SERUM CARBOHYDRATE DEFICIENT TRANSFERRIN AS A MARKER FOR ALCOHOL ABUSE

A thesis submitted for the degree of Doctor of Medicine

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Abstract

Aim: To examine serum carbohydrate-deficient transferrin (CDT) as a marker of alcohol abuse and to determine possible reasons for false positive and false negative results.

Background: Alcohol misuse constitutes a major problem for medical and social agencies world-wide. Early detection and meticulous monitoring of drinking behaviour in individuals, once identified, remain the mainstays of effective management. Objective markers are required to facilitate this process. Serum CDT has been proposed for this purpose but the performance of the available commercial kits appears to fall short of the more sophisticated HPLC techniques used for research purposes.

Methods: Two commercial assays, CDTect ® (Pharmacia & Upjohn, Sweden) and AXIS %CDT ® (AXIS Biochemicals ASA, Norway), were used.

Results: In a study population of 590 individuals with well-documented and widely divergent drinking behaviour the sensitivity/specificity of serum CDT was 47.9% and 78.4% (CDTect) and 47.9% and 90.3% (AXIS %CDT). The false positive rates in patients with non-alcoholic liver disease, 26.4% (CDTect) and 7.7% (AXIS %CDT), and the false positive and false negative rates in alcohol misusers, 33.6 / 52.1% (CDTect) and 25.5 / 52.1% (AXIS %CDT) were unacceptably high. An additional 363 individuals were studied with similar results. Monitoring performance was assessed in 40 alcohol misusers followed serially from detoxification over 6 months. Poor assay performance could not be attributed to chronic inflammation or iron status. There was evidence that some false positive results reflected changes in the transferrin isoform profile on HPLC which differed from changes associated with alcohol misuse.

Conclusion: CDT was unsatisfactory using commercial kits and when used alone. In combination with other laboratory markers of alcohol misuse improved overall performance for both screening and monitoring. Further developments are needed in order to maximise the performance of the commercial techniques for assaying serum CDT.

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I would finally like to thank all my friends and family who helped and supported me through this research and believed that it would finally be finished.

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Source of reagents

Name	Supplier & Address
25% glycerol	Sigma Chemical Co., Poole, UK
96-well plates from protein assay kit	Sigma Chemical Co., Poole, UK
Absolute ethanol	Sigma Chemical Co., Poole, UK
Acrylamide solution	Sigma Chemical Co., Poole, UK
Ammonium persulphate	Sigma Chemical Co., Poole, UK
Ampholines (ph range 4.0 – 6.5)	Pharmacia, Milton Keynes, UK
Anti-human transferrin (Rabbit polyclonal antibody)	Dako, High Wycombe, UK
AXIS %CDT turbidimetric immunoassay kit	AXIS biochemicals, Oslo, Norway
Bis Tris: Bis (2-hydroxyethyl) amino-tris (hydroxymethyl) methane	Fluka, Switzerland
Blood collecting system	Sarstedt, Numbrecht, Germany
Bovine serum albumin	Sigma Chemical Co., Poole, UK
Calcium chloride	Sigma Chemical Co., Poole, UK
Carbonic anhydrase	Sigma Chemical Co., Poole, UK
CDTect kit	Pharmacia & Upjohn, Uppsala, Sweden
Cupric sulphate	Sigma Chemical Co., Poole, UK
Dextran sulphate sodium salt	Fluka, Switzerland
Dilution buffer (phosphate buffered saline solution, ph=7.1-7.3)	Dako, High Wycombe, U
Ferric chloride	Sigma Chemical Co., Poole, UK
Folin and Ciocalteau's Reagent	Sigma Chemical Co., Poole, UK
Gel-bond PAG film	Pharmacia, Milton Keynes, UK
Glacial acetic acid	Sigma Chemical Co., Poole, UK
Glucose oxidase	Sigma Chemical Co., Poole, UK
Human serum transferrin	Sigma Chemical Co., Poole, UK
Hydrochloric acid	Sigma Chemical Co., Poole, UK
Maleic acid	Sigma Chemical Co., Poole, UK
Modified Lowry reagent	Sigma Chemical Co., Poole, UK
Nunc Maxisorp Immuno-strips	Nunc, UK
Polyacrylamide isoelectric focussing gel	Sigma Chemical Co., Poole, UK
Sodium azide	Sigma Chemical Co., Poole, UK

Sodium chloride	AnalaR BDH chemicals, UK
Sodium citrate	Sigma Chemical Co., Poole, UK
Sodium hydroxide	Sigma Chemical Co., Poole, UK
Soluble transferrin receptor assay kit	Orion Diagnostica, Espoo, Finland
Sulphosalicylic acid	Sigma Chemical Co., Poole, UK
Sulphuric acid	Sigma Chemical Co., Poole, UK
Temed	Sigma Chemical Co., Poole, UK
Transferrin Calibrator Transferrin at a concentration of 27μg/ml	Dako, High Wycombe, UK
Transferrin immunoturbidimetry	Randox, Crumlin, Co Antrim
Trichloroacetic acid	Sigma Chemical Co., Poole, UK
Tris[hydroxymethyl]aminomethane	Sigma Chemical Co., Poole, UK
Trypsin inhibitor	Sigma Chemical Co., Poole, UK
Tween 20 (polyethylene sorbitan)	Sigma Chemical Co., Poole, UK
Urea	Sigma Chemical Co., Poole, UK
β-Lactoglobulin a	Sigma Chemical Co., Poole, UK

Abbreviations

5 HTOL	5-hydroxy tryptophol
5-HIAA	5 hydroxy indole acetic acid
ADH	Alcohol dehydrogenase
AEC	Anion exchange chromatography
ALD	Alcoholic liver disease
ALDH	Aldehyde dehydrogenase
ALT	Alanine transaminase
ANCA	Anti-neutrophil cytoplasmic antibody
AST	Aspartate transaminase
AUDIT	Alcohol Use Disorders Identification Test
BAC	Blood alcohol concentration
β-Hex	β-Hexosamine
Bis Tris	Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane
BSA	Bovine serum albumin
C	Cirrhotic
CADG	Carbohydrate deficiency glycoprotein syndrome
CDT	Carbohydrate-deficient transferrin
CDTect/tf	CDTect expressed as a ratio to total transferrin
CRF	Chronic renal failure
CRP	C-reactive protein
CV	Coefficient of variation
Detox	Detoxification
DSM	Diagnostic and statistical manual of mental disorders
EIA	Enzyme-immuno assay
ESR	Erythrocyte sedimentation rate
f	Female
FPLC	Fast protein liquid chromatography
GGT	Gamma-glutamyl transpeptidase
GDH	Glutamate dehygrogenase
Hb	Haemoglobin
HPLC	High performance liquid chromatography
HV	Healthy volunteers
IB	Immunoblotting

IBD	Inflammatory bowel disease
ICU	Intensive care unit
IEF	Isoelectric focusing
IF	Immunofixation
INR	International normalised ratio
IQR	Inter-quartile range
LD	Laser densitometry
m	Male
MAb	Mouse antibody
MAEC	Micro anion exchange chromatography
MAST	Michigan Alcohol Screening Test
mAST	Mitocondrial aspartate transaminase
MCV	Mean corpuscular volume
MEOS	Microsomal oxidising system
NaCl	Sodium chloride
NALD	Non-alcoholic liver disease
NASH	Non-alcoholic steatohepatitis
NC	Non-cirrhotic
OLT	Orthotopic liver transplantation
OPCS	Office of Population Censuses and Surveys
PBS	Phosphate buffered saline
pI	Isoelectric point
PT	Prothrombin time
RA	Rheumatoid arthritis
Rec	Recommended
RIA	Radio-immuno assay
ROC	Receiver Operating Characteristic
SD	Standard deviation
SE	Standard error
Sens	Sensitivity
Spec	Specificity
STR	Soluble transferrin receptors
tAST	Total aspartate transaminase
Tf	Transferrin
TIA	Immunoturbidimetric assay

TIBC	Total iron binding capacity
Tris	Tris(hydroxymethyl)methane
WB	Western blotting
WCTU	Women's Christian Temperance Union
WHO	World Health Organisation
ZIA	Zone immunoelectrophoresis
ZIF	Zone immunoelectrophoresis

Normal ranges

Aspartate transaminase		5-40 U/l
AXIS %CDT		0-6.0 %
CDTect	males	0-20 U/l
	females	0-26 U/l
Ferritin		39-300 μg/l
Gamma-glutamyl transferase		10-48 U/l
Haemoglobin		11.5-15.5 g/dl
International normalised ratio		0.9-1.2
Iron		11-36 μmol/l
Mean corpuscular volume		80-95 fl
Saturation %		20-40 %
Soluble transferrin receptors		1.3-3.3 mg/l
Total iron binding capacity		53-85 μmol/l
Total transferrin		200-400 mg/dl

1. INTRODUCTION

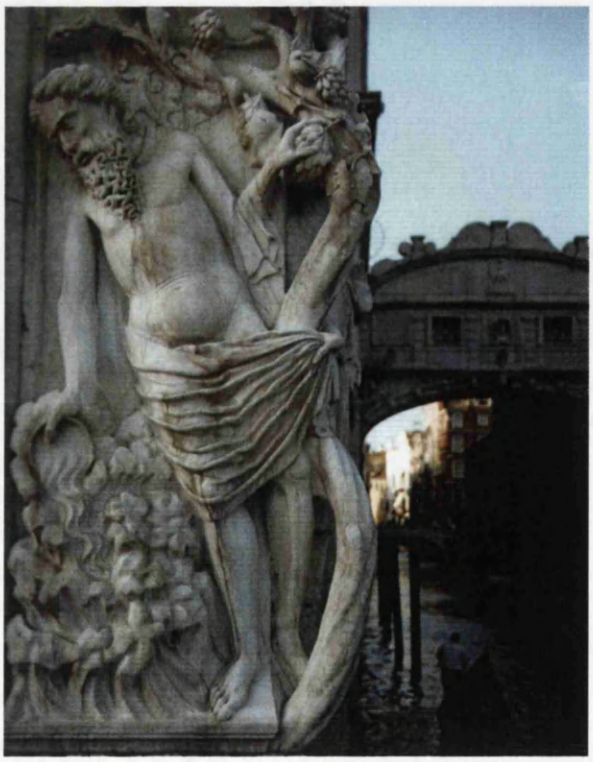
Alcohol has been used in society over centuries for its mood-lifting properties and taste. It is probably, however, the commonest drug of abuse world-wide and unfortunately causes considerable morbidity, mortality and social disruption. In 1990 the cost to the UK was almost £2.5 billion (The Institute of Alcohol Studies, 1998) and in the USA cost more than $100 billion and 100,000 lives (Lieber, 1995).

1.1 Historical Perspective

1.1.1 BACKGROUND

The relationship between alcohol and mankind is well documented from the earliest times. Wine-making equipment was found in the remains of an early neolithic village in Northern Iran dated about 5,000 BC (McGovern et al. 1996). In the Bible Noah 'planted a vineyard: and he drank of the wine, and was drunken' (Genesis 9^{20-21}).

Figure 1.1: Noah's Drunkenness



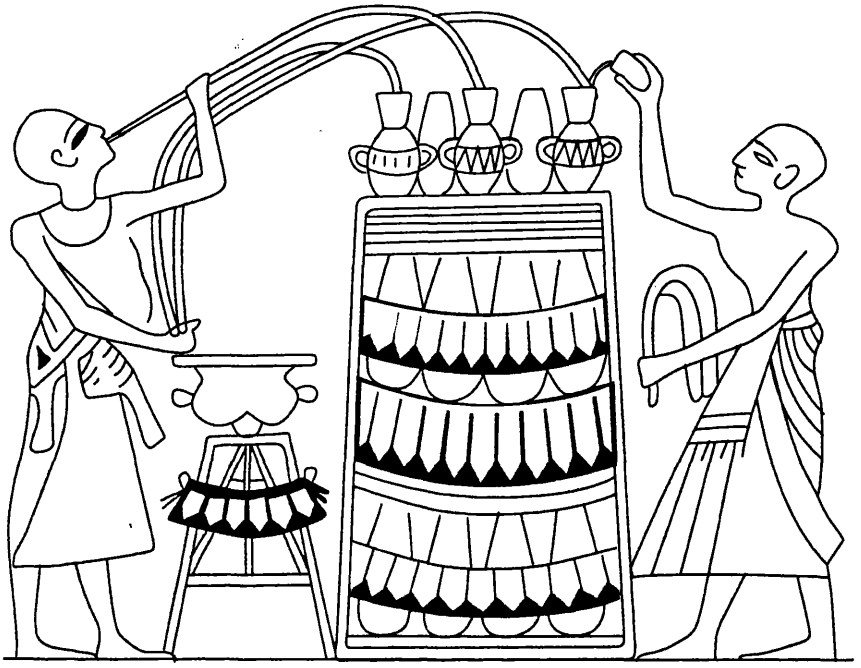
14-15th century sculpture on the façade of the Doge's Palace, Venice

Beer was first produced in Ancient Mesopotamia, Egypt and Greece. There is evidence that alcohol was used for both religious purposes in Egypt around 3,000 and also recreationally (Figure 1.2). The Ancient Greeks worshipped the God of wine, Dionysius, and seem to have been the first to develop large-scale wine fermentation and production, with export to other countries. The Romans in turn worshipped Bacchus, their God of wine, and were significant wine producers, planting vineyards across Europe. It is not just wine production that has survived through the ages. Barley provided both bread and beer from the first agricultural communities, but mead, made from fermented honey, was the preferred choice for most of Western Europe until Tudor times. Beer, brewed with hops, was introduced later from Germany.

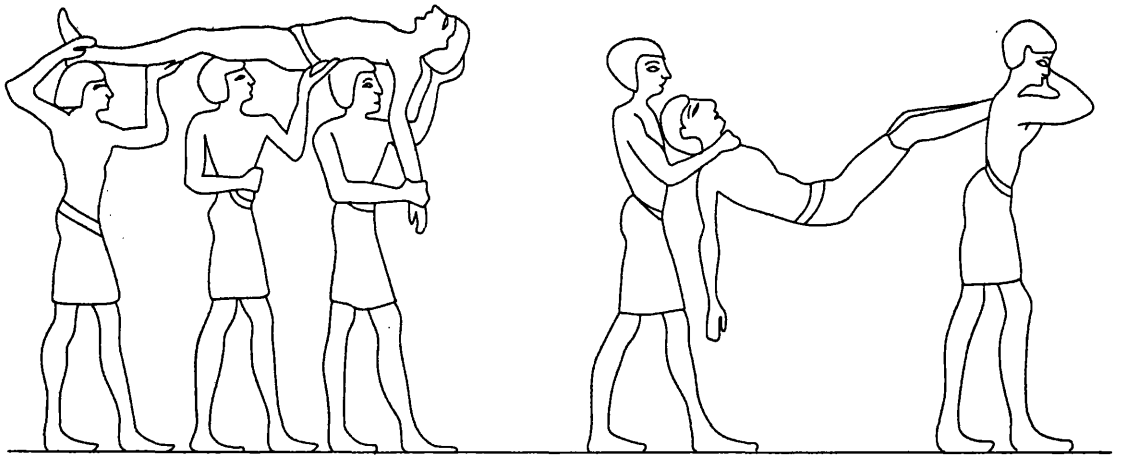
A twelfth century English country house could contain 'in the cellar or storeroom...pure wine, cider, beer, unfermented wine, mixed wine, claret, nectar, mead,...pear wine, red wine, wine from Auvergne, clove-spiced wine for gluttons whose thirst is unquenchable.' (Amt, 1993, page 151).

