralisation of the antiserum by Lewis substances present in plasma traces on unwashed red cells. Blood groups were assigned on the basis of known agglutination patterns, as shown in Table 8.

Table 8. *Interpretation of Lewis phenotypes.* The Lewis phenotypes were assigned, based on reaction of red cells with anti-Le^a and anti-Le^b.

anti-Le^a	anti-Le^b	Lewis phenotype
+	-	Le(a+b-)
-	+	Le(a-b+)
-	-	Le(a-b-)
+	+	Le(a+b+)

3.3.3 Genotyping

Secretor phenotyping was not undertaken because this would have required saliva from the study participants, and saliva is not routinely collected at ASHS. In addition, determination of secretor phenotype using saliva is unreliable (Henry et al, 1995). Consequently, all samples had a Secretor genotype performed. The techniques are discussed in sections 3.3.5, and 3.3.6. In addition to serological Lewis typing of red cells, each participant whose Lewis phenotype showed the reaction Le(a-b-), was genotyped. This strategy ensured that the presence of the Lewis gene was not missed due to inadequate Lewis typing sera, or due to not having washed the red cells.

3.3.4 DNA Extraction Method

Genomic DNA can be extracted from any nucleated cell. White blood cells were obtained after ABO and Lewis typing. DNA was extracted and stored on each participant specimen. The method used was a Proteinase K digestion/salting out method, with subsequent DNA precipitation in ethanol (Miller et al, 1988).

- The buffy coat was removed from the separated blood sample to a sterile 15 ml plastic tube.
- Thirteen ml of 1x Red Cell Lysis Buffer (RCLB) was added. The RCLB aims to lyse the red blood cells, whilst leaving the white blood cells intact. 1x RCLB was prepared by diluting 100 ml of 5 x RCLB with 400 ml millipore water. 5 x RCLB was prepared with saccharose (274 g), triton X-100 (25 ml), 1 M tris-HCl pH 7.5 (30 ml), and magnesium chloride $MgCl_2.6H_2O$ (2.54 g), in a total volume of 500 ml of millipore water. Both the 1 x and the 5 x RCLB solutions were stored at 4°C.
- After 2-3 manual inversions of the tube to mix the blood with the RCLB, tubes were centrifuged at 1000 g for 20 minutes. The lysed red cells were manually decanted away, leaving the white cell pellet.
- A further 7 ml of RCLB was added to wash the white cell pellet. Tubes were again centrifuged at 1000 g for 20 minutes.
- The lysate was again manually decanted away, leaving the white cell pellet.
- To each white cell pellet was added;
  160 μl 5 x proteinase K buffer (11 mg calcium chloride, 5 ml 1 M tris-HCl pH 7.5, 15 ml millipore water)
  220 μl millipore water
  160 μl 10 % sodium dodecyl sulphate (Gibco BRL 15553035)
  40 μl of 10 mg/ml proteinase K (Sigma P6556, 90K8604).
  This mixture aims to digest the cellular protein for subsequent removal.
- The mix was incubated in a waterbath at 55° C for 2 hours.
- The tubes were brought to room temperature and 200 μl 6 M sodium chloride solution (35.1 g NaCl in a total volume of 100 ml millipore water) was added. The saturated sodium chloride solution is used to precipitate the digested protein.
- The tubes were shaken vigorously for 15 seconds, to ensure thorough mixing of the solutions.
- The tube contents were transferred to 1.8 ml micro-centrifuge tubes for the remainder of the method.
- The 1.8 ml micro-centrifuge tubes were spun at 10,000 g for 10 minutes in a high-speed micro-centrifuge.
- The supernatant was transferred to two clean micro-centrifuge tubes, and then a volume of 99 % ethanol (BDH 15338) was added. This volume was double that of the volume of the supernatant.
- The tubes were gently inverted until DNA precipitated in the ethanol.

- The precipitated DNA was transferred manually with a pipette tip to a clean micro-centrifuge tube containing 1 ml of 70 % ethanol. The purpose of the 70 % ethanol is to wash away any residual salt into the water component.
- The DNA in 70 % ethanol was centrifuged at 10,000 g for 5 minutes.
- The supernatant was decanted, and the tube was left open in a dessicator overnight, to let the residual ethanol evaporate.
- The following morning, a volume of Tris-EDTA (TE) buffer (5 ml 1 M tris-HCl pH 7.5, 1 ml 0.5 M EDTA pH 8.0 in a total volume of 500 ml millipore water) was added. The volume of TE buffer added was dependent on the observed size of the DNA pellet. Quantitation was not performed.
- The DNA dissolved in TE buffer was stored at -20° C until the PCR batch was performed.

3.3.5 Secretor genotyping strategy

The DNA of individuals whose red cell phenotyping result was Le(a-b+) was screened by a PCR assay designed to detect the secretor weak mutation (Svensson et al, 2000). This was to ensure this genotype was not missed with inadequate antisera. The secretor weak mutation consists of an A-T substitution at position 385 of the secretor FUT-2 gene, which codes for an unstable enzyme that produces the partial secretor phenotype. The assay produces a 1033 bp fragment, together with a 659 bp fragment, in wild type secretors. Individuals with the secretor weak mutation show a 427 bp fragment in addition to the 1033 bp fragment (homozygous for the A385T secretor weak mutation) or show a 427 bp fragment in addition to the 1033 bp and the 659 bp fragment (heterozygous for the A385T secretor weak mutation)

In the Le(a-b+) group:

- If the PCR revealed only the 1033 bp and the 659 bp bands, the presence of the secretor weak mutation was eliminated, and the samples were tested no further. These individuals were recorded as “secretors”.
- If the PCR revealed a 427 bp band in the presence of the 1033 bp band, the homozygous presence of the secretor weak mutation was confirmed, and the samples were tested no further. These individuals were recorded as “partial secretors”.

- If the PCR revealed a 427 bp band in the presence of the 1033 bp and the 659 bp band, the heterozygous presence of the secretor weak mutation was confirmed, and the samples were taken on to restriction enzyme digestion, for elucidation of the other allele.

The DNA of individuals whose red cell phenotyping showed Le(a+b-) was screened by the PCR assay, and then subjected to restriction enzyme digestion of the PCR products. This is because the genetic basis of the "non-secretors", particularly those of non-European backgrounds, can be due to any combination of 6 mutations, which produce an inactive *Se* gene. It also checks for the presence of the secretor weak mutation in the presence of a gene producing an inactive enzyme.

The DNA of individuals whose phenotyping showed Le(a-b-) was screened by the PCR assay, and then subjected to restriction enzyme digestion of the PCR products. This is because we cannot determine the secretor status of Le(a-b-) individuals without genetic typing, or secretor phenotyping. The restriction enzyme digestion of the PCR products provides analysis by restriction fragment length polymorphism (RFLP), and allows identification of 6 mutations, in 11 different possible combinations. Table 9 (Svensson et al, 2000) summarizes selected combinations, and the size of the fragments expected in each.

Table 9. *Expected RFLP fragments in combinations of selected Secretor genotypes* (Svensson et al, 2000).

Bands	wild/wild	428/428	wild/428	385/428	571/685	571/385	wild/385	385/385	849/849
456bp					✓				
413bp		✓	✓	✓					
330bp	✓	✓	✓	✓	✓	✓	✓	✓	
283bp	✓		✓	✓	✓	✓	✓	✓	✓
266bp				✓		✓	✓	✓	
200bp		✓	✓	✓					
171bp									✓
159bp									✓
129bp	✓	✓	✓	✓	✓	✓	✓	✓	✓
117bp					✓	✓			
106bp	✓	✓	✓	✓	✓	✓	✓	✓	
70bp	✓		✓	✓	✓	✓	✓		✓
43bp	✓	✓	✓	✓	✓	✓	✓	✓	✓
Phenotype	Se	sese	Se	Se^w	sese	Se^w	Se	Se^w	sese

Se secretor
sese non-secretor
Se^w partial secretor phenotype
wild non-mutated gene

Using this strategy of genotyping, together with phenotyping in samples where interpretative error could arise, allowed us to more accurately assign a "secretor", "non-secretor", or "partial secretor" status to each sample. The strategy is summarised in Table 10.

Table 10. *Secretor genotyping strategy.* The table summarises the strategy used to determine whether or not to proceed to restriction endonuclease digestion of PCR products. Only alleles detected in this study are listed. The table also shows how Secretor status was assigned.

Red cell Phenotype	PCR bands (bp)	Post-restriction Secretor genotype	Secretor status
Le(a-b+)	1033 + 659	Not performed	Secretor
Le(a-b+) Le(a+b-) Le(a-b-)	1033 + 659 + 427	*385/wild* (*Se^{w}/wild*)	Secretor
Le(a-b+) Le(a+b-) Le(a-b-)	1033 + 659 + 427	*385/428* (*Se^{w}/se*)	Partial secretor
Le(a-b+) Le(a+b-) Le(a-b-)	1033 + 427	Not performed	Partial secretor
Le(a+b-) Le(a-b-)	1033 + 659	*428/428* (*se/se*)	Non-secretor
Le(a+b-) Le(a-b-)	1033 + 659	*428/wild* (*se/wild*)	Secretor
Le(a-b-)	1033 + 659	*wild/wild*	Secretor

3.3.6 Secretor genotyping method

The secretor genotyping method was developed by Svensson, Petersson and Henry, (Svensson et al, 2000) and was followed very closely, with minor modifications. It is noted that amplification of the Secretor locus by this method requires a high degree of optimisation. In particular, the magnesium concentration is critical (Svensson et al, 2000). The researcher also found that robust amplification of products was more reliable with 1.5 U of Taq polymerase, rather than 1 U, as stated in the published method. The fact that the DNA was not quantitated may have contributed to initial problems in optimising the assay. Once the method was well optimised, very high throughputs were achieved.

The method was controlled by analysing 8 control DNA samples from a variety of known secretor genotypes. These samples were previously typed by Svensson and Henry (Svensson et al, 2000).

The control genotypes represented were:

wild/wild Secretor

wild/428 Secretor

385/385 Partial secretor

428/428 Non-secretor

wild/385 Secretor

DNA extracted as described was subjected to PCR amplification. The mix was as follows:

- 5 pmol of each of primers:
  9802 s(5'-GCCTTTCTCCTTTCCCATGGCCCAC-3')
  9803 as (3'-CGTGATTACGACCGGGCAGGAAACT-5')
- 0.3 pmol of each of primers:
  II-16 s (5'-ACTGGATGGAGGAGGAATACCGCCACA-3')
  II-9801 as (3'-AAGGGCCCCCTCATGCAGGCGAAGTGG-5')
  Primers were custom made by Invitrogen
- Approximately 0.5 μg of genomic DNA
- 3.75 nmol of each dNTP (Roche 1969064)
- 1.5 Units of Taq polymerase (Qiagen 201203)
- 2.5 μl Qiagen 10 x reaction buffer (Qiagen 201203)
- 25 nmol magnesium chloride (Qiagen 201203). This was additional magnesium chloride, and was found to be critical to the performance of the assay (Svensson et al, 2000)
- 11.3 μl sterile millipore water

The PCR mix was cycled as follows in a total reaction volume of 25 μl in a thermal cycler:

94°C for 120 seconds

5 cycles of:

94°C for 30 seconds
68°C for 15 seconds
72°C for 30 seconds

20 cycles of:

94°C for 30 seconds
64°C for 15 seconds
72°C for 45 seconds

Eight μl of PCR product, and 5 μl of 10 x orange G loading dye (58 mg orange G, 5 g sucrose in a total volume of 10 ml millipore water) were electrophoresed

on a 1.5 % agarose gel (Agarose MS, Roche 1 816 586) at 130 V for 1 hour. 100 bp ladder was used (New England BioLabs N3231S). Bands were visualized and photographed on a UV transilluminator (BioRad GelDoc).

Samples to be taken on to restriction enzyme digestion were treated as follows:

Ten μl of PCR product was mixed together with:

- 1 U of Ava II (Roche 740748)
- 2 U of BstE II (Roche 404233)
- 2 U of Dde I (Roche 835293)
- 2 U of PinA I (Roche 1464841)
- 1.5 μl of SuRE/Cut Roche Buffer A. (the buffer supplied with Ava II. It is important to note that Buffer A provides a suitable environment for the buffers to multiplex in, whereas other commercial buffers may not)

The mix was incubated at 37°C for 90 minutes, followed by a further incubation at 60°C for 90 minutes. A separate thermal cycler was used for this two-step enzyme digestion incubation. 37°C is the optimal cleavage temperature for Ava II and Dde I, and PinA I, and 60°C is optimal for BstE II.

The resulting RFLP products were electrophoresed on a 3 % agarose gel (Agarose MS, Roche 1 816 586) at 70 V for 2 hours. Fifteen μl of RLFP product, and 8 μl of 10 x Orange G loading dye was loaded in each lane. The ladders used were 100 bp ladder (New England BioLabs N3231S) and Marker ∇I (Roche 1062590). The two ladders are necessary to differentiate between small differences in fragment sizes in the RFLP products. The bands were visualized and photographed on a UV transilluminator (BioRad GelDoc).