In the thirteenth century, wine was an integral part of religious life. Wine remained the predominant vehicle for alcohol and the vineyards were located primarily in the monasteries or nunneries where there seems to have been considerable use, 'There were nunneries where holy sisters allowed themselves seven litres a day' (Toussaint-Samat, 1994). However, moderation crept in with regulations; 'let none of the sisters secretly buy wine or in any way obtain it; but if any does receive some wine, let the porteresses receive it in the presence of the abbess or the prioress, and let them hand it over to the wine mistress...it will be the concern of the holy abbess to provide such wine as shall soothe those who are ill or who were raised more delicately' (Amt, 1993, page 227). But moderation was clearly not universal. In 1249, on a visit to La Salle-aux-Puelles, a convent near Rouen, the Chaplain and the Cardinal Protector reported that 'the prioress is drunk nearly every night.' (Amt, 1993, page 249). Joan of Arc (1412-1431) liked 'wine put in a silver cup, into which she put only half the amount of water, and five or six sops, which she ate, and she ate nothing else' (Toussaint-Samat, 1994). English wine production came to an abrupt halt when Henry VIII abolished the monasteries, and thus their vineyards, in 1534.

Figure 1.2: Egyptian Pictures, circa 1400 B.C.



EGYPTIAN PAINTINGS show alcohol as integral to the lives of the nobility. This depiction of wines being blended is from Amanemhat's Tomb, circa 1400 B.C.



INEBRIATED REVELERS have accompanied the presence of alcoholic beverages for millennia. This painting from Khety's Tomb, circa 2100 B.C., shows guests being carried away from a banquet after too much wine. Although drinking to excess was, and is, an unsafe practice, drinking any quantity of water 4,100 years ago was probably a much riskier undertaking.

Taken from Vallee, 1998

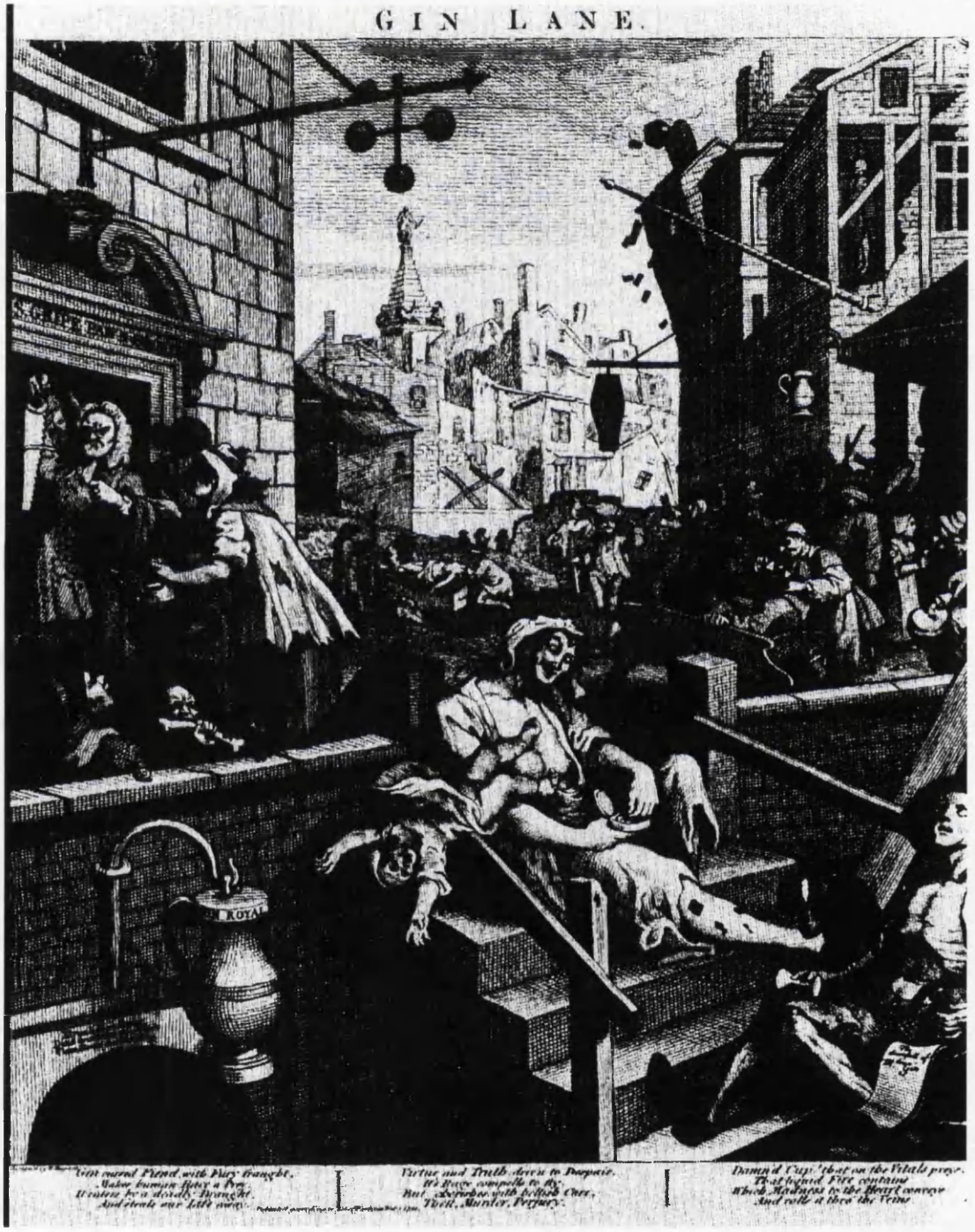
Alcohol has long been an accepted part of human daily life, and throughout the centuries there has been evidence that, for both men and women, consumption gradually increased. This in itself provided a potential source of revenue. The first taxes on alcohol were introduced in England by King Charles I in 1643 to pay for the civil war, and taxation has continued to this day. As a result it is possible to estimate adult consumption from Inland Revenue sources since records began about 300 years ago, but only for 'officially' produced beverages, not including home-made beers or wines. Beer consumption reached a peak in 1689 when consumption was 832 pints per person per annum. There is the first evidence of a fall in consumption with an increase in tax: the beer duty was tripled in 1690 and there was a dramatic fall in sales which was then partly reversed once the tax had been reduced (Spring and Buss, 1977). To this day taxation remains one of the best disincentives to alcohol consumption.

In 1688 William of Orange became King of England. Since both he and his wife, Queen Mary, were Dutch they were familiar with the hitherto relatively unknown spirit, gin. William ordered surplus grain to be used for gin production, and in 1690 stopped all import of foreign spirits. In the early eighteenth century, in Queen Anne's reign, the production of spirits was deregulated and in consequence consumption soared (Spring and Buss, 1977). Although the quality of the spirits produced was probably, at best, variable, it was extremely cheap: gin sold for 1 penny per pint, the equivalent of £0.60 today (Goodwin, 1994). At this time living conditions were very poor and the cheap spirits made drunkenness an attractive option. In 1726 the Royal College of Physicians issued this statement to the House of Commons on 'a great and growing evil' (Minutes of a meeting of the Royal College of Physicians, 1726):

> 'We the President and College or Commonalty of the faculty of Physicians in London, who are appointed by the Laws of this Kingdom to take care of the health of his Majesties Subjects in London and within seven miles circuit of the same, do think it our Duty most humbly to represent that we have with concern, observed, for some years past, the fatal effects of the frequent use of several sorts of distilled Spiritous Liquors upon great numbers of both Sexes, rend(e)ring them diseased, not fit for business, poor, a burden to themselves and neighbours and too often the cause of weak, feeble and distempered children, who must be, instead of an advantage and strength, a charge to their country. We crave leave further most humbly to represent, that this Custom doth every year increase, notwithstanding our repeated advices to the contrary.
>
> We therefore most humbly submit to the Consideration of Parliament, so great and growing evil.'

Attempts were made to reduce the consumption: in 1729 licences were introduced at £20 per year each to retailers and in 1733 the sale of spirits was limited outside houses (Spring and Buss, 1977) so that 'gin shops' developed.

Figure 1.3: Gin Lane by William Hogarth, 1751



This is probably the first depiction of Foetal Alcohol Syndrome, which results from maternal alcohol intake during pregnancy, and has features of microcephaly, upturned nose and a hypoplastic jaw. It also shows child neglect and abuse, and the financial and social decay resulting from alcoholism.

The inscription beneath Gin Lane is as follows:

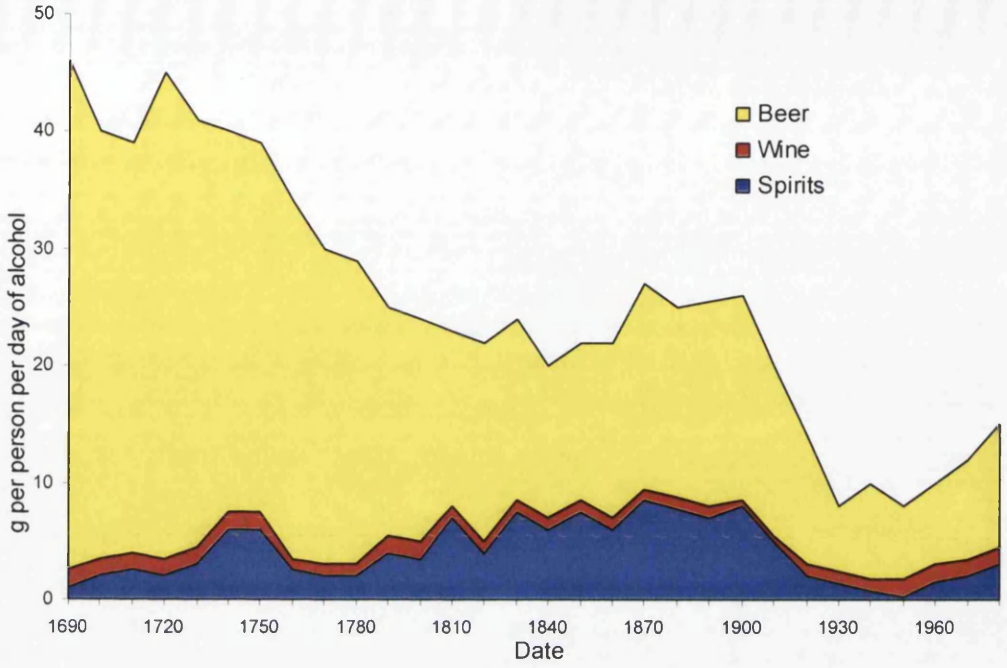
Gin cursed Friend, with a Fury fraught, Makes human Race a Prey;
It enters by a deadly Draught, And steals our Life away.
Virtue and Truth, driv'n to Despair, It's Rage compells to fly,
But cherishes, with hellish Care, Theft, Murder, Purjury.
Damn'd Cup! That on the Vitals preys, That liquid Fire contains
Which Madness to the Heart conveys, And rolls it thro' the Veins.

By the mid eighteenth century there was social pressure to curb drunkenness and in 1751 taxes on spirits were dramatically increased from one shilling to five shillings ten ¾ pence per gallon, at a time when the average family earnings were £32 per year (Spring and Buss, 1977); this caused a reciprocal drop in the national alcohol consumption.

Wine consumption as a part of everyday life had stopped with the Reformation of Henry VIII. However about one hundred and seventy years later in 1703 a British/Portuguese treaty allowed the import of Portuguese wine and port. This resulted in the gradual increase in consumption of port to alarming levels, particularly amongst the upper classes (four bottles per day was an average for men that could afford it). Records of beer intake are incomplete for the nineteenth century, as the tax was transferred to the constituents of beer (malt, hops and sugar) rather than beer itself. In 1880 Mr Gladstone changed the taxation so that duty was charged on the original specific gravity of beer. This caused the price to drop and intake to increase directly to about one pint per person per day (Spring and Buss, 1977).

In the nineteenth century the general public found drinking socially acceptable. Of Eliza Doolittle it was said that 'Gin was mother's milk to her' (Shaw, 1914) and by the turn of the century there was considerable concern for the social toll that it caused. Lloyd George addressed factory workers during the first World War with 'we are fighting Germany, Austria, and drink; and, as far as I can see, the greatest of these three deadly foes is drink' (Clark, 1994). There was concern that the war effort was being compromised by drunkenness amongst troops and munitions factory employees. The Central Control Board was created to limit the strength of alcoholic beverages and the number of hours during which they could be served (Greenaway, 1998). This was part of The Defence of the Realm Act, 1914 and was the first time that licensing laws were introduced in Britain.

Figure 1.4: Relative consumption of beer, wine and spirits over 300 years



Adapted from: Spring & Buss, 1977

The celebrations following the first World War resulted in an immediate increase in consumption of beer in the UK. In 1921 The Licensing Act removed the Central Control Board but kept the tax levels on alcohol and the licensing hours (Greenaway, 1998). Alcohol consumption again dropped at the beginning of the second World War due to rationing. Spirit consumption dropped in part because grain was diverted for food rather than for distillation.

Post-war consumption gradually increased until a large tax increase was imposed in 1964. Consumption remained steady until there was a tax reduction in 1973 (Spring and Buss, 1977). In subsequent years the taxation on wine has fallen and the sales of wine has risen, so that today, in 1998, UK wine consumption is at its highest ever, estimated at 2.03 litres of pure alcohol per person per year (Brewers' Society, UK).

1.1.2 MEDICINAL USE

That alcohol has long been part of the diet and way of life is due, at least in part, to the boiling of the water in the brewing process. An alcoholic beverage was well-known as an adjunct in the treatment of cholera. For example, in the seventeenth century Nottingham's fresh water supply derived from the River Leen which contained sewage. However, there was a plentiful

supply of ale-houses (about one for every eighty people) thus ensuring some relatively clean fluid to drink (Williams, 1962).

Alcohol is also well-known as an antiseptic, anaesthetic and medication. In the twelfth century alcohol was used for a wide variety of ailments including:

- Foulness of breath: 'tops of myrtle be grated and cooked in wine until reduced to one half. Let the wine be drunk on an empty stomach'
- Deafness: 'take the cooked fat of fresh eels, juice of caprifolium, juice of Jove's beard, and a handful of ant's eggs...After cooking add vinegar or wine in sufficient quantity to make it more penetrating. Pour it into the sound ear and stop up the defective ear.'
- Swollen tonsils: 'Mugwort grated...mixed with wine and cooked in honey' (Amt, 1993, page 106).

Part of the 'medicinal' properties of alcohol may derive from its mood-lifting effects. During epidemics and plagues alcohol consumption tended to increase. In a description of a smallpox epidemic in Ulster in 1750: 'The increased consumption of spirits by the young and by women particularly alarmed Henry. Most of the children of the lower people, who were seized with it first, died; which was occasioned by an unhappy practice of giving the children whiskey in order to strike out the pox as they termed it' (Malcolm, 1986, page 43).

In 1886 Dr Brosius presented a paper at the International Psychiatrical Congress and described some of the nervous and psychiatric diseases attributable to alcohol:

1. The moderate indulgence in spirituous liquors is in itself never hurtful in psychoses or nervous diseases.
2. Larger doses (a pint of Rhine wine, sherry etc.) are calmatives and even hypnotics in states of excitation.
3. In all patients refusing nourishment, in dyspepsia, and especially in paralytics, alcohol is a respiratory nutriment, as defined by Binz.
4. In all cases of withdrawal after morphine addiction etc. to prevent collapse, a liberal administration of alcoholic beverage is necessary (Brosius, 1886, pages 506).

In the late nineteenth century substances (mercury, cocaine) were frequently prescribed for 'neurotherapy' and in particular for the commonly diagnosed neuralgias (headache, insomnia). Of these, the most popular, particularly amongst ladies, was laudanum in a 1 part to 10 parts wine and opium mixture.

Figure 1.5: Illustration from Hieronymus Brunschwig's *Lieber de arte distillandi, 1500*

DISTILLATION created alcoholic drinks of unprecedented potency. This distillation apparatus appeared in Hieronymus Brunschwig's *Liber de arte distillandi,* the first book published on the subject, in A.D. 1500. The book featured these claims for distilled alcohol: "It causes a good colour in a person. It heals baldness ... kills lice and fleas.... It gives also courage in a person, and causes him to have a good memory."

Taken from Vallee, 1998

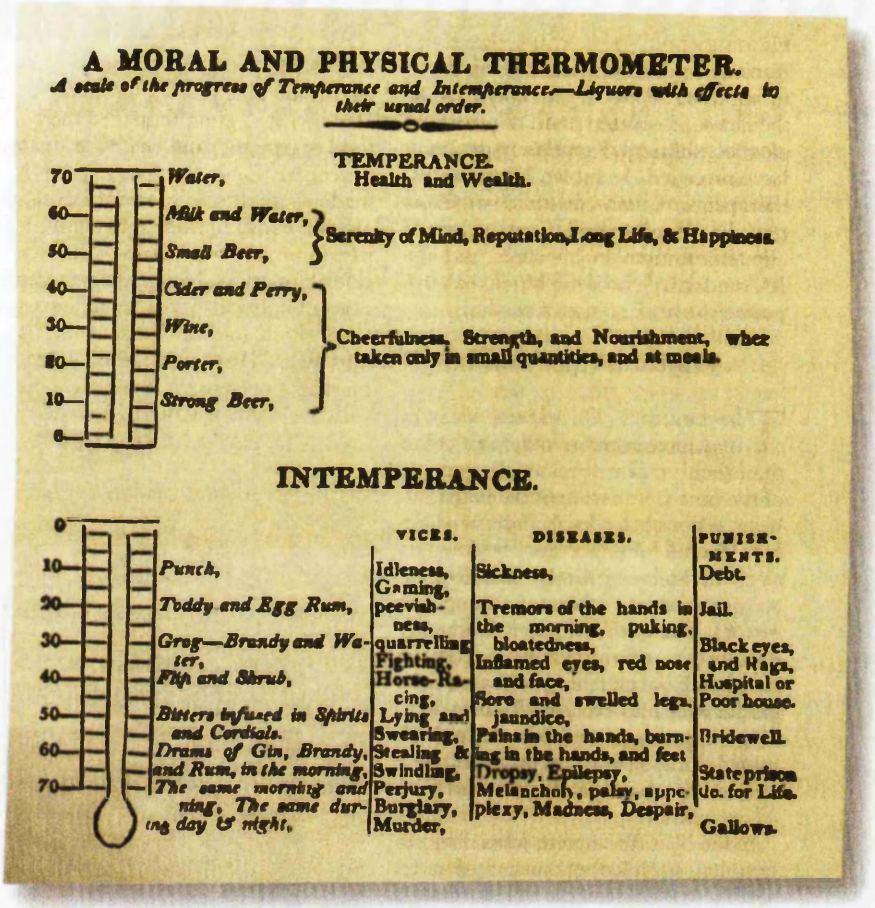
1.1.3 COUNTER ATTACK: THE TEMPERANCE MOVEMENT

The long-term side effects and problems with excessive drinking had been well known for centuries. The preacher in Ecclesiasticus (The Apocrypha, Ecclesiasticus 31^{29-31}) stated that: 'Wine drunken with excess maketh bitterness of the mind, with brawling and quarrelling. Drunkenness increaseth the rage of a fool till he offend: it diminish strength, and maketh wounds. Rebuke not thy neighbour at the wine, and despise him not in his mirth: give him no despiteful words, and press not upon him with urging him [to drink]'. St Paul in his letter to the Ephesians exorts them 'be not drunk with wine' (Ephesians 5^{18}).

There was a backlash in the formation of the Temperance Movement. The Temperance Movement began in North America in New York in 1808. In Britain the Temperance and Prohibitionist movements were particularly powerful in the north of England and Scotland, backed by wealthy industrialists. This group had an immediate incentive for obtaining a stable and disciplined work force. They gained some political influence in the Liberal party after 1870 (Greenaway, 1998) but no real political changes were effected until the First World War.

In America the movement lost momentum during the American Civil War (1861-1865) but there was a resurgence of interest following it, particularly as the drinks industry was seen to be expanding and gaining some political influence. The Prohibition Party was established in 1869 and the National Women's Christian Temperance Union (WCTU) in 1874. The Anti-Saloon League of America sought to close saloons or prevent the licensing of them for sale and consumption of alcohol, as they were regarded as dangerous establishments threatening the family. These organisations remained relatively small but were able to influence the major political parties. One of the ways they achieved this was by publicising evidence of over-indulgence, particularly effective through a collaboration of the WCTU and the Anti-Saloon league, so that by 1916, 23 out of the then 48 states had anti-saloon laws. In 1919 the 18th Amendment to the Constitution prohibited the sale of alcohol of greater strength than 0.5%, and this remained in place until it was repealed in 1933, since when there has been negligible Temperance activity. During the time of Prohibition the annual consumption per capita fell from 9.8 litres to 3.4 litres.

Figure 1.6: Thermometer analogy by physician and politician Benjamin Rush (1746-1813)



Taken from Vallee, 1998

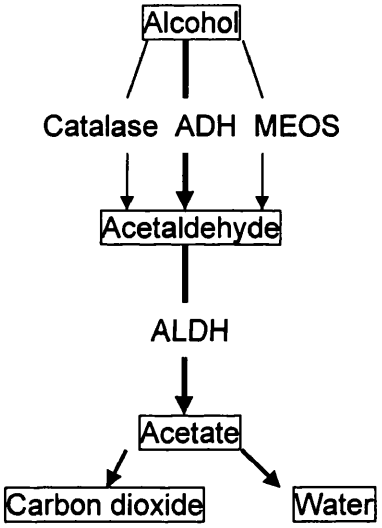
Even the ban on alcohol in 1886 in America did not include 'medicinal tonics', and during Prohibition 'intoxicating liquors with an alcohol content of more than 0.5% were permitted for medical, sacramental and industrial purposes'. This loophole was used in Birmingham, England, in a female Temperance Society which in 1836 set rules that included 'We agree to abstain from all intoxicating liquors, except for medicinal purposes and in religious ordinances'. Lydia Pinkham was a famous American Temperance campaigner and was known for her loud demonications against drink. Her contribution to the health and welfare of America was 'Pinkham's Tonic', however the alcoholic content was 20.6%.

In summary, the use of alcohol in the past has been influenced by availability, culture, religion, medicinal use and political factors. Of these, the political factor is probably the strongest, with the balance between the affluence of consumers and the taxation of alcohol regulating demand.

1.2 The metabolism of alcohol

Alcohol is essentially consumed in beverages. Following intake the majority is oxidised in the liver, although about 2% is eliminated in breath and urine. Hepatocyte oxidation is primarily by alcohol dehydrogenase (ADH) but also by the microsomal oxidising system (MEOS) and the catalase pathway located in peroxisomes (Figure 1.7). The MEOS activity has been attributed to CYP2EI, an isoform of cytochrome P450, and is increased by chronic alcohol misuse and drugs and is one of the explanations for alcohol tolerance.

Figure 1.7: The metabolism of alcohol



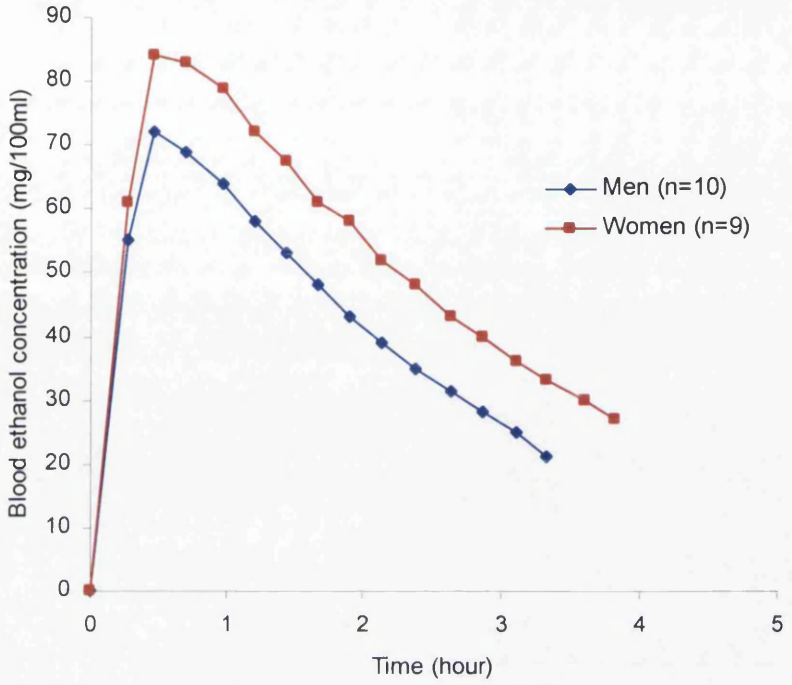
Acetaldehyde is highly toxic and is oxidised by aldehyde dehydrogenase (ALDH), found in many tissues. Acetate is produced which is metabolised to carbon dioxide and water.

1.2.1 GENDER DIFFERENCES

Female alcohol misusers are physiologically disadvantaged with regard to the effects of alcohol. If an oral test dose of alcohol is standardised for total body water no gender difference is observed. However women have a smaller total body water than men (Goist and Sutker, 1985) and so the same amount of alcohol results in a higher blood level in women (Figure 1.8). There is also reduced gastric acetaldehyde dehydrogenase activity and first-pass metabolism in women, although the effect is small and these contribute less than 5% of the total metabolism.

Figure 1.8: Gender differences in ethanol metabolism

Following ethanol ingestion of 0.5 g/kg



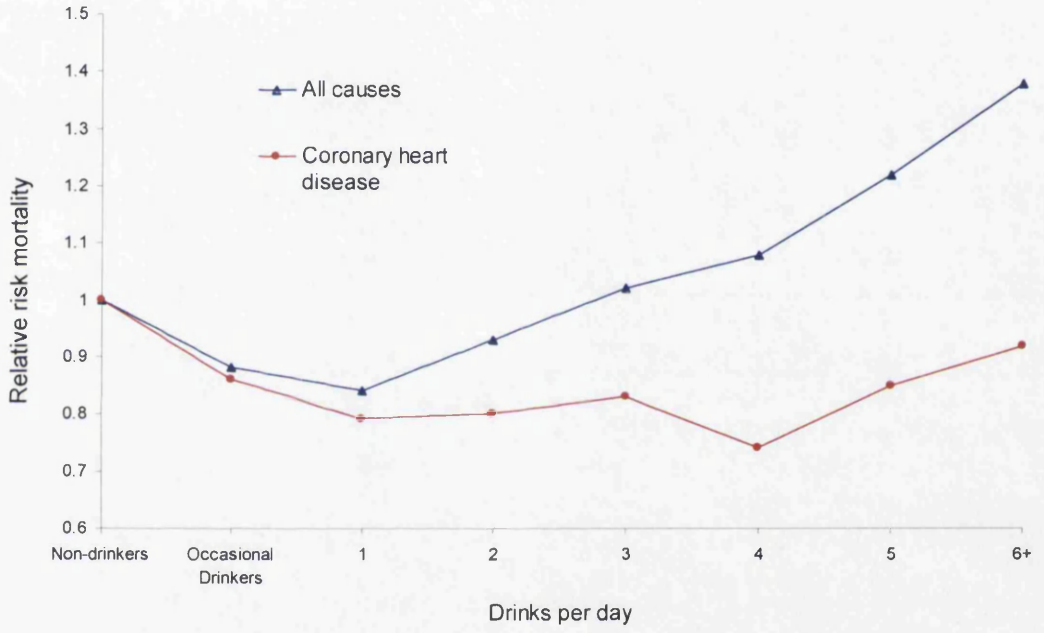
Adapted from Marshall et al, 1983

1.3 Benefits of Alcohol

For most social or moderately drinking individuals, the primary reason for drinking alcohol is its mood-lifting properties. Other than the mild euphoria there is the much promoted (by the alcohol industry) effect of 'alcohol is good for your heart'. This originated from some studies showing that the mortality in moderate drinkers (1 or 2 drinks per day) was less than in abstainers or heavy drinkers (Boffetta P. and Garfinkel L., 1990; Shaper et al. 1988; Gronbaek et al. 1994; Power et al. 1998). There was an observed lower mortality (all causes) in those drinking one drink per day than total abstainers or high alcohol consumers - the 'J or U-shaped effect': for all-cause mortality Boffetta and Garfinkel showed a relative risk of 0.84 for those drinking one drink per day, compared to 1.0 for total abstainers. Gronbaek (Gronbaek et al. 1994) found a U-shaped curve in the Copenhagen Heart Study and showed that this was independent of sex, age, body mass index and smoking, with the lowest mortality (relative risk set at 1.0) for those drinking 1-6 beverages per week and the highest (relative mortality risk 2.29) for those drinking more than 70 beverages per week. Total abstainers had a relative risk of 1.37.

Although Shaper (Shaper et al. 1988) demonstrated a U-shaped curve, it was attributed to pre-existing disease and it was concluded that, overall, there was no protective effect from alcohol. Boffetta and Garfinkel (Boffetta P. and Garfinkel L., 1990) showed in their study of men aged 40-59 followed over 12 years, a reduced mortality amongst moderate drinkers from coronary heart disease, with a relative risk of 0.74 for those drinking drinks per day compared to 1.0 for total abstainers. This J-shaped mortality curve was seen for total mortality, total cancer mortality, coronary heart disease and cardiovascular disease (see Figure 1.9).

Figure 1.9: Alcohol consumption and mortality



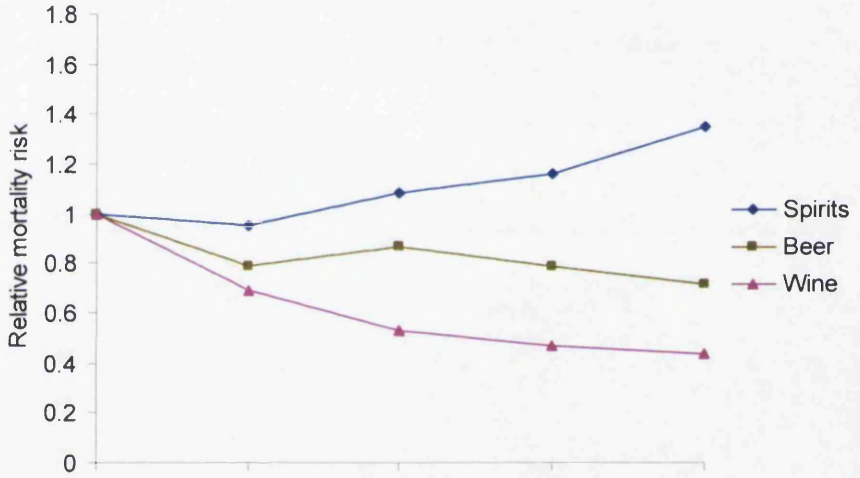
The mortality relative risk has been adjusted for age and smoking. Adapted from Boffeta and Garfinkel, 1990

Thus there does seem to be a protective effect associated with a moderate (1 drink per day) intake of alcohol. The underlying mechanism for this has been suggested to be that alcohol (or its metabolite, acetaldehyde) causes a reduction in the formation of atherosclerotic lesions and so reduces the incidence of coronary heart disease. There is an alcohol-associated increase in the level of high density lipoproteins, a reduction in platelet adhesion (from the effect of prostacyclins released by the effect of acetaldehyde) and fibrinogen formation, an increase in fibrinolysis and a reduction in lipoprotein A and insulin (Stampfer et al. 1988). All of these processes have been implicated in atherosclerosis.