3.3.7 Confirmatory work

It was sometimes difficult to distinguish between *428/428* homozygotes (non-secretors), and *wild type/428* heterozygotes (secretors). This is due to the fact that there is only one band on the post restriction-enzyme digestion gel that distinguishes the *428/428* homozygote from the *wild type/428* heterozygote. This is the 283 bp band, which is present in the *wild type/428* heterozygote, but absent in the *428/428* homozygote. The 283 bp band is close to the 330 bp band, and the 330 bp band is present in both the *428/428* homozygote and the *wild type/428* heterozygote. If the post restriction-enzyme digestion gel is not run for long enough, or if the gel conditions are not favourable, it is possible to miss the presence of the 283 bp band. Twenty-one samples of DNA were subjected to a method which aimed only to amplify a single PCR product; the

1033 bp band. This 1033 bp product was then cut with the restriction enzyme Ava II; specific for the *428* mutation. The method was carried out to distinguish between *428* heterozygotes and *428* homozygotes, as the presence or absence of the 283 bp band was sometimes difficult to determine in the full secretor genotyping method. In this method, using only Ava II, two bands are possible. If a single 413 bp band is present, the sample can be determined to be a *428/428* homozygote. If a 130 bp band is present in addition to the 413bp band, the sample is a *wild/428* heterozygote.

Of 21 confirmatory samples, two were selected because they were showing a Lewis red cell phenotype Le(a-b+), which was discrepant with the non-secretor genotype. It was suspected that the presence of the 283 bp band in the original secretor genotyping might have been missed in these two samples.

Nineteen samples with the red cell phenotype Le(a+b-) were selected for the Ava II method to confirm that they were true 428 homozygotes (to check that the presence of the 283 bp band in the original secretor genotyping had not been missed).

The method was as follows:

DNA extracted as described was subjected to PCR amplification. The mix was as follows:

- 5 pmol of each of primers:
  9802s(5'-GCCTTTCTCCTTTCCCATGGCCCAC-3')
  9803as (3'-CGTGATTACGACCGGGCACCAAACT-5')
- 0.5 μg of genomic DNA
- 3.75 nmol of each dNTP (Roche 1969064)
- 1.5 U of Taq polymerase (Qiagen 201203)
- 2.5 μl Qiagen 10 x reaction buffer (Qiagen 201203)
- 25 nmol magnesium chloride (Qiagen 201203)
- 15.2 μl sterile millipore water

The PCR mix was cycled using the same cycle described in 3.3.6. Eight μl of PCR product, and 5 μl of 10 x Orange G loading dye were electrophoresed on a 1.5% agarose gel (Agarose MS, Roche 1816586) at 130 V for 1 hour. The ladder used was 100 bp ladder (New England BioLabs N3231S). The bands were visualized and photographed on a UV transilluminator (BioRad GelDoc).
Samples showing the 1033bp band were digested with:

- 1U of AvaII (Roche 740748)
- 1.5μl of SuRE/Cut Roche Buffer A

The mix was incubated for 90 minutes at 37º C. The resulting products were electrophoresed on a 3% agarose gel (Agarose MS, Roche 1816586) at 70 V for 2 hours. Fifteen μl of RLFP product, and 8 μl of 10 x Orange G loading dye was loaded in each lane. The ladders used were 100bp ladder (New England BioLabs N3231S).

3.3.8 Lewis genotyping

The DNA of individuals whose phenotyping result was Le(a-b-) was screened by polymerase chain reaction with sequence specific primers (PCR-SSP). This assay was used to check Lewis negative status of 32 individuals. The Lewis genotyping method was developed by Grahn (Grahn et al, 2001) and was performed by Svensson in Sweden. DNA was extracted as previously described. Each DNA sample was subjected to PCR amplification with 10 primer mixes. The sequence of the primers is shown in Table 11, and the pairing and concentration of the primers in Table 12. Each mix contained:

- 5 μl of each primer pair (Invitrogen)
- 100 μM dNTP (Roche 1969064)
- 0.25 U of Taq polymerase (Biotaq, Bioline)
- 1.9mM $MgCl_2$
- 0.1% (v/v) Tween 20
- 16.6 mM $(NH_4)_2SO_4$
- 67mM TRIS base pH 8.8
- 0.4 μM sense and antisense primers for amplification of human growth hormone. This served as an internal control, to ensure the PCR had worked, even in the absence of a specific FUT-3 product.
- To each of the 10 mixes was added 0.1 μg of DNA

The PCR mix was cycled as follows in a total reaction volume of 15 μl:

96°C for 60 seconds
5 cycles of:
96°C for 20 seconds
70°C for 45 seconds
72°C for 25 seconds
21 cycles of:
96°C for 25 seconds
65°C for 50 seconds

72°C for 30 seconds

4 cycles of:
96°C for 30 seconds
55°C for 60 seconds
72°C for 90 seconds

Thirteen μl of PCR product, and 2 μl of 10 x Orange G loading dye were electrophoresed on a 1.0 % agarose gel (Agarose MS, Roche 1 816 586) at 130 V for 1 hour. The ladder used was Marker ∇I (Roche 1062590). The bands were visualized and photographed on a UV transilluminator.

Table 11. *FUT-3 sense (s) and antisense (as) primers.* The table shows the nucleotide sequence, and the melting temperature (Tm) of each sense and antisense primer used in Lewis genotyping.

Primer	Position	Mutation	Sequence	Tm (ºC)
III-48s	43-59	59wt	5′-CGCTGTCTGGCCGCACT-3′	58
III-47s	44-60	59T>G	5′-GCTGTCTGGCCGCACgG-3′	60
III-50s	185-203	202wt	5′-CCCTCCTGATCCTGCTATG-3′	60
III-49s	184-202	202T>C	5′-ACCCTCCTGATCCTGCTAc-3′	60
III-52s	297-315	314wt	5′-GTACCCACAGGCAGACACG-3′	60
III-51s	296-314	314C>T	5′-TGTACCCACAGGCAGACAt-3′	58
III-56s	490-508	508wt	5′-GACATCTTCACGCCCTACG-3′	60
III-57s	490-508	508G>A	5′-GACATCTTCACGCCCTACa-3′	58
III-54s	1050-1067	1067wt	5′-CCAGACGGTGCGCAGCAT-3′	60
III-53s	1050-1067	1067T>A	5′-CCAGACGGTGCGCAGCAa-3′	60
III-55as	1212-1229	wt	5′-TTCTGGAGGGGAGAGGCT-3′	58

Table 12. *PCR-SSP FUT-3 primer pairs and PCR product sizes.* For PCR-SSP Lewis genotyping, each one of the sense primers (s) is used with the same antisense (as) primer III-55as. The table shows the pairing of the primers to amplify either the wild type (wt) allele, or the mutated allele, together with the concentration of each primer. The expected size of the PCR product in base pairs (bp) is shown for each amplified allele.

Mix	Allele	Sense primer	Conc (μM)	Antisense primer	Conc (μM)	Product size (bp)
1	59wt	III-48s	0.5	III-55as	0.5	1186
2	59T>G	III-47s	0.5	III-55as	0.5	1185
3	202wt	III-50s	0.5	III-55as	0.5	1045
4	202T>C	III-49s	0.5	III-55as	0.5	1044
5	314wt	III-52s	0.3	III-55as	0.3	932
6	314C>T	III-51s	0.5	III-55as	0.5	933
7	508wt	III-56s	1.6	III-55as	1.6	740
8	508G>A	III-57s	1.6	III-55as	1.6	740
9	1067wt	III-54s	0.5	III-55as	0.5	180
10	508G>A	III-53s	0.5	III-55as	0.5	180

3.4 Statistical Methods

All phenotyping and genotyping results were recorded in an Excel spreadsheet. For statistical analysis, data from the Excel spreadsheet was transferred into a Statistical Package for the Social Sciences (SPSS) workbook. SPSS was used to generate counts and graphs of demographic data sets, Chi-Square tests and p values, to compare the significance of incidence of blood groups in the gonorrhoea positive group, with incidence of blood groups in the control group. Neil Binnie and Stuart Young, Department of Applied Mathematics, Auckland University of Technology reviewed procedures for statistical analysis.

3.5 Chapter review

The study was designed in conjunction with ASHS, and has the ethical approval of the Auckland ethics committee. All technical methods were adapted from published methods, and included red cell phenotyping with commercial reagents and DNA genotyping of Secretor and Lewis.

Chapter 4: Results

4.1 Overview

The results of the study are presented in the following groupings:

- Demographic:
  - age
  - gender
  - number of sexual partners
  - ethnicity

- Sexual health results:
  - gonorrhoea status
  - complicated/uncomplicated gonorrhoea status
  - other sexually transmitted infections

- Blood grouping results:
  - ABO phenotyping
  - Secretor genotyping
  - Lewis phenotyping
  - Lewis genotyping
  - control results
  - discrepant results
  - confirmatory analyses

- Statistical analysis:
  - summary of statistical analysis, without reference to blood grouping paradigms
  - statistical analysis to test hypotheses, on a framework of accepted blood grouping paradigms

4.2 Demographic Results

As stated in section 3.2, it was planned to recruit 200 individuals with gonorrhoea, (*Neisseria gonorrhoeae* confirmed in microbiological culture and biochemical tests) and 200 individuals without gonorrhoea, (absence of *Neisseria gonorrhoeae* confirmed in microbiological culture and biochemical tests). In fact, 175 gonorrhoea positive (G+) individuals, and 211 gonorrhoea negative (G-) individuals were recruited. The G- cohort self-selected on the basis of exclusion for gonorrhoea positivity, and were therefore not a true randomly selected control group.

Three hundred and ninety-eight individuals attending the ASHS clinics participated in the study. Forty-five individuals were approached, but declined to take part in the study. One individual withdrew from the study. Twelve

individuals were of unknown gonorrhoea status, and were removed from the study, leaving a total of 386 samples.

4.2.1 Age

The gonorrhoea positive group (G+) and the gonorrhoea negative group (G-) were well matched for age distribution, which showed no statistically significant difference (p=0.087). This data is presented in Table 13.

Table 13. *Age of study participants.* The age of participants ranged from 14 to 64 years. 336 participants stated their age; 50 did not.

		Age						
	Total	10-20	21-30	31-40	41-50	51-60	61-70	Not stated
G+	175	33 (19%)	60 (35%)	34 (19%)	10 (6%)	4 (2%)	2 (1%)	32 (18%)
G-	211	21 (10%)	108 (51%)	50 (24%)	12 (6%)	1 (0.5%)	1 (0,5%)	18 (8%)

This study found that 54% (n = 93) of G+ participants were less than 31 years of age, and 19% (n = 33) of G+ participants were in the 10-20 year age bracket. These findings support national distribution figures (ESR, 2001; Turley et al, 2000) where 69% of gonorrhoea cases were found at Sexual Health Clinics in attendees under 25 years of age.

Thirteen percent (n = 50) of study participants did not state their age. The researcher speculates that this group is probably made up of some older people who did not want to reveal their age, and some very young people, who did not want to reveal their age in relation to a study of sexually transmitted infections.

4.2.2 Gender

The number of males participating in the study was almost three times as high as the number of females. This aberration is thought to be due to targetted patient recruitment practices by the male clinic. It is recognized that this may have the potential to bias the study. However, there is no statistically significant difference between the preponderance of males in the G+ group, and the G- group (p = 0.634). Selected statistical analyses were performed on males only, on the basis that glycosylation may be different between males and females. No

statistically significant differences were observed between males and females combined, and males only (data not shown).

Table 14. *Gender of study participants.*

		Gender		
	Total	Male	Female	Not stated
G+	175	128 (73%)	44 (25%)	3 (2%)
G-	211	151 (72%)	58 (27%)	2 (1%)

4.2.3 Number of sexual partners

The number of sexual partners encountered by study participants in the three months prior to participating in the study ranged from zero to many, where many is defined as more than ten. Two percent (n=9) of study participants declined to answer the question on the questionnaire on number of sexual partners. The distribution of number of sexual partners in the G+ group and the G- group is shown in Table 15.

Table 15. *Number of sexual partners.*

	Gonorrhoea status		
	G+	G-	
Sexual partners			Total
0	1 (0.5%)	14 (7%)	15
1	58 (34%)	90 (43%)	148
2	45 (26.5%)	45 (22%)	90
3-5	37 (22%)	26 (12%)	63
6-9	6 (3.5%)	6 (3%)	12
≥ 10	23 (13.5%)	26 (13%)	49
Total	170	207	377

There are more participants in the G- group (50%) with zero or one sexual partner, than participants in the G+ group (34.5%) with zero or one sexual

partner. There are more participants in the G+ group (39%) with three or more sexual partners, than participants in the G- group (28%) with three or more sexual partners. This is statistically significant ($p = 0.001$), showing that the G- group was not perfectly matched in number of sexual partners. Ideally, the G- group should have been matched with the G+ group, since then the study group and the control group would have been exposed to the same risk of catching gonorrhoea, and blood groups would have been the only variable.

4.2.4 Ethnicity

Ethnicity was known only by a pre-assigned code number. The code assigned to a particular group was determined by ASHS, and used consistently throughout the study. Participants self identified themselves as belonging to a coded ethnic group. The numbers of participants in the five coded groups is shown in Table 16. Not unexpectedly, there is not an even distribution of ethnic groups represented in the study. This simply reflects differences in numbers of ethnic groups represented in New Zealand society. Two percent (n=7) of participants reported a mixed group and one percent (n=5) did not supply an ethnicity. These participants were not used for statistical analysis requiring ethnicity.