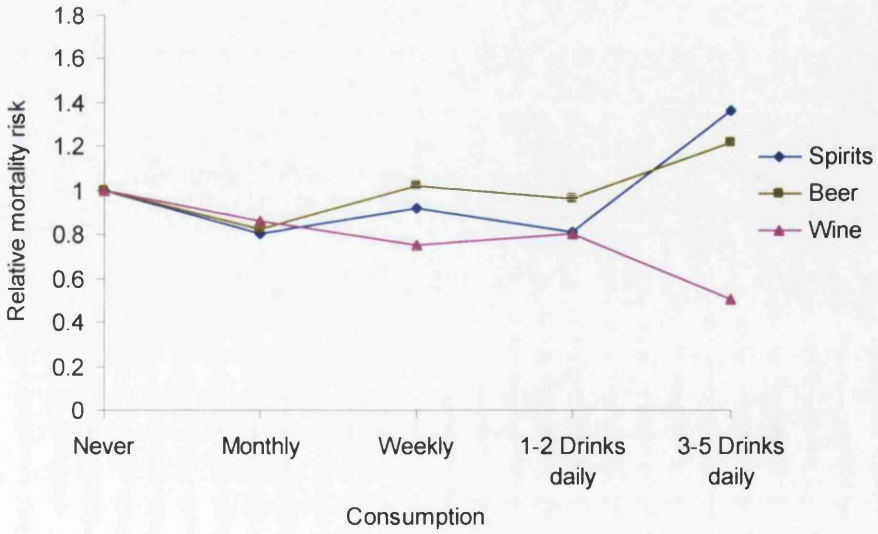
In the Copenhagen City Heart Study (Gronbaek et al. 1995a) mortality from cardiovascular causes (Figure 1.10 a) was lowest in wine drinkers and had an inverse relationship with increased wine intake. Spirit drinkers showed an increase risk with increasing intake, with a relative mortality risk of 1.35 for those drinking 3-5 drinks daily, with a risk set at 1.0 for total abstainers. Mortality from all other causes (Figure 1.10 b) again showed an inverse relationship for wine drinkers with those having the highest consumption having the lowest mortality.

Figure 1.10: Relative risk of mortality in relation to intake of wine, beer and spirits

a) From cardiovascular and cerebrovascular causes



b) From all other causes



The risk is set at 1.0 for subjects who have never drunk. Adapted from Gronbaek et al. 1995a.

However recently published research in a Scottish population (Hart et al. 1999) showed no clear evidence of a protective cardiovascular effect from drinking less than 22 units per week.

1.4 Harmful Effects Of Alcohol

Immoderate alcohol consumption may result in a broad spectrum of medical, psychiatric and social problems. These in turn are an expensive burden on the National Health Service and Society at large.

1.4.1 THRESHOLD LEVELS FOR DAMAGE

The level of drinking that constitutes misuse and results in alcohol-related damage was first studied in France. Durbec, Touyons and Pequinot tried to identify the amount required to develop cirrhosis. The risk of developing significant liver injury increases with increasing alcohol intake above the threshold levels. Bellentani *et al* showed that the threshold level for developing liver injury, with or without cirrhosis, was relatively low at 30 g per day of alcohol and that with increasing alcohol intake there is increasing risk (Bellentani et al. 1997).

The Department of Health have suggested that 'sensible limits' for drinking are 21 units per week for men and 14 units per week for women. A unit is defined as half a pint of beer, a glass of wine or a single measure of spirits. In December 1995 they changed this to 3-4 *per day* for men and 2-3 for women, and the reason given being that this removed the apparent condoning of binge drinking at weekends. However, the new limits caused considerable confusion with a general interpretation that it was now acceptable for men to increase their drinking to 28 units, and women to 21 units, per week.

The Royal Colleges of Physicians and Psychiatrists have collaborated to give indications of harmful levels of alcohol intake, and to demonstrate the relationship between alcohol intake and physical harm. It was agreed that the threshold for definitely harmful drinking is 50 units per week for men and 35 units per week for women, while that for heavy or hazardous drinking is >35 units per week for men and >25 units per week for women (The Royal College of Physicians, 1987, page 108).

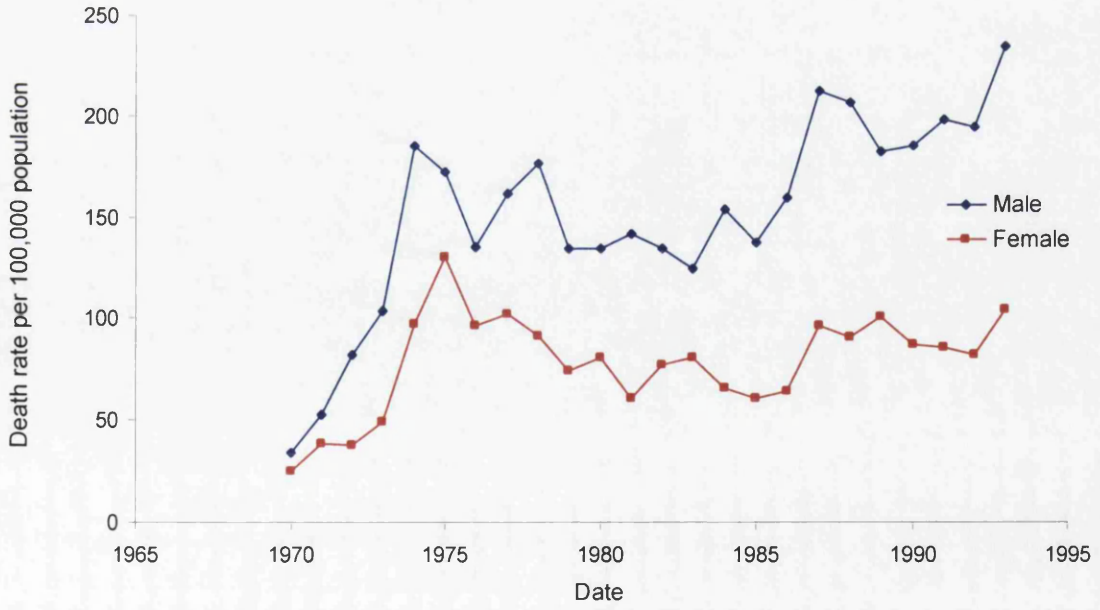
Table 1.1: The Royal College of Physicians issued advice on 'safe' and at risk drinking (The Royal College of Physicians, 1987, page 108)

	Men (units per week)	Women (units per week)
Low risk	21	14
Hazardous	21-49	14-35
Harmful	50+	36+

1.4.2 MEDICAL CONSEQUENCES

Regular excessive alcohol consumption is known to cause a wide range of diseases and disorders and there has been a reported increase in mortality over the last 25 years (Figure 1.11). This increased reporting is unreliable as there are disincentives to putting 'alcohol' as the cause of death on a certificate, due to problems from either insurance or family sensibilities, and also there are very low post-mortem rates.

Figure 1.11: Number of recorded alcohol related deaths, rate per 100,000 population in England and Wales (Scottish Council on Alcoholism)



Alcohol-related deaths: alcoholic psychoses, alcohol dependence syndrome, non dependent abuse of alcohol, alcoholic cardiomyopathy, alcoholic liver disease, alcoholic poisoning.

Trends in the mortality from alcohol may be estimated by looking at death rates from alcohol-specific causes: alcoholic cirrhosis, alcohol dependence syndrome and alcoholic

cardiomyopathy. There appears to have been an increase in all three during recent years (Table 1.2), although this may be in part a consequence of a small increase in the willingness to document alcohol on death certificates. Until July 1984, alcoholism on a death certificate necessitated a Coroner's Inquest (Maxwell and Knapman, 1985).

Table 1.2: Total recorded number of deaths from alcohol-specific causes

	1984	1989	1994
Alcohol dependence syndrome	97	148	230
Non-dependent abuse of alcohol	108	124	139
Alcoholic cardiomyopathy	58	104	108

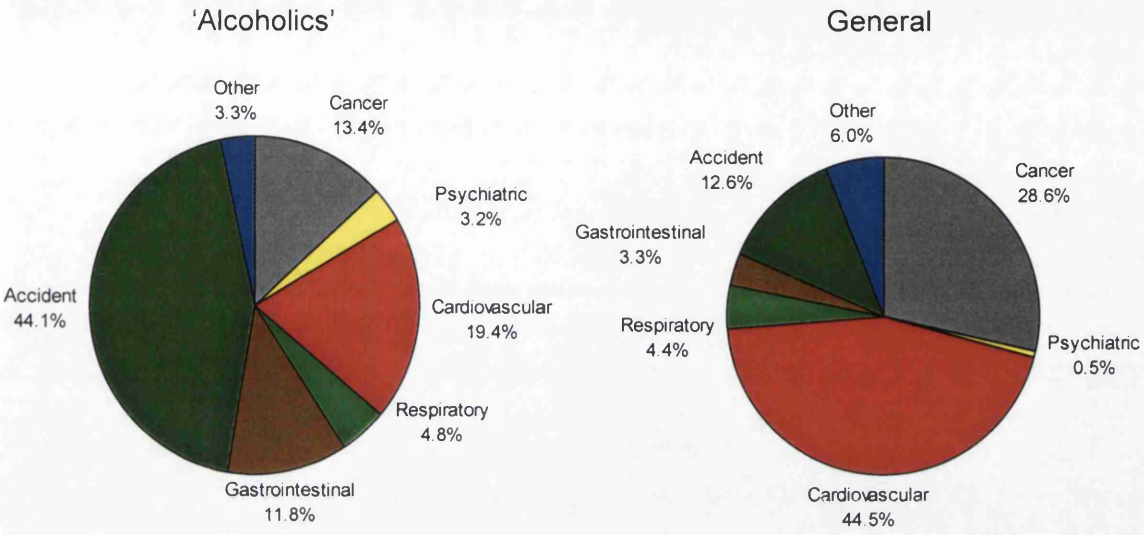
Adapted from MCA newsletter Vol 16 (2) 1997

However, these specific causes provide only a small component of the overall contribution of alcohol to mortality. There are several other alcohol-related categories such as alcohol psychosis, alcoholic liver damage, toxic effect of alcohol, and more. Alcohol contributes to accidents, suicides and other deaths, but may remain unrecorded on death certificates. The effect of alcohol on morbidity is considerably higher.

Alcohol permeates every system in the body as it is water soluble. Every system in the body is therefore liable to alcohol-induced damage, and the spectrum of deaths attributed to alcohol misuse reflects this (Figure 1.12).

The commonest causes of death in the general population are cardiovascular causes, cancer and then accidents. These remain the three commonest causes for alcohol misusers, but accidents constitute a much larger proportion (44.1%) in this group than in the general population (12.6%). The overall mortality in individuals misusing alcohol is three and a half times that for the general population (Adelstein and White, 1976).

Figure 1.12: Causes of death in male 'alcoholics'

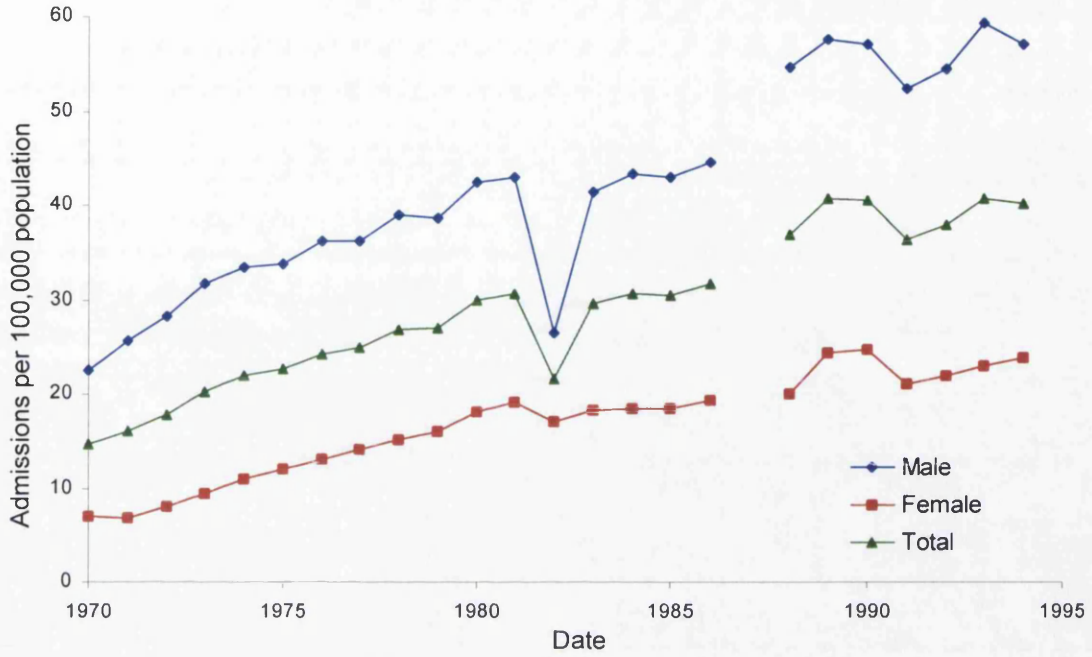


Adapted from Adelstein and White, 1976

1.4.2.1 Alcohol dependence

Alcohol dependency refers to those individuals who 'have a compulsion to drink...the same amount each day...and suffer withdrawal symptoms on stopping', Diagnostic and Statistical Manual of Mental Disorders (DSM III) (American Psychiatric Association, 1987). This is the most obvious immediately attributable disorder directly related to alcohol misuse. The number of individuals admitted to hospital for alcohol dependence over the past few years has been increasing (Figure 1.13). There is an annual increase in the rate of admissions to hospital for alcohol dependence of 1.38, 0.71 and 1.04 admissions per 100,000 population for men, women and the total population respectively.

Figure 1.13: Admissions to mental hospitals and psychiatric units in England and Wales, for alcohol psychoses, alcohol-dependence syndrome and non-dependent abuse of alcohol, per 100,000 population



From: 1997 UK alcohol statistics (The Scottish Council on Alcohol), derived from Health and Personal Social Services Statistics for England-Department of Health. A new system was introduced in 1987 and there are no accurate statistics for this year.

1.4.2.2 Alcoholic liver disease

Alcoholic liver disease forms the largest component of gastrointestinal causes of alcohol related mortality. The first histologically identifiable change seen in alcoholic liver disease is termed fatty liver. It is usually asymptomatic and can develop within days of heavy drinking (Lieber, 1995). Histologically, droplets of triglyceride can be seen within the hepatocytes. This can progress to alcoholic hepatitis, severe fibrosis and finally cirrhosis. However only 20% of chronic alcohol misusers progress to cirrhosis and the reasons for this have been postulated to be a combination of genetic and environmental factors. Cirrhosis can develop without the intervening stage of alcoholic hepatitis.

Cirrhosis may develop after only a minimal alcohol intake over a short period of time, or, despite drinking considerable amounts over a life-time, never develop. The reasons for this remain unclear. Acetaldehyde, a metabolite of alcohol, results in an increase in collagen production. Long term excessive alcohol consumption induces hepatic stellate cells to deposit collagen. Further, alcohol has been shown to cause a decrease in the amount of

phosphatidlycholine which results in a decrease in collagenase activity and so an increase in collagen formation. Therefore the development of cirrhosis may be regarded as essentially an imbalance in the degradation and production of collagen (Lieber, 1995).

Savolainen *et al* (Savolainen et al. 1993) showed that in a series of 400 male autopsies no significant features of alcoholic liver disease were seen in those drinking less than 40 g per day. Those drinking 40-80 g per day had an increased incidence of fatty liver and alcoholic hepatitis, while those drinking more than 80 g per day had an increased incidence of liver cirrhosis. The threshold for liver damage was seen to be 60 g per day, or 49 units per week.

1.4.2.3 Malnutrition

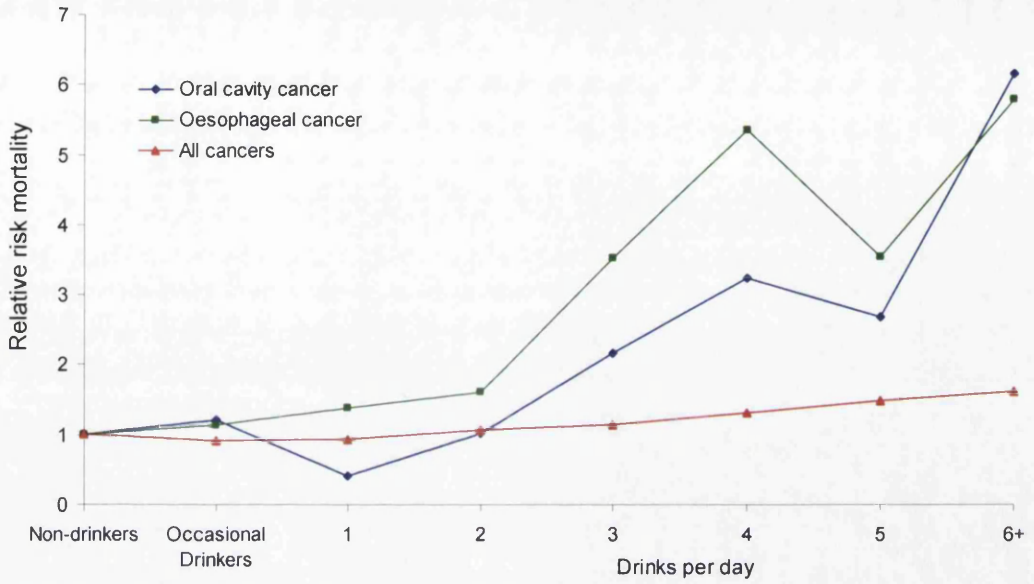
Alcohol has a calorific value of 29.7 KJ per gramme, twice the energy of carbohydrate or protein (Lieber, 1984). If the alcohol forms a substantial part of the dietary energy intake chronic alcohol misusers will have a nutritional intake deficiency, with resultant vitamin reductions and deficiency syndromes, particularly of folate and thiamine.

There is also the possibility of secondary malnutrition because of pancreatitis, with a reduction in the absorption of fat soluble vitamins (Vitamin A, D, E and K), and impaired hepatic metabolism.

1.4.2.4 Cancer

There is an association of alcohol with carcinoma of the gastrointestinal tract, breast and pharynx, and primary liver cell cancer (Sabroe, 1998). This is synergistic with the effects of smoking (Tuyns et al. 1988). This may be due to secondary activation of carcinogens via cytochrome P4502E1. There is a causal relationship between carcinoma of the mouth and oesophagus and alcohol intake (Boffetta P. and Garfinkel L., 1990) (Figure 1.13), and this relationship is particularly strong for spirits, as opposed to beer or wine drinking (Gronbaek et al. 1995a). It has been suggested that this may be due to the fact that beer and spirits have higher quantities of nitrosamines, which are known to be carcinogenic, than wine (Berger, 1998).

Figure 1.14: Relationship between alcohol intake and cancer



Adapted from Boffetta P. and Garfinkel L., 1990

The effect of differences in type of alcoholic beverage has also been explored. Wine has been shown not to increase the risk of upper gastrointestinal cancers, while a moderate intake of beer and spirits result in an increased risk (Gronbaek et al. 1998). It has been suggested that this may be a result of one of the compounds in wine, resveratrol, inhibits the initiation, promotion and progression of tumours (Jang et al. 1997).

Hepatocellular carcinoma is rare in the general population but does occur in about 10% of alcoholic cirrhotics, particularly in those who are abstinent (Lee, 1966; Morgan and Sherlock, 1977). Breast cancer is a common cancer but it has been suggested that alcohol may be associated with a small increase in the risk (Rimm et al. 1991; Schatzkin et al. 1987), although this remains controversial.

1.4.2.5 Accidents

Accidents remain the commonest cause of death for alcohol misusers (Figure 1.12) and for those aged under 40 in the general population. As such they represent a major health problem. There may be accidents in the home, work or in road traffic and alcohol is a major factor in fatal outcome. Accidents can occur with any level of consumption in any individual, in particular those who are not chronic misusers. It is estimated to be a contributory factor in 20-

30% of all accidents (Table 1.3). In young people (< 30 years) about half of accidents are road traffic accidents.

Table 1.3: Number of deaths recorded by Birmingham Coroner in 1980 which were related to alcohol consumption

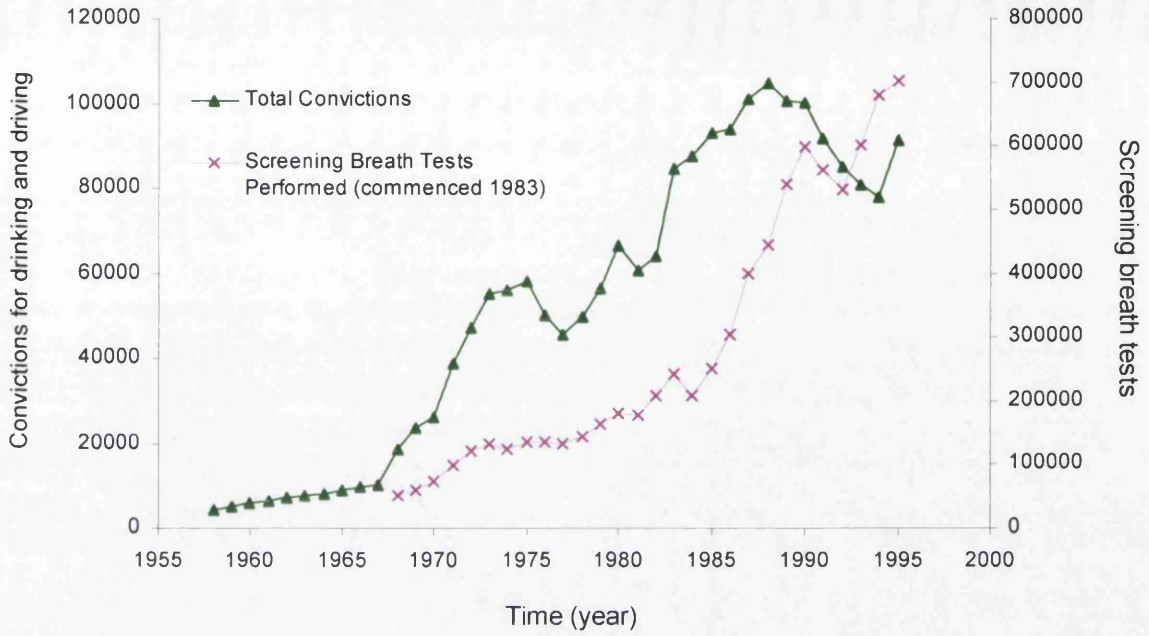
		Alcohol related deaths	
Coroner's verdict	Total No of deaths	No	%
Alleged homicide	27	*17*	*63*
Various 'other'	23	*8*	*35*
Road traffic accidents	111	*38*	*34*
Open verdict	27	*8*	*30*
Suicide	96	*20*	*21*
Total	284	*91*	*32*
Home accidents	165	*19*	*12*
Accidents elsewhere	57	*7*	*12*
Natural causes	72	*2*	*3*
Work accidents	11	*0*	*0*
Total	305	*28*	*9*
Grand total	589	*119*	

Adapted from Whittington, 1982

Road traffic accidents

This is the commonest alcohol-related accident. In Great Britain, in 1996, there were 3,598 road traffic accidents and 300,000 injuries (Department of Transport, 1997). There has been a steady reduction since 1966 which may be due to action on drink driving from 1967 with the introduction of 80 mg% legal limit for driving, traffic 'calming' measures, and car safety procedures such as seat belts. The increasing seriousness of accidents has been shown to be related to increasing level of alcohol involvement (Glucksman, 1994). A survey conducted at Grand Rapids in the USA in 1962-3 showed that an increased risk of being involved in an accident is related to increased blood alcohol concentration (BAC): at 80mg% BAC there is a two fold increase in accidents, at 100mg% there is a seven fold increase, at 150mg% there is a ten fold increase and at 200mg% there is a twenty fold increase.

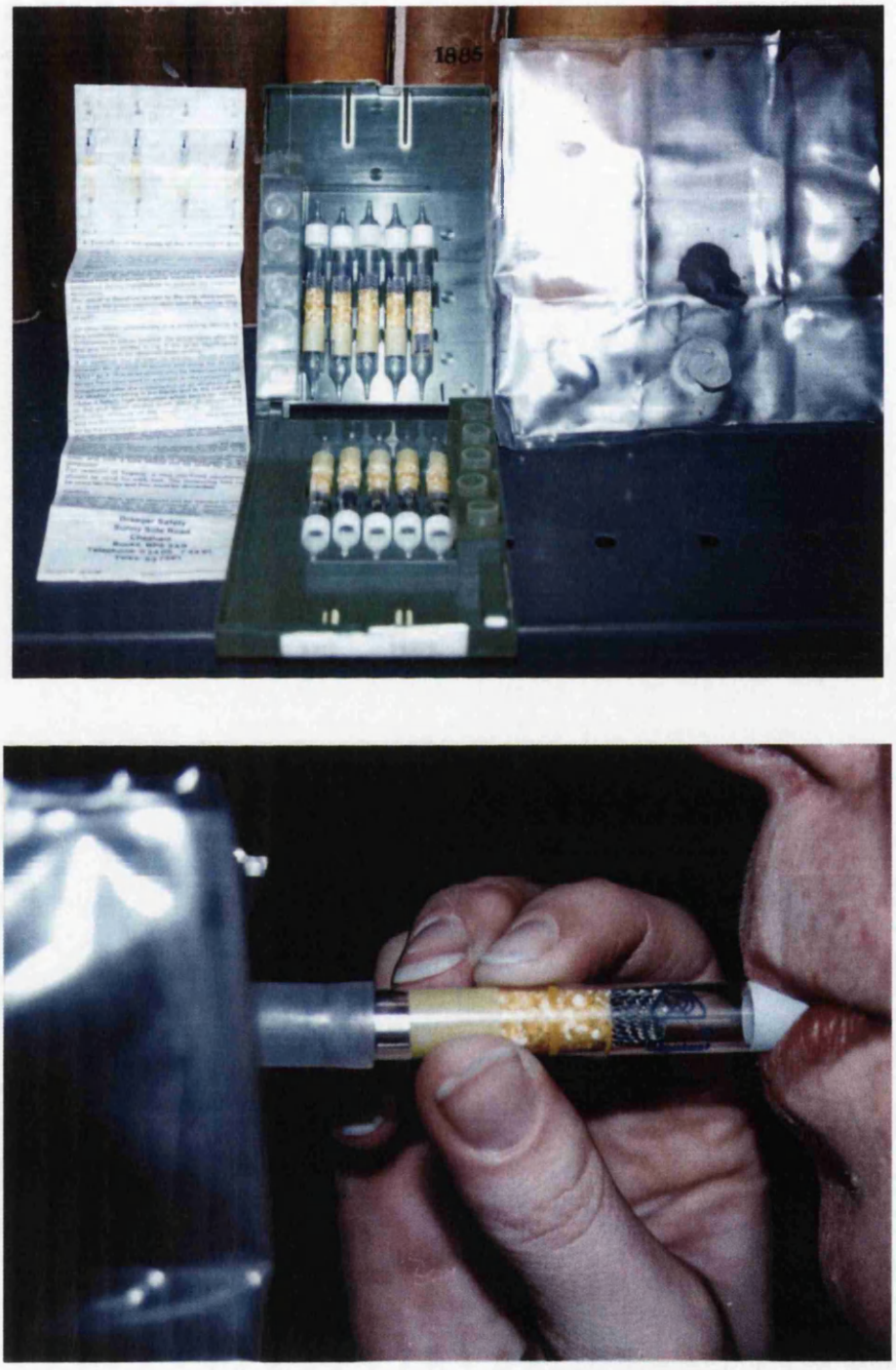
Figure 1.15: Convictions for drinking and driving, England and Wales.



Adapted from Department of Transport, 1997

There has been an increase in the number of convictions for drink driving until 1989, and since then a small decrease until 1995 (Figure 1.15). On the 9th October, 1967 an 80mg% level limit of alcohol for driving was brought into force with the Road Safety Act of that year. In order that roadside estimates could be made of BAC, various devices were evaluated. With much publicity, one was adopted that related measurements of breath alcohol to blood alcohol. However these devices could not be used as sole evidence in court cases. The officially approved breathalyser was the Alcotest (Figure 1.16). Although there was a reduction in casualties briefly after 1967, by 1972 there was an increase. Following an inquiry set-up in 1974, known as the Blennerhassett after its chairman, evidential breathalysers were introduced on 6th May, 1983.

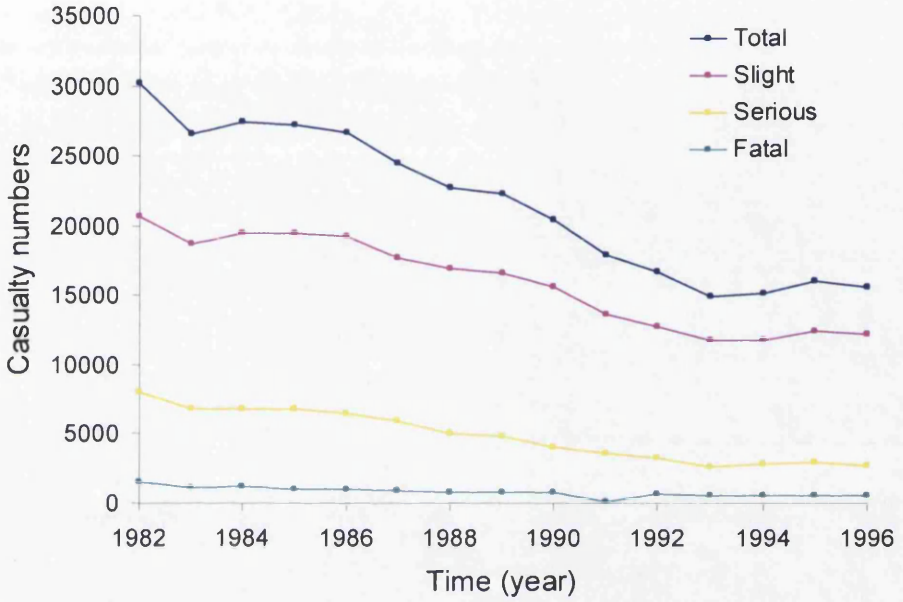
Figure 1.16: The Alcotest (Breathalyser), 1967



By kind permission of The Metropolitan Police Museum

Although there has been an increase in the number of convictions for drink driving, there has, since the introduction of evidential screening, been a reduction in casualties related to alcohol (Figure 1.17). Other reasons for this may be media coverage of the breathalyser and a campaign from 1967 to inform the public of the drink driving regulations.

Figure 1.17: Estimates of total casualties in road accidents involving illegal alcohol levels.



Adapted from Department of Transport, 1997

Home accidents

It is estimated that there are about 5,000 deaths per annum from home accidents (Whittington, 1982). A further 2,200,000 non-fatal accidents are treated in hospital and 900,000 are managed by the General Practitioner, a far greater number than those involved in road traffic accidents. Alcohol probably contributes to about one third of domestic accidents, often resulting in overdoses, falls, fires and poisoning (Editorial BMJ, 1983). Alcohol is also a factor in drownings. As on the road, there is an increase in bravado and high risk behaviour with a reduced ability to evade problems.

Industrial accidents

There has been a reduction in the number of industrial accidents in recent years, possibly due to the shift in work pattern from the traditionally heavy occupations, such as coal mining, to the

lighter service industries. There has also been a significant increase in Health and Safety at work expectations, driven by litigation.

Alcohol is implicated in accidents occurring after lunch and late in the evening. Those people identified as alcohol misusers were shown to have 50% more accidents than the rest of the work force (Beaumont and Allsop, 1983). It has been shown that increased alcohol intake results in an increase in likelihood of a road traffic accident, and a parallel detrimental effect would be expected furthermore on operating machinery. If it remains acceptable to drink at lunchtime or come to work with a hangover, the incidence of accidents is unlikely to decrease further.

1.4.3 SOCIAL CONSEQUENCES

Alcohol is involved in many aspects of social harm, although it may not be the sole or even the most important component. Other members of the family and colleagues may be affected more than the index person.

1.4.3.1 Alcohol and crime

Alcohol is a major factor in the perpetration of crime, particularly violent crime. In a study surveying all those arrested over a five month period in an English seaside resort, the number of those who had been drinking during the four hours before the crime was recorded (Jeffs and Saunders, 1983). Overall 64% had been drinking (Table 1.4).