Table 16. *Ethnicity of study participants.*

		Ethnic group						
	Total	1	2	3	4	5	Mixed	Not stated
G+	175	28 (16%)	80 (46%)	6 (3%)	44 (25%)	9 (5%)	5 (3%)	3 (2%)
G-	211	12 (6%)	146 (69%)	22 (10%)	18 (9%)	9 (4%)	2 (1%)	2 (1%)

Statistical tests were applied to test whether or not the G- group was well matched to the G+ group in terms of ethnicity. There was a significant statistical difference between ethnic distributions in the G+ group, as compared to the G- group ($p < 0.001$), showing that the G- group was not perfectly matched for ethnicity. In ethnic groups 1 and 4, the number of G+ samples is greater than G- samples. In ethnic groups 2 and 3, the number of G- samples is greater than G+ samples (Figure 10). The statistically significant differences in ethnicity and

gonorrhoea positivity were expected, as it is known that gonorrhoea is more prevalent in some ethnic groups (ESR, 2001; Turley et al, 2000). Ideally, the G- group should have been matched, since the G+ group and the G- group would have been matched for blood group distribution. The ethnic distribution in the G- group, as compared to the G+ cohort, is illustrated in Figure 10.

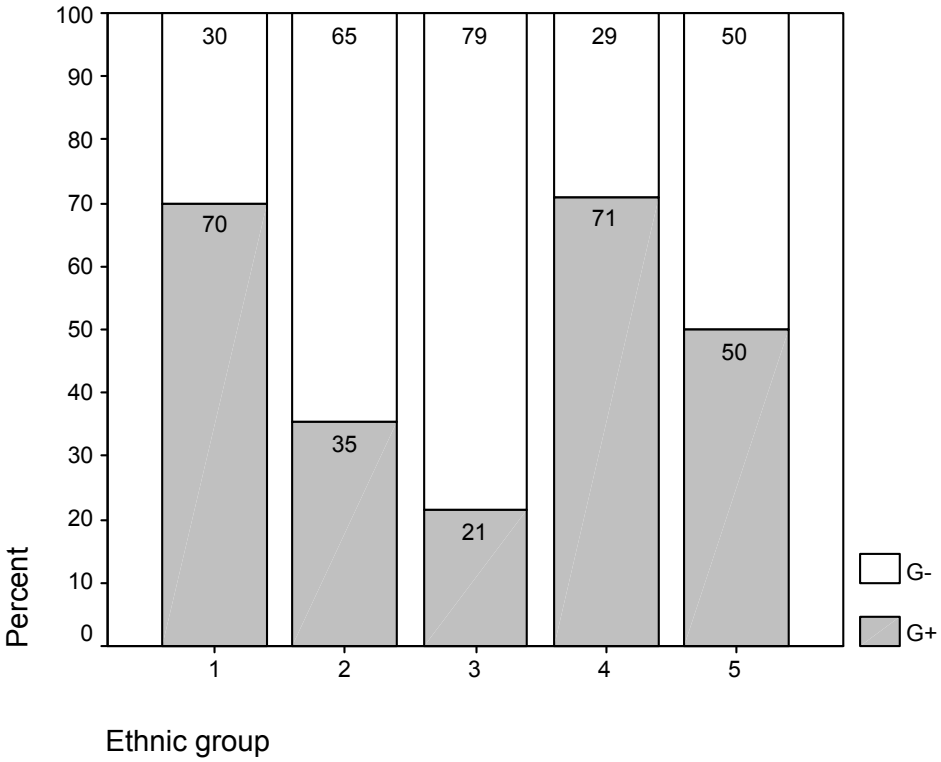

Figure 10. *Ethnicity in the G+ cohort and the G- cohort*. In ethnic groups 1 and 4, the number of gonorrhoea positive samples is greater than gonorrhoea negative samples. In ethnic groups 2 and 3, the number of gonorrhoea negative samples is greater than gonorrhoea positive samples. In ethnic group 5, there are an equal number of gonorrhoea positive, and gonorrhoea negative samples.

4.3 Sexual Health Results

4.3.1 Gonorrhoea results

G+ is defined as presence of *Neisseria gonorrhoeae* confirmed by microbiological culture and biochemical tests. G- is defined as confirmed absence of *Neisseria gonorrhoeae* in culture and biochemical tests. The number and percentage of G+ and G- individuals is shown in Table 17.

Table 17. *Gonorrhoea status of study participants where status was determined*.

Gonorrhoea	Positive	Negative
n	175	211
%	45.0	55.0

There were 12 samples where the gonorrhoea status was “not known”, because the numbered checklist was lost at the ASHS clinic, or the participant’s unique identifying number was not attached to the checklist, so that there was no link. These samples were discarded, and not included in the study.

The gonorrhoea status results cannot be used to comment on infection rates within Auckland, as there was a deliberate attempt to collect 200 gonorrhoea positive individuals and 200 gonorrhoea negative individuals for this study.

4.3.2 Complicated / uncomplicated gonorrhoea

The number and percentage of gonorrhoea positive individuals (G+) having complicated infection is shown in Table 18. Complicated infection is defined as confirmed presence of *Neisseria gonorrhoeae*, together with clinical PID, epididymo-orchitis, or DGI. It would have been of interest to look at the “complicated” infection subset of results, to see if this was associated with a blood group, as it is not clear why some individuals proceed to complications, and some do not. However, since the number was so small (n=4) it was not possible to perform statistical analysis.

Table 18. *Complicated gonorrhoea infection amongst G+ participants.* Complicated infection is defined as confirmed presence of *Neisseria gonorrhoeae*, together with clinical PID, epididymo-orchitis, or DGI.

	Infection		
	Complicated	Uncomplicated	Not known
n	4	168	3
%	2.3	96	1.7

4.3.3 Other sexually transmitted infections

Table 19 shows the number of sexually transmitted organisms other than *Neisseria gonorrhoeae* in the G+ group and the G- group. There is a statistically significant difference (p=0.005), with the G- group having a lower incidence of any STI, other than non-gonococcal urethritis (NGU). By definition, participants with NGU have had gonorrhoea excluded from diagnosis. Ideally, the G- group should have been matched, since this would be another indicator that the G+ group and the G- group had similar risks of catching an STI, including gonorrhoea, and blood groups would have been the main variable.

Table 19. *The counts of other organisms found in G+, and G- participants.*

Organism	Gonorrhoea status		Total
	Positive	Negative	
C trachomatis	40 (77%)	12 (23%)	52
T vaginalis	3 (75%)	1 (25%)	4
NGU	0	5 (100%)	5
C trachomatis and *T vaginalis*	2 (67%)	1 (33%)	3

NGU= Non-gonococcal urethritis

4.4 Blood grouping results

4.4.1 ABO phenotyping results

The breakdown of ABO blood grouping results for all samples (G+ and G-) is shown in Table 20. The samples which were “not tested” for ABO blood group were not tested because the sample was not collected, or the sample was lost, or the sample was inappropriately stored. These samples were excluded from the study.

Table 20. *ABO phenotyping results*. Percentages are calculated from 384 ABO phenotyped samples (14 not tested).

ABO group	A	B	AB	O
n	149	41	18	176
%	38.8	10.7	4.7	45.8

The breakdown of ABO blood grouping results for all samples (G+ and G-) is compared to Australian Red Cross Blood Service (ARCBS) statistics, and is shown in Figure 11. The blood group percentages in this study are representative of a normal multicultural population (Australian Red Cross Blood Service, 2003). No statistical difference in distribution of ABO blood groups between this study and ARCBS is observed (p=0.92). ARCBS statistics were chosen for comparison, because recent New Zealand statistics are not available, either from sexual health clinics, or from blood donor services. Australian blood donors do not represent an ideally matched group for comparison, however they do represent multiple ethnicities, and are therefore a reasonable comparison group for this study.

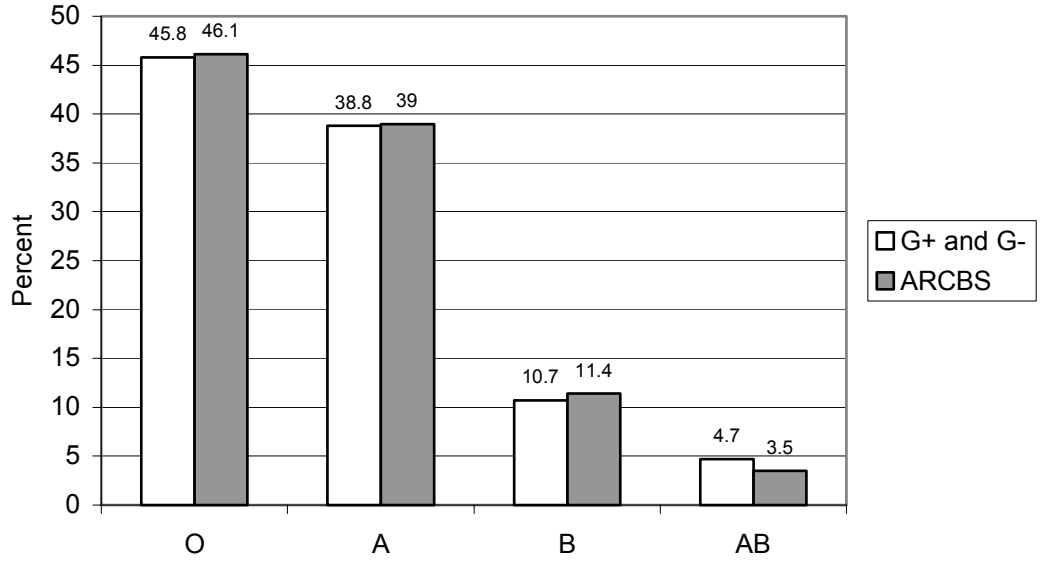

Figure 11. *ABO phenotyping results.* The study ABO percentages are compared with Australian Red Cross Blood Service statistics. No statistical difference in distribution is observed (p=0.92).

ABO status versus gonorrhoea

The difference in ABO blood group distribution between the G+ cohort and the G- cohort was statistically analysed. There is no statistically significant difference between the ABO blood groups alone and gonorrhoea (p=0.952). Results are shown in Table 21.

Table 21. *Comparison of gonorrhoea status by ABO blood group*. There is no statistically significant difference between the ABO blood groups alone and gonorrhoea. (p=0.952)

	A	B	AB	O
G+	68	17	7	78
G-	78	23	10	93
$\chi 2$	0.34			
p	0.952			

As blood group B has been reported to be linked with gonorrhoea infection in previous studies (Foster et al, 1976; Miler et al, 1977; Kinane et al, 1983a), the significance of group B alone was examined, and the results are shown in Table 22. As can be seen from the p value, the result was not significant.

Table 22. *Statistical test for group B*. The test compares group B with non group B, in the G+ and the G- cohort.

	B	vs	Non-B
ABO	B		A, AB, O
G+	17		153
G-	23		180
$\chi 2$	0.171		
p	0.679		

B and AB were also considered together, since the B antigen is expressed in both. The results are shown in Table 23 and once again do not reach statistical significance (p=0.581).

Table 23. *Statistical test for group B and AB*. The test compares group B and AB with group O and A, in the G+ and the G- cohort.

	B and AB	vs	A and O
ABO	B and AB		A and O
G+	24		146
G-	33		171
$\chi 2$	0.304		
p	0.581		

4.4.2 Secretor genotyping results

Results presented in section 4.4.2 represent a sub-section of the 386 total samples. Complete results are available on 306 samples. This was due to failure to extract (n=53) DNA, or failure to amplify DNA (n=27). Sixty-one percent of participants (combined G+ and G-) were found to possess a wild-type secretor gene, either in heterozygous or homozygous form, and were designated “secretors”. Nineteen percent of participants (combined G+ and G-) were homozygous for a gene coding for a non-functional transferase, and were designated “non-secretors”. Twenty percent of participants (combined G+ and G-) were either homozygous for the weak secretor gene, or were heterozygous for the weak secretor gene and a gene coding for a non-functional transferase, and were designated “partial secretors” (Figure 12).

The number of secretors reported in this study (61%) is low compared to the percentage of 80% often quoted (Harmening, 1999). However, if one adds the 20% of partial secretors, determined to have the gene *Se*^{w}, to the 61% of secretors, the figure becomes 81%. As Henry points out (Henry et al, 1996a) the partial secretor phenotype is under-recognised in most studies. Genotyping must be performed in order to accurately report secretor frequencies in a population. To our knowledge, this has not often been the case in population studies.

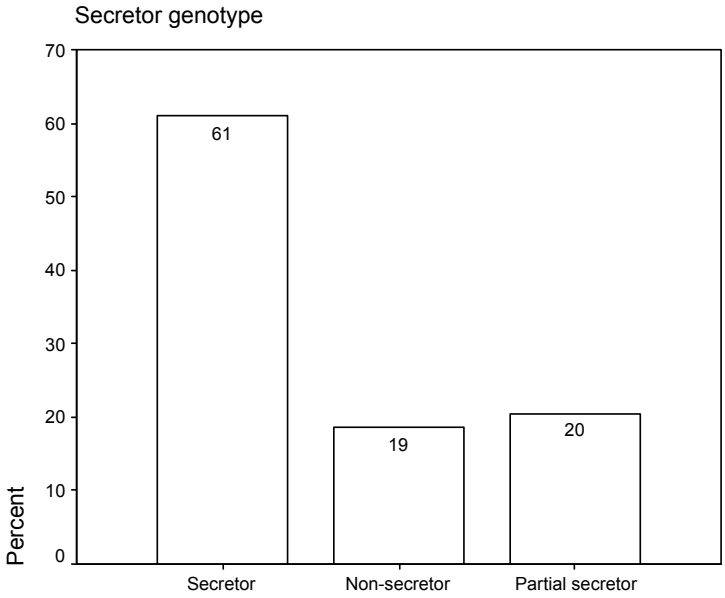

Figure 12. *Secretor genotypes.* 61% of participants were designated “Secretors”. 18.5% of participants were designated “non-secretors”. 20.5% were designated “partial Secretors”.