There has been a steady increase in violent crime in recent years and in 1990 a Home Office study cited increased consumption of beer as the most important factor (Home office research study, 1997), however causality has not been proven. In a study in Western Scotland, half either of those found guilty of murder, or their victims, were intoxicated at the time of the event (Gillies, 1976).

Table 1.4: Reported alcohol consumption in the four hours prior to the offence

Offence	% reporting alcohol intake
Drunkenness	100
Drink driving	100
Criminal damage	88
Breach of the Peace	83
Assault	78
Theft	41
Miscellaneous	37
Burglary	26
Total % reporting alcohol intake	64

Adapted from Jeffs and Saunders, 1983

1.4.3.2 Alcohol and employment

Alcohol misuse affects not only the employee, but also the employer and other work colleagues. The employer is affected by a profit reduction secondary to decreased efficiency from absenteeism and increased accidents. The employee experiences the effects of increased illness and injuries and reduced work performance. This results in reduced pay and promotion, poor self-esteem, and finally unemployment. The non-drinking work colleagues are inconvenienced by the poor time-keeping and an increased risk of accidents.

Occupations most associated with a risk of drinking are those with increased access to alcohol, or where the individual is removed from the family to an environment with increased peer pressure to drink. The top three occupations associated with alcohol misuse are (Plant, 1987):

1. Publicans
2. Deck/engineroom hands, bargemen, lightmen, boatmen
3. Barmen.

Alcohol misuse has also been attributed to a stressed lifestyle; 60% of junior doctors exceeded 'safe-limits' (Birch et al. 1998).

1.4.3.3 Alcohol and the family

Alcohol misuse is a major factor in marital and family problems. In one third of divorce petitions alcohol is cited as a major factor (Editorial BMJ, 1983). In a study involving 100 battered wives (Gayford, 1975), 52 had partners who were 'frequently drunk' and a further 22 had partners who had 'episodes of heavy drinking with drunkenness'.

Children are also affected. They may be direct victims of violence and abuse. In one third of child abuse cases one parent drinks heavily (Editorial BMJ, 1983). Abused children are also affected by neglect, poverty and social isolation, which are often secondary effects of parental alcohol misuse.

1.4.4 COST TO SOCIETY

Alcohol misuse is a major socio-economic problem, costing the UK an estimated £2.46 billion per annum (Table 1.5). The greatest proportion of this is from the cost to industry (£2.1 billion) in absenteeism, unemployment and premature deaths.

In addition are unquantifiable costs of loss of productivity at home and work, accidents which do not reach formal medical attention, alcohol-related fires and criminal damage, social services involvement from child neglect and family problems, and last but not least the huge emotional distress.

However, in economic terms this may be balanced against the revenue to both the government and the alcohol industry. In 1990, while alcohol misuse was estimated to cost the UK at least £2.46 billion, the government received £7.85 billion in taxes from alcohol, 5.1% of the total government revenue. Although the cost to the country of alcohol misuse may well be an underestimate, there is a clear net profit to the government. The government gains by revenue from excise duty and Value Added Tax from alcohol sales.

The alcohol industry employs about 750,000 individuals and, further, provides substantial revenue to the advertising industry and sponsorship of sporting events. Currently the UK is a net exporter of alcohol, but if wine consumption and sales continue to increase this may change.

Table 1.5: The annual social cost of alcohol misuse in the UK (1990 prices)

The social cost to industry	**£m**
Sickness absence	964.37
Housework services	64.78
Unemployment	222.23
Premature deaths	870.76
Total	**2122.14**
Social costs to the National Health Service	
Psychiatric hospitals, inpatient costs (alcoholic psychosis, alcohol dependence syndrome, non dependent use of alcohol)	26.51
Non psychiatric hospitals, inpatient costs, (alcoholic psychosis, alcohol dependence syndrome, alcoholic cirrhosis and liver disease)	10.64
Other alcohol-related inpatient costs	109.41
General practice costs	2.79
Total	**149.35**
Society's response to alcohol related problems	
Expenditure by national alcohol bodies	0.44
Research	0.80
Total	**1.24**
Social cost of material damage	
Road traffic accidents (damage)	138.62
Social costs of criminal activities	
Police involvement in traffic offences (excluding road traffic accidents)	6.53
Police involvement in road traffic offences (including judiciary and insurance administration)	19.36
Drink related court cases	24.18
Total	**50.07**
Total Excluding unemployment and premature death	**1368.43**
Total Including unemployment and premature death	**2461.42**

Adapted from Social Costs of Alcohol: is it helpful to measure the social costs of alcohol use? Alan Maynard in The Institute of Alcohol Studies, 1998, page 33.

Overall, in 1995, the UK voluntarily spent £26.1 billion on alcoholic drinks (6.0% of total consumer expenditure) and the government received £9.75 billion in revenue (4.9% of total government revenue) (Office of National Statistics and Brewers' Society, 1996). While the government continues to be such a net gainer, it is unlikely to take drastic steps to reduce alcohol misuse.

In summary, alcohol misuse is responsible for a considerable, and apparently increasing, social and medical burden. The medical profession has a responsibility both to detect alcohol misuse and manage its consequences.

1.5 Current UK Drinking

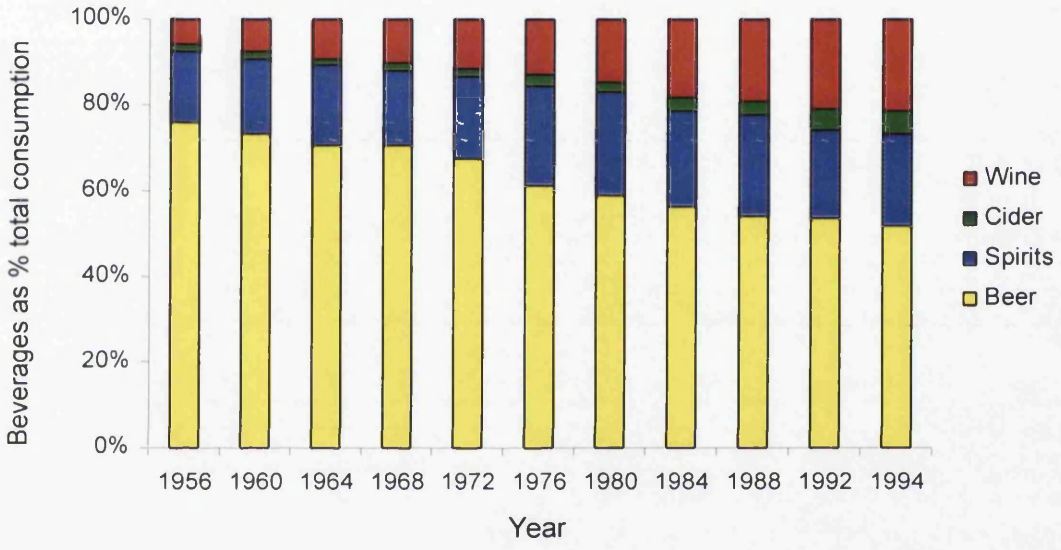
Despite the common usage of alcohol, exact quantification of the amount drunk on an individual or population basis is difficult to estimate. Information on alcohol consumption comes from two main sources: customs and excise data and population surveys.

Customs and excise data have some confounding factors: they do not include 'home-brew', which is probably a small component of our intake except in economically disadvantaged communities, or alcohol brought in from overseas, in particular the European Union, where taxation is lower than UK. This is an ever-increasing source of supply. The figures do include alcohol consumed by overseas visitors in the UK, but this is unlikely to be a significant contributing factor.

Population surveys consistently produce lower figures than the excise data. The Office of Population Censuses and Surveys (OPCS) survey in 1987 estimated alcohol consumption to be 4.2 litres absolute alcohol per head per year, while excise data estimated this at 7.4 litres per head per year (Goddard and Ikin, 1988). Population surveys are difficult because, by their nature, they are based on several elements of subjectivity. There is a significant non-response rate which could be amongst the higher or problem drinkers. In general, individuals tend to underestimate their consumption to within, or close to, 'sensible limits'. There is a theoretical consideration that some individuals may overestimate their consumption. This has been considered in specific groups, for example the armed forces, who may exaggerate their intake for macho reasons (Bisson and Milford-Ward, 1994). However these small groups are unlikely to affect population figures significantly.

Surveys may underestimate overall intake, but they provide useful information on age, social class and gender differences in consumption not available from excise data. The consumption per head of the population is now 7.5 litres per person per year, or 9.3 litres per adult (those aged 15 and over). These figures have been stable for beer for some time but the proportion of alcohol intake due to wine is increasing at the expense of beer, while spirits remain stable (Figure 1.18).

Figure 1.18: Beverages as a percentage of total consumption.

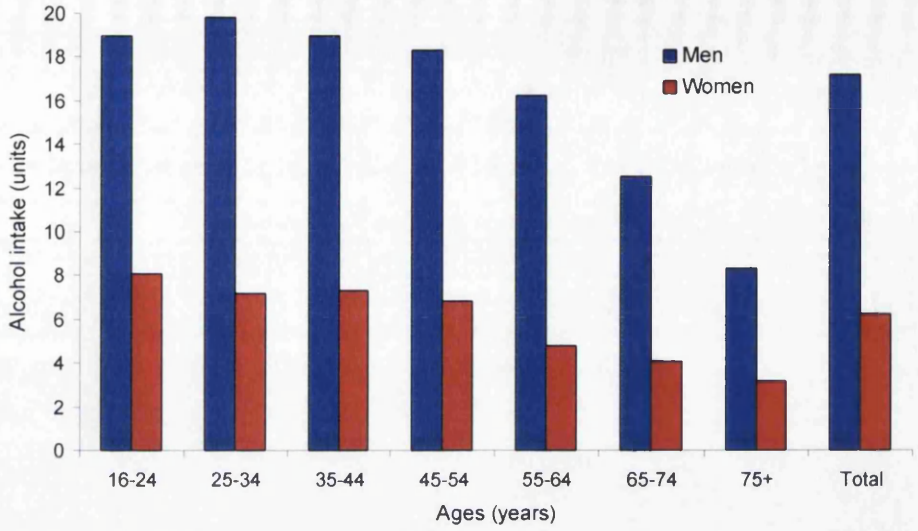


Adapted from Brewers' Society, UK

1.5.1 AGE DIFFERENCES

In the 1930s individuals under 25 years of age were considered most likely to be abstinent or light drinkers; in the 1990s this group constitute the highest drinkers in the population (Figure 1.19). This may be because, until the 1960s, young people tended to meet in cafés and coffee bars. However pubs and clubs have now become the most popular recreational meeting places. This is reflected in recent marketing and advertising. The young have been targeted with 'alcopops', although the preferred beverage remains lager.

Figure 1.19: Mean weekly intake at different ages, for men and women



Adapted from OPCS, 1995

The major problem arising from alcohol intake in this younger age group is accidents from drunkenness. Metropolitan Police convictions/cautions for drunkenness (Table 1.6) are considerably higher in men than women, being greatest at age 18 and then decline during the 20s. This is likely to be due more to adaptation to alcohol than a reduction in intake, which is currently highest in the late 20s' and 30s'.

Table 1.6: Drunkenness convictions/cautions in England and Wales: *rate per 100,000 population*

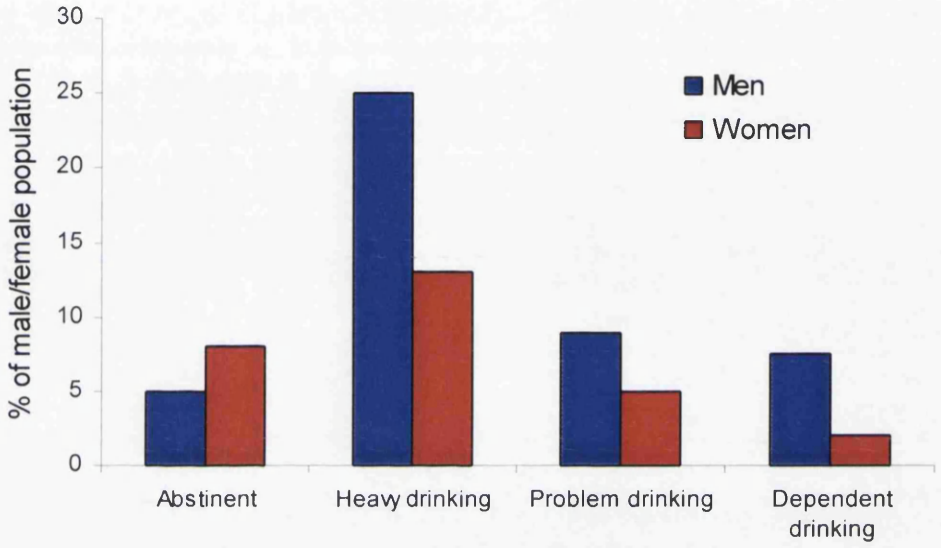
	Age 16	Age 18	Age 19	Age 20	Age 21-29	Age 30-59
Men	21.7	71.0	70.1	67.3	37.4	15.1
Women	3.5	5.7	4.9	4.5	3.0	1.7

Adapted from Alcohol as a Medical and Social problem, The Institute of Alcohol Studies

1.5.2 GENDER DIFFERENCES

Whatever the reason, men drink more at higher levels than women and women are more likely to be abstinent (Figure 1.20).

Figure 1.20: Gender differences in level of alcohol intake



Adapted from OPCS, 1995

In recent years, while male alcohol consumption has remained stable, female consumption is increasing. This is seen particularly in the younger age groups. This is probably the result of sociological changes. It is now not only more acceptable but expected that women will drink alcohol. In addition, a greater proportion of women are economically more independent. They have the disposable income, coupled with more readily available sources of supply in the form of supermarkets, cinemas, cafés and local shops, all of which have facilitated the increased consumption. Advertising of alcohol has targeted women, portraying them as independent, glamorous and fun-loving.

Figure 1.21: The Times, 18th June, 1998

Cognac drinkers splutter at trendy image

FROM ADAM SAGE IN PARIS

FRENCH cognac drinkers yesterday expressed outrage

Cognac-makers are hoping to boost flagging sales by a marketing campaign aimed at the fashion-conscious

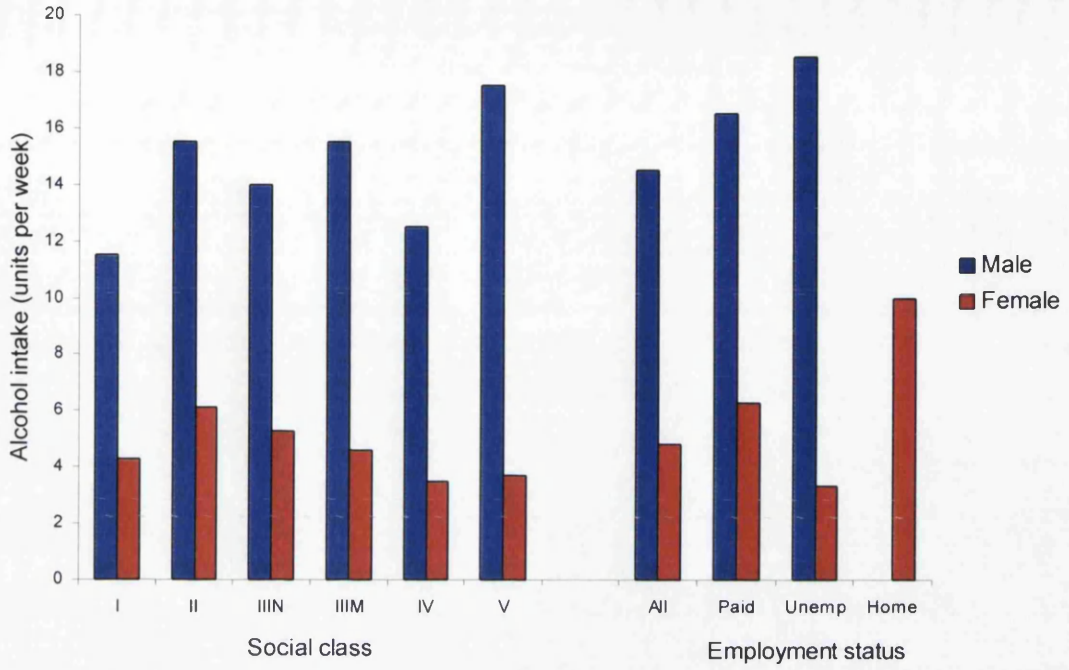
Attempts to control advertising have been made. Both the European Community directive on television broadcasting and Independent Broadcasting Authority code of conduct do not allow alcohol to be targeted at the young (under 25s), or to be associated with sexual gratification or enhanced physical performance, including driving (The Royal College of Physicians, 1991, page 180, (Anderson P and Lehto G, 1994).

In the past the women most likely to be drinking heavily were those with multiple roles (mother, wife, wage earner) but now women with 'role deprivation' (women living alone, usually young, professional and single) are also drinking more heavily (Plant, 1997). Whatever the reason for the increased consumption of alcohol in women, it is of particular concern as the physical effects of alcohol on health occur earlier in women than men (see section 1.2.1).

1.5.3 Social Differences

The 1993 OPCS Health Survey shows that in men alcohol misuse and dependency is much more likely to occur in social class 5 and in the unemployed, while in women it is less clear-cut, the highest rate surprisingly being amongst housewives who cannot readily be classified (Figure 1.22).

Figure 1.22: Alcohol consumption by social class, employment status and gender



Adapted from OPCS, 1995

The corollary of this is that it is difficult to maintain a full-time job if one is alcohol-dependent. The problem in men may be part cause and part effect: alcoholism is commoner in those living alone and the unemployed. Of those in work, alcohol misuse is greatest in those with best access: publicans and barmen (Plant, 1987).

The problems related to alcohol misuse are preventable. There is a need for an effective screening method for the early detection of alcohol misuse so as to provide support services and then the monitoring of progress. To do this effectively there is a need for objective markers of alcohol misuse.

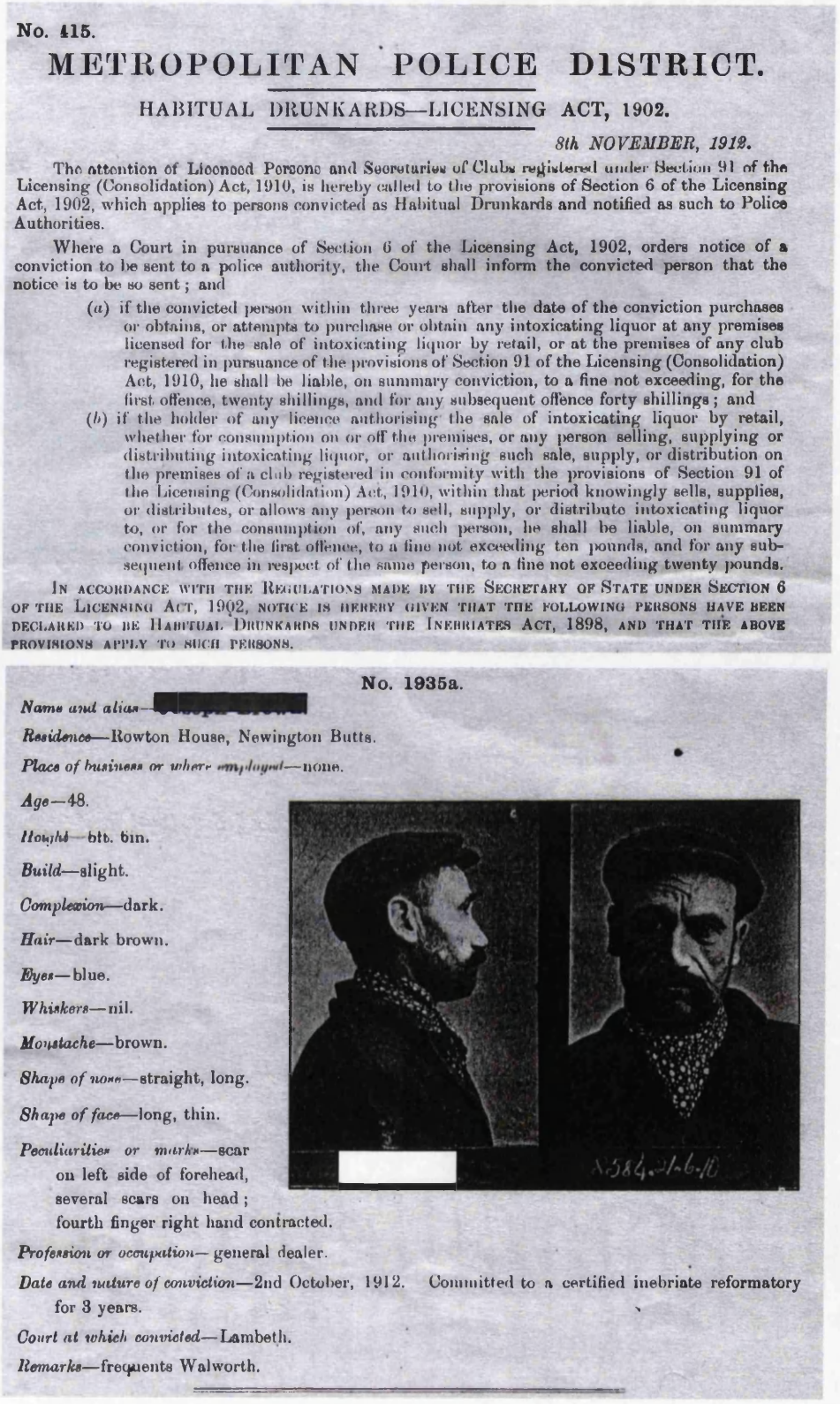
2. DETECTING ALCOHOL MISUSE

The early detection of alcohol misuse is vital, so that the physical and psychological damage can be limited and reversed where possible. Those drinking at misuse levels need to be identified by screening, and then these individuals need to be monitored through treatment. The Royal College of Physicians recommend that 'Every person seen in general practice or in hospital should be asked about his or her alcohol intake as a matter of routine, along with questions about smoking and medication, and the answers recorded' (The Royal College of Physicians, 1987, page 103).

2.1 Historical detection

Earlier this century the detection of alcohol misuse rested with the police. They arrested those found repeatedly drunk. The Habitual Drunkards-Licensing Act of 1902 (Figure 2.1) fined and banned such individuals from buying or selling alcohol. They were photographed for police records (Figure 2.1), and were often committed to a certified inebriates' reformatory (Figure 2.2). These were often 'farms' located in the country. The Farmfield Reformatory was opened in 1900 in Surrey and the maximum sentence was three years. Rehabilitation consisted largely of experimenting in teaching new skills to individuals in a new environment.

Figure 2.1: Page from the portraits and description of Habitual Drunkard and example of portrait and description of habitual drunkard, 1912



No. 415.

METROPOLITAN POLICE DISTRICT.

HABITUAL DRUNKARDS—LICENSING ACT, 1902.

8th NOVEMBER, 1912.

The attention of Licensed Persons and Secretaries of Clubs registered under Section 91 of the Licensing (Consolidation) Act, 1910, is hereby called to the provisions of Section 6 of the Licensing Act, 1902, which applies to persons convicted as Habitual Drunkards and notified as such to Police Authorities.

Where a Court in pursuance of Section 6 of the Licensing Act, 1902, orders notice of a conviction to be sent to a police authority, the Court shall inform the convicted person that the notice is to be so sent; and

(*a*) if the convicted person within three years after the date of the conviction purchases or obtains, or attempts to purchase or obtain any intoxicating liquor at any premises licensed for the sale of intoxicating liquor by retail, or at the premises of any club registered in pursuance of the provisions of Section 91 of the Licensing (Consolidation) Act, 1910, he shall be liable, on summary conviction, to a fine not exceeding, for the first offence, twenty shillings, and for any subsequent offence forty shillings; and

(*b*) if the holder of any licence authorising the sale of intoxicating liquor by retail, whether for consumption on or off the premises, or any person selling, supplying or distributing intoxicating liquor, or authorising such sale, supply, or distribution on the premises of a club registered in conformity with the provisions of Section 91 of the Licensing (Consolidation) Act, 1910, within that period knowingly sells, supplies, or distributes, or allows any person to sell, supply, or distribute intoxicating liquor to, or for the consumption of, any such person, he shall be liable, on summary conviction, for the first offence, to a fine not exceeding ten pounds, and for any subsequent offence in respect of the same person, to a fine not exceeding twenty pounds.

IN ACCORDANCE WITH THE REGULATIONS MADE BY THE SECRETARY OF STATE UNDER SECTION 6 OF THE LICENSING ACT, 1902, NOTICE IS HEREBY GIVEN THAT THE FOLLOWING PERSONS HAVE BEEN DECLARED TO BE HABITUAL DRUNKARDS UNDER THE INEBRIATES ACT, 1898, AND THAT THE ABOVE PROVISIONS APPLY TO SUCH PERSONS.

No. 1935a.

Name and alias—[redacted]

Residence—Rowton House, Newington Butts.

Place of business or where employed—none.

Age—48.

Height—5ft. 6in.

Build—slight.

Complexion—dark.

Hair—dark brown.

Eyes—blue.

Whiskers—nil.

Moustache—brown.

Shape of nose—straight, long.

Shape of face—long, thin.

Peculiarities or marks—scar on left side of forehead, several scars on head; fourth finger right hand contracted.

Profession or occupation—general dealer.

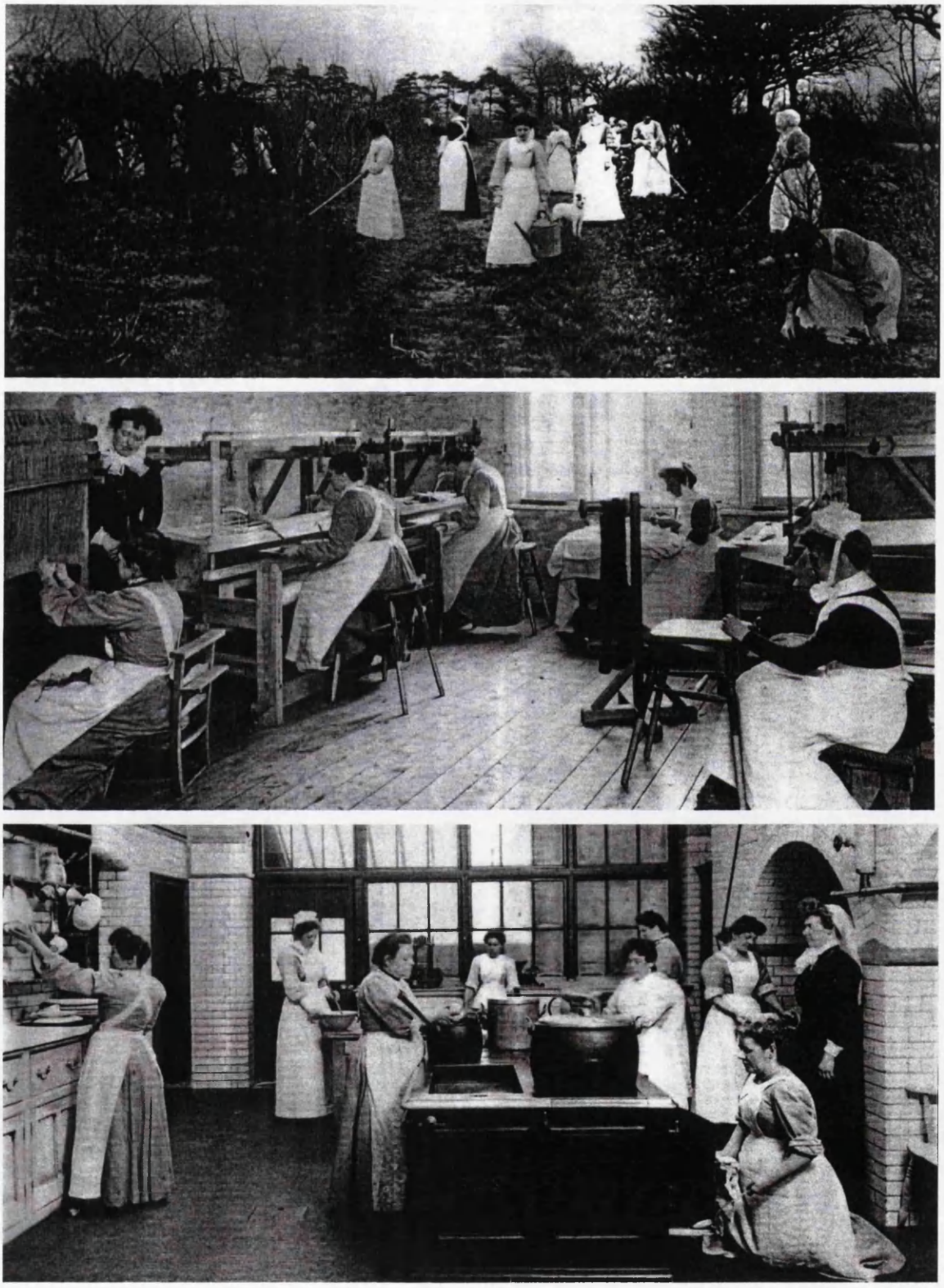
Date and nature of conviction—2nd October, 1912. Committed to a certified inebriate reformatory for 3 years.

Court at which convicted—Lambeth.

Remarks—frequents Walworth.

By kind permission of The Metropolitan Police Museum.

Figure 2.2: Scenes from Farmfield reformatory for female inebriates, 1900



By kind permission of the Metropolitan Police Museum

2.2 History and Questionnaires

The history is the most important means for detecting alcohol misuse (O'Connor and Schottenfeld, 1998). The history should cover current and past alcohol intake, and identify quantity and frequency of intake. Unfortunately, although self-report has been shown to be reliable and reproducible, it is subjective, and often is, intentionally or unintentionally, an underestimate.

Questionnaires have been used to try to improve the identification of alcohol misuse. The CAGE (Ewing, 1984) consists of four questions; two or more positive answers warrant further investigation:

1. Have you ever felt that you should **C**ut down your drinking?
2. Have people **A**nnoyed you by criticising your drinking?
3. Have you ever felt **G**uilty about your drinking?
4. Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover? (an **E**ye opener).

This tends to be too sensitive, having a high false positive rate but, if combined with self-report, can detect 90% of alcohol misusers (Seppa et al. 1992). The MAST (**M**ichigan **A**lcohol **S**creening **T**est) is best at detecting alcohol misusers who have had complications and has been modified to the MmMAST (**M**almo **m**odified **M**ichigan **A**lcoholism **S**creening **T**est). The AUDIT (**A**lcohol **U**se **D**isorders **I**dentification **T**est) was developed by the WHO collaborative group and was designed to detect early heavy drinking. It consists of ten questions to be used in primary care. The CAGE, MmMAST and AUDIT were compared in Occupational Health and detoxification clinic settings and compared with self-report for alcohol intake (Seppa et al. 1995). Overall the sensitivities for the CAGE and MmMAST were 100% and for the AUDIT was 91% amongst the alcohol-misusers, but the AUDIT had the best performance in the Occupational Health setting.

2.3 Clinical signs

There are a number of cutaneous signs of chronic alcohol misuse and may be found in those with no significant liver disease. They include spider naevi, telangiectasiae, palmar erythema, gynaecomastia and Dupuytren's contracture. The mechanisms by which these develop are

unknown, and with the exception of Dupuytren's contractures, they may all regress with abstinence.

2.4 Biological Markers of Alcohol Misuse

These are particularly valuable to screen or confirm a suspicion of alcohol misuse as they are objective, are useful serially for monitoring, and may be helpful in motivating the patient. These markers can be divided into those indicating recent alcohol drinking, and those of chronic alcohol misuse.

Sensitivity is the true positive rate or the proportion of individuals testing positive of all the active alcohol misusers. This is adversely affected by the false negative rate.

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False negatives}}$$

The specificity is the true negative rate or the proportion of non-misusers who tested negative. This is adversely affected by the false positive rate.