All the non-secretors were homozygous for the G428A mutation. This is not surprising, given that this is the most common of the non-secretor mutations in Europeans. As shown in Figure 13, ethnic group 2 accounts for the majority of non-secretors, and the remaining ethnic groups had a very low incidence of non-secretors.

Secretor status versus ethnicity

The distribution of Secretor genotypes in the five ethnic groups was statistically analysed. The results are illustrated in Figure 13. The results show a statistically significant difference ($p<0.001$). Ethnic groups 1 and 3 show approximately equal numbers of secretors and partial secretors, and very few non-secretors. In ethnic group 2, there are many more secretors than non-secretors, and very few partial secretors. In ethnic group 4, there are more partial secretors than secretors, and very few non-secretors. In ethnic group 5, there are only small numbers, but there are more secretors than non-secretors, and very few partial secretors. Ethnic group 1 shows that 50% of participants (both G+ and G-) are partial secretors. This is higher than previously reported, where incidence of the Le(a+b+) red cell phenotype (without secretor genotyping) reported the incidence of partial secretor at an average of 20.9% in this ethnic group (Henry, et al 1988). Ethnic group 3 shows that 48% of participants (both G+ and G-) are partial secretors. This is higher than previously reported, where incidence of the

Le(a+b+) red cell phenotype (without secretor genotyping) reported the incidence of partial secretor at 25% in this ethnic group (Broadberry et al, 1991). Ethnic group 4 shows that 58% of participants (both G+ and G-) are partial secretors. This is higher than previously reported, where incidence of the Le(a+b+) red cell phenotype (without secretor genotyping) reported the incidence of partial secretor at 15.9% in this ethnic group (Henry et al, 1988). The higher incidence of partial secretors in this study almost certainly reflects the use of secretor genotyping, as compared to Lewis red cell phenotyping in the reference studies.

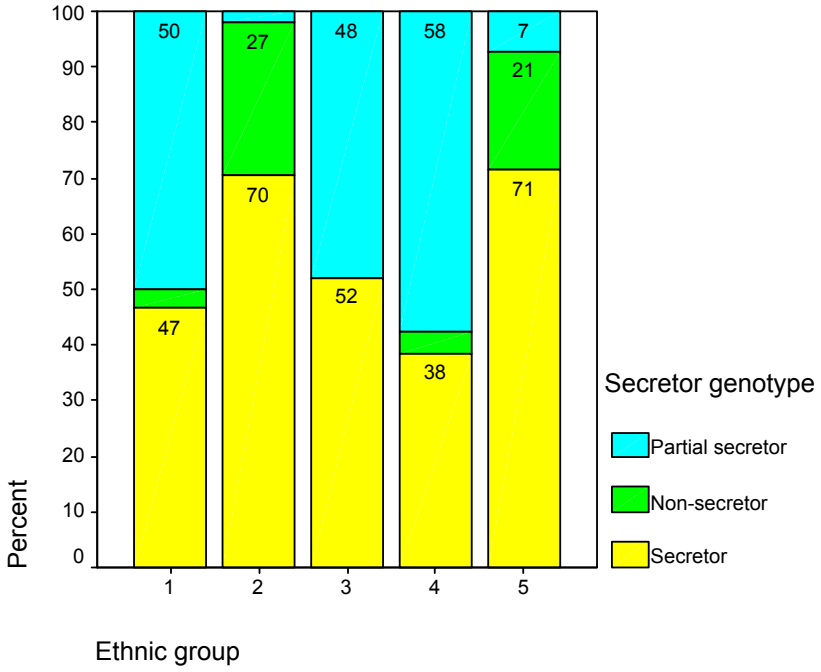

Figure 13. C*omparison of secretor genotype by ethnicity.* The results show a statistically significant difference (p<0.001).

These results suggest that the secretor data cannot be tested in a combined analysis, rather individual ethnic groupings must be separately considered. Ethnic groups 1 and 4 were combined, because both possess a high incidence of partial secretor phenotype and a high incidence of gonorrhoea (see later section 4.5).

4.4.3 Confirmatory results

Twenty-one apparent 428/428 homozygotes (non-secretors) were re-tested by the method outlined in section 3.3.7, which amplified a single PCR product, the 1033 bp band. This product was cut with the restriction enzyme Ava II; specific for the 428 mutation cut. Of these 21 samples, 20 gave a single 413 bp product,

and were confirmed as 428 homozygotes. One gave the 130bp product in addition to the 413bp product, and was therefore a wild-type/428 heterozygote. This sample had originally been called a 428 homozygote, because the 283bp band had not been detected. This sample was re-classified as a “secretor”.

Secretor status versus gonorrhoea

The relationship between secretor status in the G+ cohort and the G- cohort was statistically analysed. The results are presented in Table 24, and Figure 14, and show a statistically significant difference (p=0.05). Fifty-six percent of partial secretors are G+ and forty-four percent of partial secretors are G-. Thirty-eight percent of non-secretors are G+ and sixty-three percent of non-secretors are G-

Table 24. *Secretor genotypes.* These were assigned from the PCR and restriction endonuclease digestion genotyping, by the methods described in section 3.3.5 and 3.3.6. The difference between Secretor genotype and gonorrhoea status is statistically significant (p=0.05).

	Genotype		
Total	Secretor *SeSe* *SeSe*^{w} *Sese*	non-secretor *sese*	partial Secretor *Se*^{w}*Se*^{w} *Se*^{w}*se*
G+	75 (57%)	21 (16%)	35 (27%)
G-	113 (65%)	35 (20%)	27 (15%)

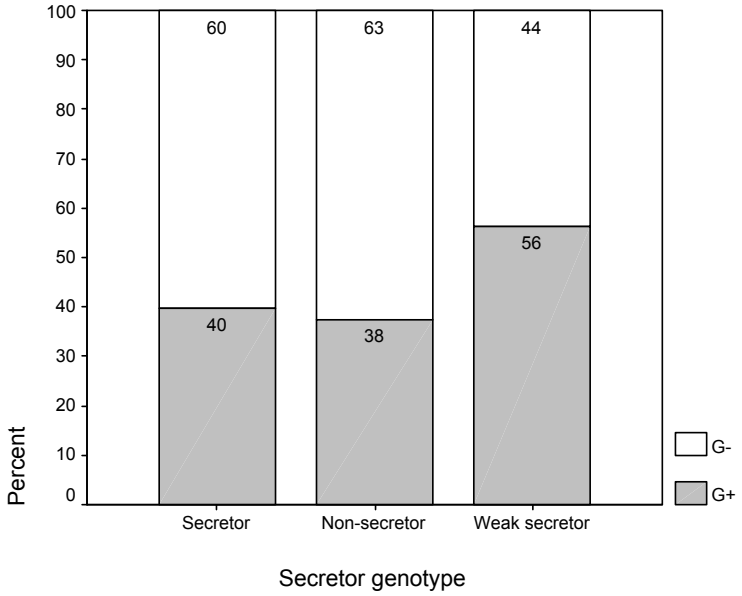

Figure 14. *Comparison of gonorrhoea status by secretor genotype.* The number of weak secretors is greater in the G+ group, and the number of secretors and non-secretors is greater in the G- group. This result is statistically significant (p=0.05).

4.4.4 Lewis results

Corrected red cell Lewis phenotypes were assigned on 306 samples with both secretor and Lewis genotypes available. These phenotypes were corrected on the basis of Lewis and secretor genotyping. A status of Lewis positive or Lewis negative was assigned on 378 samples, in which a Lewis red cell phenotype, and/or a Lewis genotype is available. These figures are shown in Table 25.

Table 25. *Lewis results.* These results represent the final Lewis types assigned on the basis of Lewis and secretor genotyping. Corrections based on genotyping have been made to data previously assigned by phenotyping (uncorrected data not shown).

	Corrected redcell phenotype					Lewis		
Phenotype	Le(a-b+)	Le(a+b+)	Le(a+b-)	Le(a-b-)	Total	Pos	Neg	Total
n	183	59	43	21	306	351	27	378
%	59.8	19.3	14.1	6.9		93	7	

Ninety three percent of individuals in the study were Lewis phenotype positive. This is as expected, since 94% of Europeans have the *Le* gene (Harmening, 1999).

Lewis genotyping results

Thirty-six samples yielded the Lewis red cell phenotyping result Le(a-b-), suggesting they were Lewis negative. Thirty-two of these samples were suitable to be sent to Sweden for genotyping, and were tested in Sweden by the method described in 3.3.8. Of the 32 Le(a-b-) samples subjected to Lewis genotyping, one sample could not be genotyped; despite repeated attempts, the DNA failed to amplify. Results of this sample are not shown in Table 26. Results are available on 31 Le(a-b-) samples and are shown in Table 26. Seven of the Lewis red cell phenotyping Le(a-b-) samples were genetically Lewis positive.

These seven were shown to possess two wild type Lewis alleles, coding for an active α-4-L fucosyltransferase (FUT-3). These samples yielded a Lewis negative red cell phenotype, but were genetically Lewis positive. This was not an unexpected result, especially as the red cells were not washed prior to typing with anti-Le^a and anti-Le^b. Lewis substances present in plasma traces on unwashed red cells may have neutralized the antisera. In addition, it is well recognized that anti-Le^b may give false negative reactions in group A, B or AB, Le^b positive individuals, due to the competition of the A and B glycosyltransferases with the Lewis transferase (Henry et al, 1995). Five of the seven samples which yielded the Le(a-b-) red cell phenotype, but were genetically Lewis positive, were group A (n=2), B (n=1), or AB (n=2). The remaining two samples were group O. The false negative red cell phenotype in these two samples could have arisen from neutralizing Lewis substances in the plasma surrounding the unwashed red cells.

A further seven genetically Lewis negative samples were apparently heterozygous for the C314T mutation, and homozygous for the T202C mutation, apparently having the genotype T202C;C314T/T202C. It is unusual to see the 202 mutation without the 314 mutation (Orntoft et al, 1996). It was considered that this could be a new mutation. However, this seems unlikely, given that seven samples showed this pattern. A more likely explanation is that these samples actually have the genotype T202C;C314T/T202C;C314T, and that they are le5 homozygotes (Oriol, 2002; Oriol et al, 2003). Svensson noted on testing that the 314 wild type primer showed only a very weak band in these seven samples. The wild-type band is therefore a possible contaminant.

Sample 157, which shows the result “see text” in the Lewis genotype column of Table 26, requires discussion. Firstly, the DNA showed weak amplification, and

so was difficult to interpret. Secondly, the result highlights a limitation of Grahn's method for determining the mutations of FUT-3. The sample is heterozygous for T59G, G508A, and T1067A mutations. The most likely combination of mutations is T59G and G508A on one allele, (inactivating) and T1067A mutation on the other allele (also inactivating) (Oriol, 2002). This would represent a Lewis negative genotype. However, it is also possible that the three mutations are on one allele (as proven in sample 317 where the T59G, and the G508A mutations are homozygous and occur on both alleles). If this is the case, then the second, wild-type allele, would mean that the individual is Lewis positive. The Lewis genotype of this individual cannot be interpreted.

One further sample (317) is of particular interest and may possess a new allele. It is homozygous for the T59G mutation (GG) and homozygous for the G508A mutation (AA). In addition, it is heterozygous for the T1067A mutation, and therefore apparently has 3 mutations on one allele; 59/508/1067. This has not been previously described (Oriol, 2002). This finding was in an individual participant from ethnic group 4. The mutations of α-4-L fucosyltransferase (FUT-3) in ethnic group 4 have not been studied extensively, and it is possible that this represents a new allele. However, the remote possibility that the T1067A mutation comes from contaminant DNA cannot be ruled out, and this sample would need to be tested again to confirm the finding. Any work done outside the scope of this study would require independent ethical approval.

Table 26. *Lewis genotyping results*. The results are assigned from the PCR-SSP assay described in section 3.3.8. Shaded areas indicated mutated nucleotides. *Sample 157 cannot be designated either Lewis positive, or Lewis negative, due to a limitation of the method.

	Lewis (FUT3) Mutation					
Code	T59G	T202C	C314T	G508A	T1067A	Lewis genotype
026	TG	TC	CT	GG	TA	Negative
034	TG	TC	CT	GG	TA	Negative
044	TG	TC	CT	GG	TA	Negative
255	TG	TC	CT	GG	TA	Negative
264	TG	TC	CT	GG	TA	Negative
367	TG	TC	CT	GG	TA	Negative
155	TG	CC	CT	GG	TA	Negative
099	TT	CC	CT	GG	TT	Negative
124	TT	CC	CT	GG	TT	Negative
133	TT	CC	CT	GG	TT	Negative
160	TT	CC	CT	GG	TT	Negative
338	TT	CC	CT	GG	TT	Negative
374	TT	CC	CT	GG	TT	Negative
379	TT	CC	CT	GG	TT	Negative
185	GG	TT	CC	GG	AA	Negative
187	GG	TT	CC	GG	AA	Negative
342	GG	TT	CC	GG	AA	Negative
376	GG	TT	CC	GA	TA	Negative
393	GG	TT	CC	GA	TA	Negative
317	GG	TT	CC	AA	TA	Negative
256	TG	TC	CT	GA	TT	Negative
420	TG	TC	CT	GA	TT	Negative
132	TG	TT	CC	GG	TA	Positive
270	TG	TT	CC	GG	TA	Positive
012	TG	TT	CC	GA	TT	Positive
234	TG	TT	CC	GA	TT	Positive
003	TT	TT	CC	GG	TT	Positive
400	TT	TC	CT	GG	TT	Positive
361	TT	TT	CC	GG	TT	Positive
157	TG	TT	CC	GA	TA	See text*

Fifty-four of 306 samples (17%) had discordancy between Lewis red cell phenotyping and secretor genotyping. This result is concordant with other studies (Henry et al, 1995) and reflects the inadequacy of Lewis antisera. Six different types of discrepancies were observed. The number and definition of each type is presented in Table 27.

Table 27. *Discrepancies in secretor genotyping and Lewis red cell phenotyping.*

	Red cell Lewis phenotype	Secretor genotype	Number	Classification
I.	Le(a+b-)	Secretor	6	Secretor
II.	Le(a-b+)	Partial secretor	26	Partial Secretor
III.	Le(a+b-)	Partial secretor	10	Partial Secretor
IV.	Le(a+b+)	Secretor	7	Secretor
V.	Le(a+b+)	Non-secretor	5	Non-secretor

I. This discrepancy has a serological Lewis phenotype that suggests the individual is a non-secretor, but a genotype which suggests the individual is a secretor. There were six individuals in this category. This can be generally explained by poor performance of anti-Le^b reagents. These samples are thus classified as secretors. Techically, they should be Le(a+b+) but the Se^w genotype was absent, thus we classified them as Le(a-b+). This may be technically incorrect. However, as they are secretors, they are best defined Le(a-b+) rather than Le(a+b-) as this causes the least distortion of data and is probably the correct result.

II. Twenty-six samples had this discrepancy which has a serological Lewis phenotype which suggests the individual is a secretor, but a genotype which suggests the individual is a partial secretor. This has been previously observed. Henry notes " at one infrequent extreme, red cells (of the Le(a+b+) phenotype) appear as Le(a+b+) with a weak Le^a reaction." (Henry et al, 1995). In this case, the Le^a reaction was absent, and more frequently encountered than Henry previously found. Again it reflects performance of the reagents. These samples were classified as partial secretors.

III. Ten samples had a discrepancy with a serological Lewis phenotype that suggests the individual is a non-secretor, but a genotype that suggests the individual is a partial secretor. This discrepancy is well recognized (Henry, et al 1996a). These samples were classified as partial secretors.

IV. Seven samples had a discrepancy with a serological Lewis phenotype that suggests the individual is a partial secretor, but a genotype that suggests the individual is a wild-type secretor. This may have been due to interpreting a negative result with anti-Le^a as a weak positive. Some reactions were difficult to read, when it was difficult to discern between a

weak positive and a negative reaction. Sometimes negative reactions appeared “grainy”. It was not possible to repeat the red cell typing, as red cells were not stored in the study. These samples were classified as secretors.

V. Five samples had a discrepancy with a serological Lewis phenotype that suggests the individual is a partial secretor, but a genotype that suggests the individual is a non-secretor. These five samples had their secretor genotype repeated, and the non-secretor genotype was confirmed in all five. It was not possible to repeat the red cell typing, as red cells were not stored in the study. This discrepancy may be due to interpreting a negative result with anti-Le^b as a weak positive. The samples were classified as non-secretors.

The range of these discrepancies is not unexpected, especially in mixed ethnic populations (Henry et al, 1995). The discrepancies highlight the requirement to perform secretor genotyping, and red cell Lewis phenotyping. Secretor genotyping was used as the definitive result to classify secretor status in this study. Using the genotyping data, the frequencies of the Lewis phenotypes were corrected (Table 25).

Lewis versus gonorrhoea

There was no statistically significant difference between corrected red cell Lewis phenotype, and gonorrhoea status (p=0.150). These results are illustrated in Table 28.

Table 28. *Statistical test of Lewis red cell phenotypes*. The test compares Lewis red cell phenotypes in the G+ and the G- cohort.

	G+	vs	G-
Le(a-b+)	74		109
Le(a+b+)	33		26
Le(a+b-)	16		27
Le(a-b-)	8		13
$\chi 2$	5.31		
p	0.150		

Nor was there any significant difference between Lewis positivity, and gonorrhoea status (p=0.374). The data is presented in Table 29.

Table 29. *Statistical test of Lewis positivity.* The test compares Lewis positive and Lewis negative in the G+ and the G- cohort.

	G+	vs	G-
Le +	161		190
Le -	10		17
$\chi 2$	0.79		
p	0.374		

4.5 Statistical Analysis

4.5.1 First Summary of statistical analysis for blood groups and gonorrhoea status

Fourty-four combinations of the blood group profiles were analyzed to test for statistically significant differences between the G+ group and the G- group. This first set of statistical analysis was performed without bias to any blood grouping paradigm; hence every possible combination was analysed. This set was also performed first without reference to ethnicity; in each grouping all ethnic groups were counted as one. Then the statistical tests were repeated in ethnic group two only, where neither gonorrhoea nor partial secretor phenotype was over-represented, and then in ethnic groups one and four combined, where both gonorrhoea and partial secretor phenotype were over-represented. Any statistic that had less than 5 values in a criterium is marked with an asterisk*, and χ^2 and p values must thus be interpreted with caution. The data are too numerous to reproduce in full here, however, summary Table 30 of the results which are statistically significant at $p<0.05$, is included. There is an apparent statistically significant association between gonorrhoea and partial secretor phenotype when all ethnic groups are combined. However, when ethnic group two (in which the partial secretor phenotype is rare and there are more G- than G+ individuals) is analysed, there is no association. Similarly, when ethnic groups one and four (in which the partial secretor phenotype is common and there are more G+ than G- individuals) are analysed as a single group, there is no association. The 2 x 2 tables for each line of this table are presented in Tables 31, 32, 33, 34 and 35. Ethnic group three is also known to have a high

incidence of the partial secretor phenotype. In ethnic group three, the number of G- individuals exceeded the number of G+ individuals. It therefore would have been helpful in teasing out whether the apparent association between gonorrhoea and the partial secretor phenotype is true, or statistically biased, and not true, to test the statistical strength of the association by examining the same set of statistics in ethnic group three only. Unfortunately, the number of individuals in ethnic group three (n=29) was too small to yield informative statistics.

Table 30. *Summary of* ***statistically significant*** *associations.* In comparison of the G+ group and the G- group, the only significant p values were seen in combinations involving the partial secretor phenotype in the "all ethnic groups" category. Ethnic group 2 was chosen for analysis because neither gonorrhoea nor partial secretor phenotype were over-represented, and ethnic groups 1 and 4 were combined because both gonorrhoea and partial secretor phenotype were over-represented.

	All Ethnic groups		Ethnic group 2		Ethnic groups 1 and 4	
	χ^2	p	χ^2	p	χ^2	p
Se vs sese vs Se^w	6.0	0.05	0.59*	0.75*	1.43*	0.49*
(Se and sese) vs Se^w	5.9	0.02	0.58*	0.45*	0.29	0.59
O Se vs A Se^w	4.9	0.03	2.46*	0.12*	0.14	0.71
sese vs A Se^w	3.9	0.05	1.92*	0.17*	0.41*	0.24*
O Le Se vs A Le Se^w	4.73	0.03	2.51*	0.11*	0.44*	0.51*

Se=Secretor. sese= non-secretor. Se^w = partial Secretor. Le=Lewis positive
* The χ^2 and p value must be interpreted with caution; some cells have a count of less than 5

Table 31 shows the raw data used to generate the p value for the first line of Table 30 above; namely secretors versus non-secretors versus partial secretors. It is important to note that there were only two G+ and two G- partial secretors in this group, therefore the χ^2 and p value cannot be interpreted with confidence. As the *Se^w* gene is unknown in this ethnic group, (Henry et al, 1996a) it is presumed that these four individuals were of mixed ethnicity, but ethnic group two was the group to which the individuals self-identified. It is also important to note that there are only three G+ and no G- non-secretors in ethnic groups one and four, therefore the χ^2 and p value cannot be interpreted with confidence. This finding is consistent with the work of Henry in these ethnic groups (Henry et al, 1995).

Table 31. *2 x 2 table used to generate the p value in Table 30 above.* Numbers are shown in each ethnic grouping for the parameter secretor (Se) vs non-secretor (sese) vs partial secretor (Se^w) in G+ and G- individuals.

	All ethnic groups			Ethnic group 2			Ethnic groups 1 and 4		
	Se	sese	Se^w	Se	sese	Se^w	Se	sese	Se^w
G+	75	21	35	41	16	2*	24	3*	29
G-	113	35	27	88	33	2*	10	0*	14
χ^2	6.01			0.59			1.43		
p	0.05			0.75			0.49		

Se=Secretor. sese= non-secretor. Se^w = partial Secretor. Le=Lewis positive
* The χ^2 and p value must be interpreted with caution; some cells have a count of less than 5

Table 32 shows the raw data used to generate the p value for the second line of Table 30 above, namely (Se and sese) versus Se^w. The same comments about the small numbers of partial secretors in ethnic group two apply.

Table 32. *2 x 2 table used to generate the p value in Table 30 above*. Numbers are shown in each ethnic grouping for the parameter (Se and sese) vs Se^w in G+ and G- individuals.

	All ethnic groups		Ethnic group 2		Ethnic groups 1 and 4	
	Se + sese	Se^w	Se + sese	Se^w	Se + sese	Se^w
G+	96	35	57	2*	27	29
G-	148	27	121	2*	10	14
χ^2	5.91		0.58		0.29	
p	0.02		0.45		0.59	

Table 33 shows the raw data used to generate the p value for the third line of Table 30 above, namely group O partial secretors versus group A partial secretors. The same comments about the small numbers of group A partial secretors in ethnic group two apply. However, there are sufficient numbers in both O partial secretors and A partial secretors in ethnic groups one and four, for a robust χ^2 and p value.

Table 33. *2 x 2 table used to generate the p value in Table 30 above*. Numbers are shown in each ethnic grouping for O secretors (OSe) vs A partial secretors (ASe^w) in G+ and G- individuals.

	All ethnic groups		Ethnic group 2		Ethnic groups 1 and 4	
	OSe	ASe^w	Ose	ASe^w	OSe	ASe^w
G+	34	15	18	1*	13	14
G-	57	9	46	0*	5	7
χ^2	4.91		2.46		0.14	
p	0.03		0.12		0.71	

Table 34 shows the raw data used to generate the p value for the fourth line of Table 30 above, namely all ABO group non-secretors versus A partial secretors. All ABO groups are combined because non-secretors do not express their blood group on the mucosal tissues. The same comments about the small numbers of

group A partial secretors in ethnic group two apply. However, there are sufficient numbers in both O partial secretors and A partial secretors in ethnic groups one and four, for a robust χ^2 and p value.

Table 34. *2 x 2 table used to generate the p value in Table 30 above.* Numbers are shown in each ethnic grouping for all ABO non-secretors (A, B, AB, O sese) vs A partial secretors (ASe^w) in G+ and G- individuals.

	All ethnic groups		Ethnic group 2		Ethnic groups 1 and 4	
	A, B, AB, O sese	ASe^w	A, B, AB, O sese	ASe^w	A, B, AB, O sese	ASe^w
G+	21	15	16	1*	3*	14
G-	34	9	32	0*	0*	7
χ^2	3.98		1.92		1.41	
p	0.05		0.17		0.24	

Table 35 shows the raw data used to generate the p value for the fifth line of Table 30 above, namely group O Lewis positive secretors versus group A Lewis positive partial secretors. The same comments about the small numbers of group A Lewis positive partial secretors in ethnic group two apply. However, there are sufficient numbers in both O Lewis positive partial secretors and A Lewis positive partial secretors in ethnic groups one and four, for a robust χ^2 and p value.

Table 35. *2 x 2 table used to generate the p value in Table 30 above.* Numbers are shown in each ethnic grouping for O Lewis positive secretors (OLeSe) vs A Lewis positive partial secretors ($ALeSe^w$), in G+ and G- individuals.

	All ethnic groups		Ethnic group 2		Ethnic groups 1 and 4	
	OLeSe	$ALeSe^w$	OLeSe	$ALeSe^w$	OLeSe	$ALeSe^w$
G+	34	15	18	1*	13	14
G-	56	9	47	0*	4*	7
χ^2	4.73		2.51		0.44	
p	0.03		0.11		0.51	

4.5.2 Hypotheses to test relationship between blood groups and gonorrhoea status

The following set of hypotheses is designed to examine the statistical results on a framework of knowledge of the blood group systems. The relative expression of each carbohydrate is shown in each of the blood groups examined in the study. This semi-quantitative expression was used a basis for the following five hypotheses.

Table 36. *Comparison of relative expression of ABH, Le^a and Le^b*. The table was constructed using available and tacit knowledge to compare and contrast relative expression of molecules on the tissues, and in the secretions, of individuals with different ABO, Le and Se genetic profiles. See also Table 4.

Blood type	A	B	H	Le^a	Le^b	ALe^b	BLe^b
O Le(a-b+)	-	-	+	+	+++++	-	-
A Le(a-b+)	++	-	(+)	+	++	++++	-
B Le(a-b+)	++	+++	(+)	+	++	-	++++
O Le(a+b+)	-	-	(+)	+++	+++	-	-
A Le(a+b+)	++	-	(+)	+++	+	++	-
B Le(a+b+)	-	++	(+)	+++	+	-	++
ABO Le(a+b-)	-	-	-	++++++	-	-	-
A Le(a-b-) secretor	+++++	-	++	-	-	-	-
B Le(a-b-) secretor	-	+++++	++	-	-	-	-
O Le(a-b-) secretor	-	-	+++++++	-	-	-	-
ABO Le(a-b-) non-secretor	-	-	-	-	-	-	-

Note that red cell phenotypes are shown in each of the following 14 statistical tests of the five hypotheses. This is to ease understanding of the application of the hypotheses. However, secretor genotyping was used as the gold standard method in this study. Therefore, red cell phenotypes have been deduced from secretor genotyping results in this section, and Lewis results corrected to correlate with genotyping.

Hypothesis 1: *That the Le^a antigen is a pre-disposing factor to infection with Neisseria gonorrhoeae.*

Expression of Le^a varies between individuals, depending on the inheritance of the *Le* and the *Se* genes.

- Le^a is strongly expressed in individuals with the red cell phenotype Le(a+b-), who possess the *Le*, but not the *Se* gene.
- Le^a is moderately expressed in individuals with the red cell phenotype Le(a+b+), who possess the *Le* and the *Se^w* genes.
- Le^a is weakly expressed in individuals with the red cell phenotype Le(a-b+), who possess the *Le* and the *Se* genes.
- Le^a is not expressed in individuals with the red cell phenotype Le(a-b-), who do not possess the *Le* gene, whether or not the *Se* gene is present.
- Le^a expression in Le(a+b-) individuals is unaffected by the ABO group.

To test the hypothesis that Le^a is a predisposing factor, it is necessary to examine the incidence of gonorrhoea infection in the following groups;

- Le(a+b-) where Le^a is present in large amounts
- Le(a+b+) where Le^a is present in moderate amounts
- Le(a-b+) where Le^a is present in small amounts
- Le(a-b-) where Le^a is absent

Statistical tests one (Table 37), two (Table 38) and three (Table 39), of hypothesis one tested the possibility that the Le^a antigen is a pre-disposing factor to infection with *Neisseria gonorrhoeae.* No statistically significant differences between the G+ group and the G- group were seen. It is therefore concluded that relative presence, or absence of Le^a expression (with or without ABO) is not a pre-disposing factor to gonorrhoea infection.

Table 37. *Statistical test 1 of hypothesis 1*. The test compares high expression of Le^a in the Le(a+b-) group (all ABO groups) with no expression of Le^a in the Le(a-b-) group (all ABO groups). The numbers for both groups are shown in the G+ study cohort, and the G- cohort.

	ABO and Le(a+b-)	vs	ABO and Le(a-b-)
ABO	All		All
Lewis	Positive		Negative
Secretor	Negative		All
G+	16		8
G-	26		12
$\chi 2$	0.021		
p	0.886		

Table 38. *Statistical test 2 of hypothesis 1*. The test compares high expression of Le^a in the Le(a+b-) group (all ABO groups) with moderate or low expression of Le^a in the Le(a+b+) and Le(a-b+) groups (all ABO groups). The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	ABO and Le(a+b-)	vs	ABO + Le(a+b+) + Le(a-b+)
ABO	All		All
Lewis	Positive		Positive
Secretor	Negative		Positive and partial
G+	16		105
G-	26		131
$\chi 2$	0.593		
p	0.441		

Table 39. *Statistical test 3 of hypothesis 1*. The test compares high and moderate expression of Le^a in the Le(a+b-) and Le(a+b+) groups (all ABO groups) with low expression of Le^a in the Le(a-b+) group (all ABO groups). The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	ABO + Le(a+b-) + Le(a+b+)	vs	ABO and Le(a-b+)
ABO	All		All
Lewis	Positive		Positive
Secretor	Negative and partial		Positive
G+	48		73
G-	51		110
$\chi 2$	1.937		
p	0.164		

Hypothesis 2: *That the Le^b antigen is a pre-disposing factor to infection with Neisseria gonorrhoeae.*

Le^b is a very different antigen to Le^a, both in steric shape, and because it can exist in a shape that has either A or B modifications, for example ALe^b and BLe^b. Only individuals who possess the *Le* gene, and the *Se* gene (or the Se^w gene) can make Le^b. Red cell phenotyping for Le^b with Lewis antisera is therefore a surrogate marker of secretor status.

Because red cell phenotyping with Lewis antisera has been shown to be unreliable in many cases (Henry et al, 1995), secretor and Lewis genotyping were used in this study to determine the presence of Le^b. Generally, large amounts of Le^b are expressed on the red cells, and in the secretions, of individuals with the red cell phenotype OLe(a-b+). Conversely, no Le^b is expressed by those individuals who posses the *Le* gene, but do not possess the *Se* gene. In the middle of the range of Le^b expression are the partial secretors, who possess the A385T mutated gene $Se^{w.}$ As discussed in chapter 2, less Le^b is made in the Lewis positive partial secretor than in the Lewis positive wild-type secretor.

To test hypothesis 2, it is necessary to compare the incidence of gonorrhoea in groups expressing large amounts, moderate amounts, or no $Le^{b.}$ This was assessed in the following groups:

- Group O Le(a-b+) individuals, where Le^b is present in large amounts
- Group A Le(a-b+) individuals, where ALe^b is present in large amounts, but Le^b is present in small amounts.
- Group B Le(a-b+) individuals, where BLe^b is present in large amounts, but Le^b is present in small amounts.
- Group O Le(a+b+) individuals, where Le^b is present in moderate amounts.
- Group A, B and O Le(a+b-) individuals, where Le^b is not expressed.
- Group A, B and O Le(a-b-) individuals, where Le^b is not expressed.

Statistical test 1 (Table 40) of hypothesis 2 investigated the possibility of high expression of Le^b predisposing to infection with *Neisseria gonorrhoeae*. There was no statistically significant difference between high expression of Le^b (in the Group O secretor) and no expression of Le^b (in all ABO groups of non-secretors and Lewis negative individuals).

Table 40. *Statistical test 1 of hypothesis 2*. The test compares high expression of Le^b in the O Le(a-b+) group with no expression of Le^b in the Le(a+b-) and Le(a-b-) groups (all ABO groups). The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	O Le(a-b+)	vs	ABO + Le(a+b-) and Le(a-b-)
ABO	O		All
Lewis	Positive		Positive and negative
Secretor	Positive		All
G+	34		24
G-	54		42
$\chi 2$	0.083		
P	0.773		

Statistical tests 2 (Table 41) and 3 (Table 42) of hypothesis 2 investigated the likelihood of moderate expression of elongated chains of Le^b (seen in the Group O partial secretor) predisposing to infection with *Neisseria gonorrhoeae*. There was no statistically significant difference between high and moderate expression of elongated chains of Le^b. Nor was there any statistically significant difference between moderate and no expression of elongated chains of Le^b.

Table 41. *Statistical test 2 of hypothesis 2*. The test compares high expression of Le^b in the O Le(a-b+) group with moderate expression of Le^b in the O Le(a+b+) group. The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	O Le(a-b+)	vs	O Le(a+b+)
ABO	O		O
Lewis	Positive		Positive
Secretor	Positive		Partial
G+	34		10
G-	54		12
$\chi 2$	0.341		
p	0.559		

Table 42. *Statistical test 3 of hypothesis 2*.The test compares no expression of Le^b in the Le(a+b-) and Le(a-b-) groups (all ABO) with moderate expression of Le^b in the O Le(a+b+) group. The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	ABO + Le(a+b-) + Le(a-b-)	vs	O Le(a+b+)
ABO	All		All
Lewis	Positive		Positive
Secretor	All		Partial
G+	24		10
G-	42		12
$\chi 2$	0.848		
p	0.357		

Statistical tests four (Table 43), five (Table 44), six (Table 45), seven (Table 48) and eight (Table 49) of hypothesis 2 investigated the effect of A and B genes with expression of Le^b as a predisposing factor to infection with *Neisseria gonorrhoeae*. No statistically significant differences were observed, although in one (test six, Table 45) there was insufficient data.

Table 43. *Statistical test 4 of hypothesis 2*. The test compares high expression of ALe^b with moderate expression of ALe^b. The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	A Le(a-b+)	vs	A Le(a+b+)
ABO	A		A
Lewis	Positive		Positive
Secretor	Positive		Partial
G+	28		15
G-	32		9
$\chi 2$	1.72		
p	0.190		

Table 44. *Statistical test 5 of hypothesis 2*. The test compares high expression of ALe^b with no expression of ALe^b. The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	A Le(a-b+)	vs	O Le(a-b+) + B Le(a-b+) + O Le(a+b+) + B Le(a+b+) + ABO Le(a+b-) + ABO Le(a-b-)
ABO	A		All except A Le(a-b+)
Lewis	Positive		All
Secretor	Positive		All
G+	28		81
G-	32		121
$\chi 2$	0.821		
p	0.365		

Table 45. *Statistical test 6 of hypothesis 2*. The test compares high expression of BLe^b with moderate expression of BLe^b. The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	B Le(a-b+)	vs	B Le(a+b+)
ABO	B		B
Lewis	Positive		Positive
Secretor	Positive		Partial
G+	7		6
G-	13		4
$\chi 2$	Insufficient data		
p	Insufficient data		

Statistical tests four (Table 43) and six (Table 45) were combined to test significance, as test four was approaching significance, while test six had insufficient data. Both tests showed a reverse ratio of G+ to G- (i.e. there were more G- Le(a-b+) than G+ Le(a-b+) individuals, and less G- Le(a+b+) individuals than G+ Le(a+b+) individuals. It was considered reasonable to combine these two data sets, because Le^b is expressed in the same relative amount in both group A Le(a-b+) and group B Le (a-b+) individuals. The 2 x 2 table is shown (Table 46). The result is not statistically significant (p=0.08), although approaching significance.

Table 46. *2 x 2 test of A and B Le(a-b+) versus A and B Le (a+b+).* This is a combination of raw data from Table 43 and Table 45.

	A and B Le(a-b+)	vs	A and B Le(a+b+)
ABO	A and B		A and B
Lewis	Positive		Positive
Secretor	Positive		Partial
G+	35		21
G-	45		13
$\chi 2$	2.99		
p	0.084		

Because the p value in Table 46 was approaching significance, a further statistical test was done to compare A, B and O Le(a-b+) with A, B and O Le(a+b+). Although more Le^b is expressed in A and B Le(a+b+) individuals than in group O Le(a+b+) individuals, all express the elongated chains of the partial Secretor. This result was statistically significant (p=0.055). However, when the data was disaggregated for ethnicity, statistical significance was once again lost. The data is shown in Table 47.

Table 47. *2 x 2 test of A and B and O Le(a-b+) versus A and B and O Le (a+b+).*

	All ethnic groups		Ethnic group 2		Ethnic groups 1 and 4	
	A, B and O Le(a-b+)	A, B and O Le(a+b+)	A, B and O Le(a-b+)	A, B and O Le(a+b+)	A, B and O Le(a-b+)	A, B and O Le(a+b+)
G+	68	32	37	2	21	27
G-	96	25	78	2	7	12
χ^2	3.68		0.56		0.27	
p	0.055		0.455		0.605	

Statistical test 7 of hypothesis 2 compared high expression of BLe^b with no expression of BLe^b. The results, which revealed no significant difference, are shown in Table 48.

Table 48. *Statistical test 7 of hypothesis 2*. The test compares high expression of BLe^b with no expression of BLe^b. The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	B Le(a-b+)	vs	O Le(a-b+) + A Le(a-b+) + O Le(a+b+) + A Le(a+b+) + ABO Le(a+b-) + ABO Le(a-b-)
ABO	B		All except B Le(a-b+)
Lewis	Positive		All
Secretor	Positive		All
G+	7		111
G-	13		147
$\chi 2$	0.489		
p	0.484		

Statistical test 8 of hypothesis 2 compared high expression of BLe^b with high expression of ALe^b. The results, which revealed no significant difference, are shown in Table 49.

Table 49. *Statistical test 8 of hypothesis 2*. The test compares high expression of ALe^b with high expression of BLe^b. The numbers for both groups are shown in the G+ cohort, and the G- cohort.

	A Le(a-b+)	vs	B Le(a-b+)
ABO	A		B
Lewis	Positive		Positive
Secretor	Positive		Positive
G+	28		7
G-	32		13
$\chi 2$	0.830		
p	0.362		

No statistically significant differences were seen between relative presence, or absence of Le^b expression, in the G+ group, and the G- group. Nor were statistically significant differences seen between Le^b expression in the ALe^b and BLe^b groups in the G+ group, and the G- group. It is therefore concluded that Le^b expression is not a pre-disposing factor to gonorrhoea infection.

Hypothesis 3: *That Le^c (type 1 precursor) chains are a predisposing factor to gonorrhoea infection.*

Type 1 precursor remains unmodified in individuals who are lacking the Se gene and the Le gene (who are Lewis negative non-secretors). Le^c is strongly expressed in these individuals, and very weakly in all other groups, who modify it by the actions of FUT-2, or FUT-3. The red cells and tissues of the Lewis negative non-secretors have the phenotype Le(a-b-).

In 1990, Deal (Deal et al, 1990) demonstrated that *Neisseria gonorrhoeae* bound preferentially to some glycolipids isolated from endocervical endothelium. Amongst the sequences which bound *Neisseria gonorrhoeae*, were paragloboside (type 1 or Le^c) and lactotriaosylceramide (the molecule to which type 1 attaches on red cells). Therefore it seems reasonable to test the hypothesis that Le^c (type 1 precursor) chains are a predisposing factor to gonorrhoea infection, since Deal has already demonstrated that Le^c can bind *Neisseria gonorrhoeae*.

To test the hypothesis, it is necessary to compare the highest expression of Le^c chains (in the Lewis negative non-secretor) with the lowest expression of Le^c chains (in the Lewis positive secretor). This study tested the hypothesis that Le^c is a predisposing factor to gonorrhoea infection, in hypothesis 3 (Table 50).

Table 50. *Statistical test 1 of hypothesis 3*. The test compares high expression of Le^c in the Le(a-b-) sese group (all ABO groups) with low expression of Le^c in the Le(a-b+) group (all ABO groups). The numbers for both groups are shown in the G+ cohort, and the G- cohort .

	ABO Le(a-b-) sese	vs	ABO Le(a-b+)
ABO	All		All
Lewis	Negative		Positive
Secretor	Negative		Positive
G+	5		73
G-	7		109
$\chi 2$	0.011		
p	0.915		

No statistically significant differences were seen between relative presence, or absence of Le^c expression, in the G+ and the G- groups. The results of this

study do not support Deal's findings, at least *in vivo*, because it has been proven that high expression or low expression of Le^c showed no statistical association with gonorrhoea (p=0.915). It is therefore concluded that Le^c (type 1 precursor) is not a predisposing factor to gonorrhoea infection.

Hypothesis 4: *That H type 1 (Le^d) is a predisposing factor to gonorrhoea infection.*

H type 1 remains unmodified in individuals lacking the Le gene, but possessing the Se gene (Lewis negative secretors). It is strongly expressed in group O Lewis negative secretors, and weakly expressed in:

- Lewis positive secretors (who convert H type 1 to Le^b)
- Lewis positive non-secretors (who don't make H type 1)
- Group A/B Lewis negative secretors (who convert H type 1 to A/B type 1)

To test the hypothesis, the following were considered:

- Lewis negative secretors with Lewis positive secretors
- Lewis negative secretors with Lewis positive non-secretors

Unfortunately, these analyses did not yield p values, as there are insufficient numbers of Lewis negative secretors in the study. Hypothesis 4 remains untested, and so is inconclusive.

Hypothesis 5: *That ABO blood groups are a predisposing factor to gonorrhoea infection.*

As discussed in chapter 2, inheritance of different ABO blood group genes, together with inheritance of the *Se* gene, control expression of ABO sugars in mucosal surfaces of the reproductive tract.

- Group A secretors, where A type 1 is present in large amounts
- Group B secretors, where B type 1 is present in large amounts
- Group AB secretors, where A type 1 and B type 1 are present in large amounts
- Group O secretors, where H type 1 is present in large amounts

To test hypothesis 5, it is necessary to examine the incidence of the ABO blood groups in secretors in the G+ group, and the G- group.

Table 51. *Statistical test 1 of hypothesis 5*. The test compares expression of A, B and H. The numbers for both groups are shown in the G+ cohort and the G- cohort.

	A Se	B Se	AB Se	O Se
ABO	A	B	AB	O
Lewis	All	All	All	All
Secretor	Positive	Positive	Positive	Positive
G+	29	7	4	34
G-	33	14	9	57
$\chi 2$	2.294			
P	0.514			

Hypothesis 5 examined whether or not there was an association of the ABO blood groups in secretors expressing A, B, or H substances with gonorrhoea infection. There were no statistically significant differences found between expressed A, B, or H, between the G+ group and the G- group (p=0.514). The A, B and AB secretors were also combined, and tested against the O secretors, to examine if expression of H type 1 versus (A type 1 or B type 1) made a difference. The result was not statistically significant (p = 0.571).

4.6 Chapter review

Neither random statistical analysis of data sets, nor statistical analysis of data sets arranged by accepted blood grouping paradigms, yielded a statistically significant association of ABO, Lewis and Secretor phenotypes and genotypes with gonorrhoea that could not be refuted when the data was disaggregated for ethnicity. The study did show a statistically significant difference (p=0.05) in the incidence of the partial secretor phenotype (26.7%) in the G+ population and the incidence of the partial secretor phenotype (15.4%) in the G- population, when all ethnic groups were analysed together. However, when the data was disaggregated for ethnicity, the p values were no longer statistically significant.

The very high rate of discordance between serology and genotyping (17%) was not unexpected. In fact it highlighted the requirement to genotype and phenotype in parallel. The red cell only data was compared to phenotype corrected data (corrected on the basis of genotyping), but the difference was not statistically significant (data not shown).

Chapter 5: Discussion

The results of this study are discussed in light of the body of knowledge surrounding the work.

The hypothesis of this study was that *Neisseria gonorrhoeae* may bind preferentially to some profiles of carbohydrates over others, these carbohydrates being expressed on the mucosal surfaces of the human genital tract, as a result of inherited ABO, Lewis and Secretor genes. Deal's work (Deal et al, 1990) was used as a scientific basis for the hypothesis, since Deal found that *Neisseria gonorrhoeae* bound preferentially to some carbohydrates *in vitro*. It may be that Deal's *in vitro* findings do not correlate with *in vivo* ability to invade the epithelium, which is a multi-factorial process.

This work also re-examined conflicting findings/hypotheses of previous studies of association with gonorrhoea with blood groups, (Foster et al, 1976; Johnson et al, 1983; Kinane et al, 1983a; Kinane et al, 1983b; Matzkin, 1987; Schofield, 1966), many of which the researcher considered did not have a strong scientific basis.

The carbohydrate profile that humans express on mucosal surfaces depends on the genes that they possess in at least 3 histo-blood group systems: ABO, Lewis and Secretor. No previous study has examined the combined effects of these 3 systems. Previous studies searched for:

1. An association between gonorrhoea and one of these three histo-blood group systems (for example ABO) (Matzkin, 1987; Schofield, 1966). This is a flawed hypothesis, since A and/or B sugars are only expressed at the mucosal surface if the individual is a secretor. We analysed the data from this study in this same flawed manner in Table 21, (considering ABO without considering the effect of secretor status), and found the same finding as Schofield and Matzkin (namely no statistically significant difference in the blood group frequencies in the gonorrhoea positive, and the gonorhhoea negative groups).
2. An association between gonorrhoea and two of these three histo-blood group systems (for example ABO and Secretor) (Johnson et al, 1983). This hypothesis is sounder than considering the effect of ABO without secretor status. However, it is still flawed, for 2 reasons:

(a) Secretor status was determined by saliva testing (Johnson et al, 1983), a method which is known to be less reliable than genotyping (Henry et al, 1996b).

(b) The effect of the Lewis gene *Le* must be considered, since individuals possessing the *Le* gene produce a different set of carbohydrates at the mucosal surface to those not possessing the *Le* gene. The effects of the ABO, H, Le and the Se transferases cannot be considered separately, since all determine the carbohydrate profile produced.

In the light of knowledge of the interaction of ABO, Lewis and Secretor to produce carbohydrate profiles on the mucosal surface, this study examined:

- *ABO red cell phenotype on all samples.* ABO phenotyping is universally accepted as a reliable predictor of expressed ABO genes, and so it was not considered necessary to perform ABO genotypes.
- *Secretor genotype on all samples (with a multi-step strategy).* Secretor genotyping is the only accurate way to determine secretor status. Surrogate markers, such as saliva testing, or interpretation of red cell phenotypes with Lewis antisera, are unreliable.
- *Lewis red cell phenotype on all samples, followed by Lewis genotype on samples with the phenotype Le(a-b-).* Lewis phenotyping of red cells is a reliable predictor of the presence of the Le gene in the phenotypes Le(a+b-), Le(a+b+) and Le(a-b+). However, samples which yield the Lewis red cell phenotype Le(a-b-) may in fact be positive for the *Le* gene, and these samples should have their Lewis negative status confirmed by genotype. False negatives can arise in Lewis positive individuals who are pregnant, diseased, or transplanted (Hammar et al, 1981; Makni et al, 1987; Orntoft et al, 1991; Stigendal et al, 1984).

This is the first documented study between disease association (of any type, including gonorrhoea) and blood types of which the author is aware, in which a multi-pronged approach to typing has been used. This approach should be applied to future disease and blood group association studies, and not limited to sexually transmitted infections.

The results show that the hypothesis is not proven. The only associations where a statistically significant difference between the G+ and the G-group was observed, involved combinations of blood groups including the partial secretor

phenotype. This study shows a statistically significant difference (p=0.05) in the incidence of the partial secretor phenotype (26.7%) in the G+ population and the incidence of the partial secretor phenotype (15.4%) in the G- population, suggesting that susceptibility to gonorrhoea infection may be associated with the partial secretor phenotype. As discussed earlier, the partial secretor phenotype leads to expression of elongated chains of the ABH blood group carbohydrates on the mucosal surfaces (Henry et al, 1995). Therefore it was considered that *Neisseria gonorrhoeae*, which uses pili to attach to mucosal carbohydrates offered to ligands in the bacterial cell (Cohen et al, 1999), may have a greater chance of attaching to the mucosal wall in the partial secretor (simply because there are more sites to attach on the elongated carbohydrate chains of the partial secretor). When all ethnicities are grouped together, there is indeed a statistically significant difference (p=0.05) in the incidence of the partial secretor phenotype (26.7%) in the G+ cohort and the incidence of the partial secretor phenotype (15.4%) in the G- cohort. However, we know that the partial secretor phenotype is common in some ethnic groups (Greenwell et al, 1986; Henry et al, 1990; Henry et al, 1989; Lin-Chu et al, 1988; Sturgeon et al, 1970, Vos et al, 1967), and absent in others (Mourant et al, 1976). In this study, ethnic groups 1 and 4 are known to have a high incidence of the partial secretor phenotype, and ethnic group 2 is known to have a low or absent incidence of the partial secretor phenotype. Ethnic groups 1 and 4 were over–represented in the gonorrhoea positive group; the number of gonorrhoea positives exceeded the number of gonorrhoea negatives. In ethnic group 2, the number of gonorrhoea negatives exceeded the number of gonorrhoea positives. For this reason, it was necessary to repeat the statistical tests in ethnic group 2, and ethnic groups 1 and 4 combined. In order to control the results for ethnicity, the Ethics committee gave permission for ASHS to reveal the ethnicity codes to the researcher. When the data is disaggregated for ethnicity, the difference between the incidence of the partial secretor phenotype in the gonorrhoea positive population and the incidence of the partial secretor phenotype in the G- population loses statistical significance. It is likely that the difference is reflecting the higher incidence of gonorrhoea, together with the higher incidence of the partial secretor phenotype, in ethnic groups 1 and 4, rather than a true association between gonorrhoea and the partial secretor phenotype.

In the data which is disaggregated for ethnicity, it is noted that some cells in the 2 x 2 test has less than 5 values, and therefore the χ^2 and the p value must be interpreted with caution. This highlights the need to consider ethnicity when performing studies such as this one, otherwise false associations between disease and blood group could easily be made.

This study shows that there is no association of ABO, Lewis and Secretor phenotypes and genotypes with gonorrhoea and suggests that *Neisseria gonorrhoeae* does not bind preferentially to some blood group profiles of carbohydrates *in vivo*. There is no difference in the incidence of gonorrhoea between the ABO blood groups, nor between Lewis positive and Lewis negative individuals, nor between secretors and non-secretors, nor between any combination of these factors.

In summary, no statistically significant associations were seen between gonorrhoea, and ABO, Lewis and Secretor blood groups. The initially apparent association between the partial secretor phenotype and gonorrhoea is weak, due to the increased incidence of the partial secretor phenotype in ethnic groups 1 and 4, and its absence in ethnic group 2. This highlights the requirement to disaggregate for ethnicity in disease association studies, where populations with different distributions of blood group genes are studied.

The frequency of the partial secretor phenotype in this study in ethnic group 1 (50%), ethnic group 3 (48%) and ethnic group 4 (58%) is considerably higher than previously reported in any other study (Henry et al, 1988; Lin Chu et al, 1988). Henry and Lin Chu both used Lewis red cell phenotyping to count the Le(a+b+) partial secretors in 1988, because secretor genotyping was not readily available at that time.

If the percentage frequency of the Le(a+b-) red cell phenotype is added to the Le(a+b+) , then the frequency of Le(a+b+) in Henry's study in ethnic group 1 becomes 32.6%, and in ethnic group 4 becomes 22.2%. These figures were added on advice from Henry, (Henry et al, 1993) as Le(a+b+) is frequently mistyped as Le(a+b-) with all Lewis antisera.

Likewise, if the percentage frequency of the Le(a+b-) red cell phenotype is added to the Le(a+b+), then the frequency of Le(a+b+) in Lin Chu's study in ethnic group 3 becomes 23.3%. These figures are still lower than the frequencies reported in this study. The higher incidence of partial secretors in

this study almost certainly reflects the use of Lewis and secretor genotyping, as compared to Lewis red cell phenotyping in the reference studies. This highlights the requirement to use Lewis and secretor genotyping to correct red cell Lewis phenotyping, as the gold standard for determination of secretor phenotypes in future population studies.

In 1966, Schofield (Schofield, 1966) studied the ABO and Rh(D) types of 2,575 patients attending the venereal disease clinics (as they were then known) in Newcastle and Tynemouth. Of these patients 14.5% were diagnosed with gonorrhoea. The ABO groups of these patients were compared to ABO frequencies from blood donors in the region. No statistical differences were found between the gonorrhoea positive group and the control group. This is in accord with the findings of this thesis. However, Schofield's study design was flawed in not considering whether or not the individuals were secretors of ABH, i.e. whether they expressed ABH antigens in their mucosal tissues.

In 1987, Matzkin (Matzkin, 1987) studied the ABO blood groups of gonorrhoea positive males in the Israel Defence Forces (n=1,119). Matzkin did not find any association between ABO blood groups and occurrence of disease. This is again in accord with the findings of this study. However, Matzkin's study design was also flawed in not considering whether or not the individuals were secretors of ABH.

> Matzkin comments that "Asian and North African ethnic origin, fewer years of schooling , and low socioeconomic status were found to be important risk factors".

The fact that Asian and African ethnic origin was a risk factor is interesting to this study, because the partial secretor phenotype is know to be prevalent amongst Asian and African populations (Greenwell et al, 1986; Lin-Chu et al, 1988). It would be interesting to disaggregate Matzkin's data for ethnicity, as was done in this study. Conversely, it is more likely to that the socio-economic risk factor is making the largest contribution.

In 1976, Foster (Foster et al, 1976) also investigated the question. Foster studied 584 Black women attending a prenatal clinic. The number of white women was too small for the data to be used. The incidence of blood group B was significantly higher amongst the women who tested positive for gonorrhoea (25.6%) than it was in the women who tested negative for gonorrhoea (14.4%).

Secretor status was not studied, so this finding is potentially flawed, although dark skinned individuals are usually secretors.

In 1977, Miler (Miler et al, 1977) performed a study of 177 gonorrhoea patients.

> Miler found that “in white gonorrhea patients, there was a significantly higher frequency of group B individuals over those with group A, AB, or O. No such correlation was found in Black patients”.

This work contradicts Foster‘s paper, in which Black patients showed an increase of group B in gonorrhoea positive patients, and Whites were not commented on due to low numbers.

> Miler et al make the comment “ the argument that there is a significantly increased risk of gonorrhea in group B subjects appears to be weak, and the difference may be due to non-representative sampling”.

In 1983, Johnson and colleagues (Johnson et al, 1983) also studied the question of a relationship between ABO blood groups and gonorrhoea, largely to see if they could resolve the differences in the results in the Foster and Miler studies. They studied the blood groups and secretor status of 216 male Caucasians positive for gonorrhoea, and 2043 male Caucasians negative for gonorrhoea.

In light of the different results in the Foster and Miler studies, Johnson estimated the number of patients needed to have a 90% chance of detecting a significant difference, to be 150. Johnson was able to recruit 216 patients (in excess of the 150 required). This study also recruited in excess of 150 patients (n=175) although not for each of the different ethnic groups. Ideally greater than 150 samples for each ethnic group would be required to unambiguously assert whether or not an association exists.

Johnson drew his control population from 2043 male Caucasians, participating in a study of ischaemic heart disease (Meade et al, 1978). ABO blood group was determined by slide aggluatination of red cells with commercial antisera. Secretor status was determined by incubating boiled patient saliva with *Ulex europaeus* (anti-H) and antiserum (source not stated) and then by testing the mixtures against appropriate groups of red cells. This is a recognised method of testing for secretor status. For example, if a blood group A individual is a secretor, then she will express A substance in her saliva. This saliva, when incubated with human anti-A, should neutralise the anti-A activity, and hence should give a negative result for agglutination when mixed with human A cells.

However, the method's accuracy depends on antisera source, and the titre of the antisera used and is significantly flawed (Henry et al, 1996b). Johnson et al found no significant difference in the distribution of blood groups or secretor status between the positive and negative gonorrhoea groups. This finding is also in accord with the results of this study.

Kinane also studied the association of ABO blood groups and gonorrhoea (Kinane et al, 1983a). In 1983, Kinane and colleagues published work in which they found an increased frequency of group B in gonorrhoea positive patients attending the department of genito-urinary medicine at Royal Infirmary, Edinburgh, as compared to the control population. The control population was blood donors, and it is questionable whether this represents a matched control group, since the socio-economic status and number of sexual partners may not be comparable between these 2 groups. The difference in frequency of blood group B between these 2 groups (patients attending at the genito-urinary department and blood donors) was statistically significant ($p<0.05$). However, the difference in frequency of blood group B between the gonorrhoea positive patients and the gonorrhoea negative patients was not statistically significant ($p>0.1$).

Several researchers have also investigated the hypothesis that the absence of anti-B may be a factor in the reported susceptibility of group B individuals to gonorrhoea, without conclusive result (Foster et al, 1976; Kinane et al, 1983a; Miler et al, 1977).

In this study, the "control" samples were drawn from the same population (namely that attending ASHS) because it was considered that this represented a better-matched control group in terms of social factors. The group was not a randomly selected control group, because they self-excluded from the study group when they were found to be gonorrhoea negative. Those individuals who do not have gonorrhoea may have factors protecting against gonorrhoea. Although not ideal as a comparative control, they are very useful as they should force statistical significance by being an extreme, i.e. those who do not have gonorrhoea.

This G- group was collected from any individuals attending the clinic, agreeing to participate in the study. It is recognized that people attend the clinic for a variety of reasons. The control group does not only draw from those presenting

with symptoms of a sexually transmitted infection, or those with a high number of sexual partners. Nineteen individuals (9%) of the control group had a sexually transmitted infection other than gonorrhoea (*Chlamydia trachomatis*, *Trichomonas vaginalis*, or NGU). Therefore the rest of the control group did not have (or were not investigated for) a STI. This means that the control group is not as well matched as it was anticipated it would be, in terms of exposure to STI. Conversely, this may be a helpful factor in selecting the control group, in that many of these individuals did not necessarily have factors protecting against gonorrhoea. As shown in Table 15, 50% of the G- cohort had zero or one sexual partner, and therefore may have had similar risks of catching an STI as an average population.

It is always difficult to get a perfectly matched control group in studies on humans, and it is the opinion of the researcher that having a control group from the Sexual Health Clinic is still more valuable than having an unrelated control group (for example blood donors).

This study did not consider the influence of the P blood group system. P system antigen P_1, and the globoside collection of P, P^k, and Luke, are biochemically related to the ABO antigens. Like ABO, the P system, and globoside collection of antigens, are comprised of carbohydrates, sequentially added to precursor chains under the control of glycosyltransferases, as reviewed by Daniels (Daniels, 1995). P_1, P, and P^k antigens have been found on uroepithelial cells (Mollison et al, 1997). Although the biosynthetic pathway for the formation of the P_1 antigen, and the globoside collection, is reasonably well understood, the genes involved are not yet known. When they are, a genotyping strategy could be developed, and added to the multi-pronged approach of this study, for future work in disease associations.

Strain typing was not included in the study, due to the high cost of serotyping. It would be worthwhile to include strain typing in future studies. Although there was no association found between ABO, Lewis and Secretor, and the species *Neisseria gonorrhoeae* as a whole, individual strains may show an association.

Concluding remarks

In conclusion, it is recognized that the G- group was not perfectly matched in terms of number of sexual partners, or ethnicity. However, this study may be comparatively better matched than many other previous studies that have not used sexual health clinic attendees with similar exposure to STI.

This study is the first example of a disease association study that has examined the effect of all the relevant carbohydrate molecules expressed on the mucosal tissues. It has hopefully contributed to solving the long running debate as to whether blood group B individuals are more susceptible to infection with *Neisseria gonorrhoeae*. This study concludes that there is no association between ABO, Lewis and Secretor phenotypes and genotypes with *Neisseria gonorrhoeae* in ethnic populations based in New Zealand.

Glossary of Medical and Scientific terms

Allele: One form of a series of genes occupying a single locus on either of a pair of homologous chromosomes (Issitt et al, 1999)

Amorph: A gene that does not code for a detectable characterstic (Issitt et al, 1999)

Antiserum: Commercial, reagent antibody

Arthritic: Affected by arthritis

Arthritis: Inflammation of the joints

Bacteremia: Presence of bacteria in the blood circulation

Buffy Coat: The layer of white blood cells and platelets that rests between the red blood cells at the bottom of a tube, and the plasma supernatant, after centrifugation of blood

Cervix: The neck of the womb (Oxford, 1990)

Cervical: From the cervix

Chi-Square ($\chi 2$): A statistical method of comparing observed with expected values

Chlamydia trachomatis: Spherical, non-motile bacteria of the genus *Chlamydia*, which cause infection and inflammation in the human genital tract (Stedman, 1990)

Conjunctiva: The mucous membrane covering the front of the eye, and the inside of the eyelids (Oxford, 1990)

Conjunctivitis: Inflammation of the conjunctiva

Dermatitis: Inflammation of the skin (Oxford, 1990)

Dysuria: Painful or difficult urination (Oxford, 1990)

Ectopic pregnancy: A pregnancy where the fetus grows in the fallopian tubes, rather than the uterus (Reed, 2001)

Endocarditis: Inflammation of the endocardium

Endocardium: A layer of endothelial cells lining the inner wall of the cardiac chamber and the surface of the valve cusps (Underwood, 2000)

Enzyme: A protein acting as a catalyst in a specific biochemical reaction (Oxford, 1990)

Epididymis: A duct behind the testis, through which sperm passes (Oxford, 1990)

Epididymitis: Inflammation of the epididymis

Epididymo-orchitis: Inflammation of the epididymis, and the testis (Underwood, 2000)

Exocrine: Secreting through a duct (Oxford, 1990)

Fellatio: Oral stimulation of the penis (Reed, 2001)

Genotype: The genes of an individual, inherited from 2 parents, which may, or may not, code for a protein expressed at the phenotypic level. In the case of blood grouping, the genotype is deduced by typing the DNA of the individual with primers appropriate for the gene(s) of interest

Glycosyltransferase: An enzyme that adds a carbohydrate to a precursor substrate

Gram Stain: A staining procedure used in the laboratory to differentiate gram-positive bacteria (which retain the stain crystal violet) from gram-negative bacteria (which retain the stain safranin)

Haplotype: A half of the genotype

Linkage Disequilibrium: The genetic combination of 2 independent, but closely linked loci produces a haplotype whose frequency is more common than expected, when considering the frequencies of the individual genes

Lymphocyte: A class of white blood cell of the adaptive immune system. T lymphocytes present bacteria to B lymphocytes, which then produce specific antibodies against the bacteria

Meninges: The membranes of the central nervous system; namely the pia, the arachnoid, and the dura mater (Underwood, 2000)

Meningitis: Inflammation of the meninges, which may be due to bacterial or viral infection

Menorrhagia: Abnormally heavy menstrual periods

Neutrophils: A class of white blood cells, characterized by a multi-lobed nucleus, which have the ability to engulf bacteria

Opthalmia neonatorum: Inflammation of the eye, especially the conjunctiva, in newborns (Oxford, 1990)

Orogenital: Contact of the mouth with the genitals

Ovarian: Of the ovaries

Ovaries: Two small bodies on either side of the uterus, which produce the female ova, and the female sex hormones (Reed, 2001)

p value: The probability that a variate would assume a value greater than or equal to the observed value strictly by chance (Weisstein, 1999). A p value of < 0.05 is often regarded as significant, meaning that there is a less than a 5% probability that the variate has occurred by chance.

Pelvic Inflammatory Disease (PID): Acute or chronic inflammation in the female pelvic cavity, and particularly the fallopian tubes, uterus, and/or ovaries.

Pharyngeal: Of the pharynx

Pharynx: The wide air passage which connects the nose to the throat (Reed, 2001)

Phenotype: The observable characteristics of an individual. In the case of blood grouping, the phenotype is deduced by the reactions of an individual's red cells with commercial antisera

Plasma: The straw-coloured, non-cellular portion of the blood, which is found as supernatant after centrifugation. Plasma contains all the clotting factors of blood necessary for coagulation, inactivated by the addition of an anticoagulant

Polymerase Chain Reaction (PCR): A multiple cycle process, with each cycle comprised of 3 stages, which allows a gene of interest to be amplified a billion-fold, in order to allow the detection of that gene of interest

Primers: Small segments of DNA, used in the PCR, complementary to the flanking regions of the gene of interest

Prostate: A gland around the neck of the male bladder. The gland forms fluid which makes up part of the seminal fluid (Oxford, 1990)

Prostatitis: Inflammation of the prostate

Rectal: Of the rectum

Rectum: The final section of the large intestine, meeting the anus (Oxford, 1990)

Salpingitis: Inflammation of the fallopian tubes (Oxford, 1990)

Secretor: An individual expressing his or her ABH antigens in their body fluids

Serum: As for plasma, except that serum is depleted of clotting factors, as it is formed as blood clots

Sexually Transmitted Infection (STI): An infection of the human reproductive tract, caused by a bacterial, viral, fungal, or parasitic pathogen, transmitted by 1 human to another during sexual intercourse

***Trichomonas vaginalis*:** A parasitic protozoan flagellate, frequently found in the vagina and urethra of women, and the prostate gland of men. The parasite causes vaginitis in some women, with pathogenicity dependent on the strain of the species (Stedman, 1990)

Urethra: The duct by which urine is discharged from the bladder (Oxford, 1990)

Urethral: From the urethra

Urethritis: Inflammation of the urethra

Vagina: The canal between the uterus and the vulva of a woman (Oxford, 1990)

Vaginal: From the vagina

Vaginitis: Inflammation of the vagina (Stedman, 1990)

Vestibule: A small cavity at the entrance of a canal. In the context of this thesis, refers to the vaginal vestibule; the space behind the glans clitoridis and between the labia minora, containing the openings of the vagina, urethra, and ducts of the glands (Stedman, 1990)

White Blood Cells: The neutrophils, lymphocytes, and monocytes in human peripheral blood

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