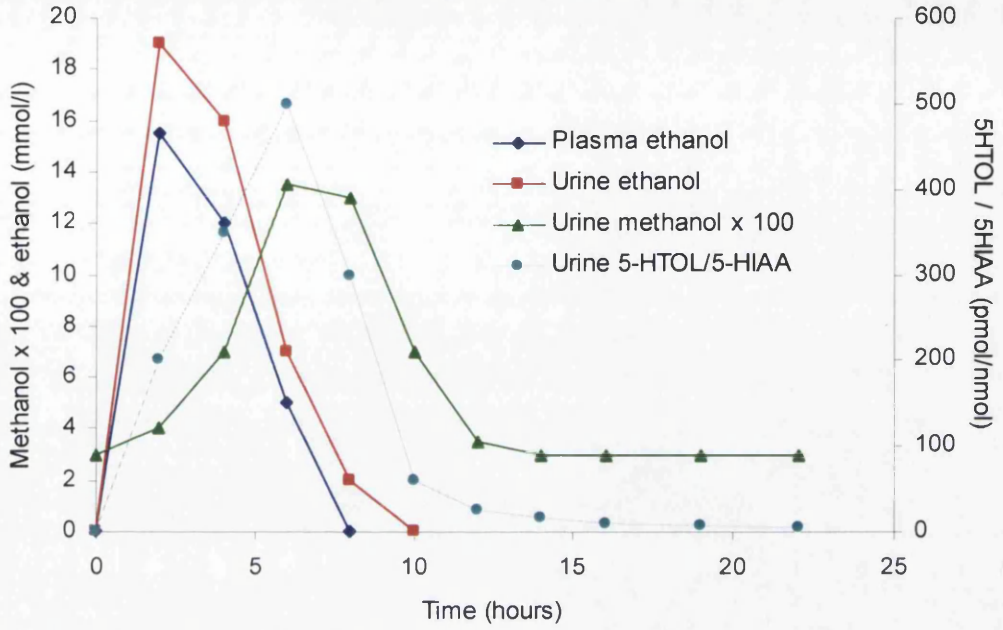
$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}$$

2.4.1 Markers of Recent Alcohol Consumption

2.4.1.1 Ethanol

This is the most obvious confirmation of recent drinking and can be detected in breath, serum or urine. It is useful to validate self-report, if positive. Alcohol is eliminated at the rate of 1g/kg/hr, usually in 4-6 hours from breath and blood, and within 8 hours from urine (Figure 2.3), although the elimination rate is affected by the chronicity of alcohol misuse. Most patients know this and adapt their habits so that their clinical value is limited.

Figure 2.3: Concentration-time profiles of ethanol (plasma and urine), urinary methanol and urinary 5-HTOL/5-HIAA in healthy volunteers after oral ethanol



Adapted from Helander et al. 1996a

2.4.1.2 Methanol

This is present in the body and in small amounts in alcoholic beverages as a congener. Both ethanol and methanol are metabolised via alcohol dehydrogenase. However alcohol dehydrogenase has a much higher affinity for ethanol, so this is preferentially metabolised. The level of methanol therefore accumulates during ethanol metabolism and does not start to fall until ethanol has been removed (Figure 2.3). In practice this is at least 2-6 hours after ethanol has ceased to be detectable (Helander et al. 1996a).

2.4.1.3 Serotonin metabolites

The urinary metabolites of serotonin (5-hydroxytryptamine) are 5-hydroxytryptaphol (5-HTOL) and 5 hydroxy indole acetic acid (5-HIAA). These are natural substrates. Normally 5-hydroxytryptamine (5-HT) is metabolised predominantly to 5-HIAA by aldehyde dehydrogenase, but a small amount is metabolised to 5-HTOL by alcohol dehydrogenase. However after alcohol ingestion, alcohol is metabolised to acetaldehyde, which than inhibits aldehyde dehydrogenase. Therefore there is a shift towards 5-HTOL and an increase in the 5-HTOL:5-HIAA ratio. The increase is dose-dependant and it can be detected 5-15 hours after the ethanol has been eliminated (Figure 2.3). In urine, methanol and serotonin metabolites can

be detected up to 18 hours after drinking, long after the ethanol is cleared (Helander et al. 1996a). The sensitivity and specificity of the 5HTOL:5HIAA ratio is proportional to the alcohol intake above 200 μmol/L. This is however affected by serotonin containing foods, for example bananas, and disulfiram which both increase the 5HTOL level but not the 5HIAA level (Oneta et al. 1998). This can be resolved by using the 5HTOL/creatinine ratio in addition to the 5HTOL/5HIAA ratio.

In summary, the best marker is the 5-HTOL:5-HIAA ratio; however it is laborious to perform and not routinely available. Ethanol remains the most frequently used, whether by breath, urine or serum.

2.4.2 MAKERS OF CHRONIC MISUSE

The markers most commonly evaluated are those readily available as part of routine screening: erythrocyte mean corpuscular volume (MCV), serum aspartate aminotransferase and alanine aminotransferase (AST, ALT), and gamma glutamyl transferase (GGT).

Table 2.1: Sensitivity and specificity for detection of hazardous and dependant alcohol use

		Sensitivity (%)	Specificity (%)
MCV	Hazardous consumption	20-30	64-100
	Dependence/alcoholism	40-50	64-100
AST	Hazardous consumption	10-30	>90
	Dependence/alcoholism	35-50	>90
ALT	Hazardous consumption	10-20	>80
	Dependence/alcoholism	20-50	>80
GGT	Hazardous consumption	20-50	55-100
	Dependence/alcoholism	60-90	55-100

Adapted from Conigrave et al. 1995

2.4.2.1 Erythrocyte mean cell volume (MCV)

This is thought to be elevated as a result of direct toxicity by ethanol (Morgan et al. 1981). It increases after about six weeks of drinking and has a dose-dependent relationship to ethanol

(normal range 80-100 fl). It normalises after about three months of abstinence and so has a limited use in monitoring alcohol intake. The sensitivity is higher in women (86.3%) than in men (63.0%) (Morgan et al. 1981). False positives are found in hypothyroidism, vitamin B_{12} and folate deficiency, non-alcoholic liver disease and, a minor effect of smoking (Sillanaukee, 1996).

2.4.2.2 Serum aspartate amino transaminase

Serum aspartate amino transaminase (AST) and serum alanine transaminase (ALT) are also markers of liver damage as opposed to alcohol misuse. Both transaminases are found in hepatocytes but AST is also found in skeletal and myocardial cells. In alcohol-related liver damage, the AST is elevated more than the ALT, at least in part as a reflection of the alcohol-related skeletal damage. This is the reverse of the normal pattern in acute hepatocellular disease (for example acute viral hepatitis) where the ALT exceeds the AST. The normal range for both is 5-40 U/L.

False positive results are found in non-alcoholic liver disease, muscle damage and myocardial damage. Despite these the specificity is reasonably high at >90% (Table 2.1).

AST itself has a mitochondrial (mAST) and cytosolic component. It appears that alcohol selectively affects the mitochondrial component following damage to this organelle so that the serum increase in alcohol misusers is mAST. This has been proposed as a more sensitive marker of alcohol misuse. There is also a small increase in non-alcoholic liver disease and it has therefore been suggested it should be used as a ratio of mAST to total AST (Goldberg and Kapur, 1994).

2.4.2.3 Serum gamma glutamyl transferase (GGT)

Serum GGT increases in alcohol misuse in a dose-dependent manner, and is often the first marker to be elevated (Conigrave et al. 1995). It is less sensitive in women than men (Anton and Moak, 1994; Helander et al. 1996b). The exact mechanism of elevation of GGT in alcohol misuse is unclear. The enzyme may be released by hepatic cell injury or by induction following exposure to alcohol. In alcoholic liver disease a component of the increase is also from hepatocyte cholestasis and hepatocyte damage. It increases after five weeks of drinking more than 50g per day. It usually increases to three times the upper reference limit, but will

normalise within five weeks of abstinence, with a half-life of 26 days, although this is lengthened in chronic liver disease (Rosman, 1992).

Some individuals misusing alcohol never have an elevated GGT; in some chronic alcohol misusers initially high levels fall despite continued drinking. False positives are seen in non-alcoholic liver disease, including fatty liver, biliary tract disease, obesity, diabetes, pancreatitis, hyperlipidaemia, trauma and heart failure, and with microsomal inducing drugs such as anti-epileptics (Sillanaukee, 1996).

The varying sensitivity and specificity makes it an unsuitable marker to be used for screening, but it is useful to confirm a clinical suspicion of alcohol misuse. Several isoforms of GGT exist and can be separated by electrophoresis. The pattern in alcohol abuse is distinctly different from not only that of healthy volunteers, but also from non-alcoholic liver disease. It is, however, the same as that in those taking anti-epileptic drugs since both result in enzyme induction. It has been suggested that the analysis of GGT isoforms may improve the specificity of GGT for alcohol misuse (Bellini et al. 1997).

As can be seen from Table 2.1, none of the routinely available markers have sufficient sensitivity or specificity to be used alone, and in practice a combination is usually used. If only one of the markers in a combination is positive, then the overall sensitivity is increased and specificity decreased. If all the markers used in the combination are positive, then the specificity is increased and sensitivity decreased (Rosman, 1992). Thus Chick *et al* showed that if either GGT or MCV are positive the sensitivity was 63% and specificity was 78%, but if both were positive then the sensitivity was 17% and specificity 98% (Chick et al. 1981).

The clinical case mix affects the test performance of any given marker. The sensitivity of the test is highest where there are a high number of severe alcohol misusers, in for example the alcohol treatment centre. The sensitivity is lowest in the general community (Conigrave et al. 1995). Chick *et al* found a sensitivity of 40% for in-patient alcohol misusers and 23% for those in the setting of employment screening (Chick et al. 1981). Sillanukee *et al* found a sensitivity of 47% for MCV for detecting alcoholics in the context of a detoxification centre, but only 22% for detecting heavy drinkers voluntarily attending for health screening. Similarly using GGT there was a sensitivity of 65% in the detoxification centre, but only 35% within the community (Sillanaukee et al. 1993).

2.4.2.4 Other routinely available markers

Serum urate may be elevated in 40% of male and 25% of female alcohol misusers. False positives results are seen in gout, renal disease and some drugs.

Serum triglycerides increase after one week of drinking in 40% of alcohol misusers, and normalise within one week of abstinence. False positives are seen in hyperlipidaemia, diabetes, obesity and with some drugs.

In summary, detection of alcohol misuse is probably best achieved by the combination of self-report, validated by an acute marker, together with at least one chronic marker.

3. CARBOHYDRATE DEFICIENT TRANSFERRIN AS A MARKER FOR CHRONIC ALCOHOL MISUSE

It can be seen that the currently available routine markers do not have sufficient sensitivity or specificity to be used alone to detect alcohol misuse. Considerable research has been undertaken to try to find any other potentially more useful markers. One such marker is carbohydrate deficient transferrin (CDT).

3.1 Structure of Transferrin

Transferrin is a glycosylated iron-binding protein whose main function includes the transport of iron in the plasma. It also participates in the regulation of iron absorption. Under normal conditions it has a serum concentration of 3 to 3.66 g/l and is approximately 30% saturated with iron. It usually binds to cell surface receptors, the transferrin-receptor complex is endocytosed, and the iron is removed intracellularly in a pH dependent process. The apotransferrin is then recycled to the cell surface and released into the circulation.

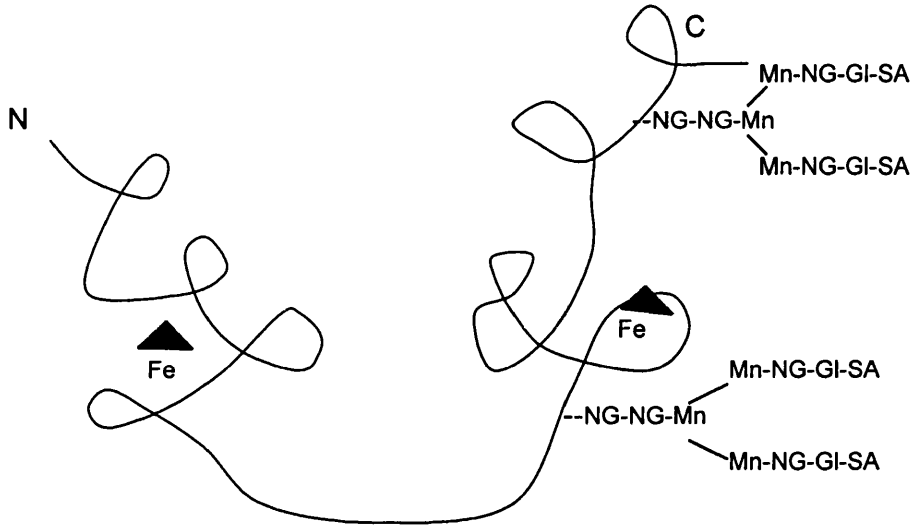
Transferrin has a molecular mass of 79,570 Da. The protein moiety consists of a single polypeptide chain containing 679 amino acid residues. There are two N-linked oligosaccharide chains, each of which has biantennary glycans and terminal sugars that form a carbohydrate unit. Transferrin can be divided into two parts: the N and C domains. Each of these has a specific metal binding site so that transferrin has the potential to carry two ferric ions. Each carbohydrate unit has a sugar composition of two residues of sialic acid and galactose, and four residues of mannose and N-acetylglucosamine. The sialic acid is situated terminally and the usual total number of sialic acids residues is four, *tetrasialotransferrin* (Putnam, 1975).

Each of the two biantennary glycans is linked N-glycosidically to the peptide sequence: Tyr-Asn-Lys-Ser or to Gly-Ser-Asn-Val-Thr (Spik et al. 1975). There is variation in the branching, so that it is possible to have bi-, tri- or tetra-antennary glycans (van Eijk and de Jong, 1992) and these can vary in their terminal sugars and may be incomplete, terminating in galactose rather than the more usual N-acetylglucosamine (Marz et al. 1982).

The carbohydrate function of transferrin may be to enhance water solubility, and assist in binding of transferrin to cell membranes during iron exchange and in the metabolism and

clearance of apotransferrin. However they are neither essential for maintaining tertiary structure, nor required for the uptake and exchange of iron.

Figure 3.1: Schematic representation of the transferrin molecule



Fe = Iron; NG = N-acetyl-glucosamine; Mn = Manose; Gl = Galactose; SA = Sialic acid

Adapted from Van Eijk and de Jong, 1992

3.2 Historical perspective

Following a daily alcohol intake of greater than 60g for at least two weeks, the transferrin produced is predominantly of a-, mono- and disialotransferrin rather than the normal tetrasialotransferrin. In view of the lack of terminal sialic acids these are collectively termed carbohydrate deficient transferrin (CDT). This was initially observed in cerebrospinal fluid of alcohol-misusers with neurological problems, by Helena Stibler in 1976 (Stibler and Kjellin, 1976) and then found to be also present in plasma. Subsequently Landberg *et al* showed that CDT lacked not just sialic acid, but one or both of their entire carbohydrate chains, (Landberg et al. 1995) and also the other terminal carbohydrates galactose and N-acetyl glucosamine (Stibler and Borg, 1986). The reduction in the negatively charged sialic acid results in predominantly a-, mono-, and disialotransferrin, and an increase in pI to $\geq$5.7.

3.3 Synthesis and glycosylation of transferrin

Transferrin is mainly synthesised in the hepatocyte, although some production takes place in Sertoli cells, oligodendroglial cells, ependymal cells in the choriod plexus and capillary endothelial cells in the brain. Serum transferrin concentrations are increased in chronic iron-deficiency, pregnancy and during oestrogen treatment and sometimes in the acute phase of hepatitis (Aisen and Brown, 1977). Conversely serum concentrations are reduced in malnourished individuals, in patients with chronic liver disease (Potter et al. 1985) and in those with iron overload, for example genetic haemochromatosis. This reduction may be a direct effect of the liver damage as the transferrin is synthesised in the hepatocyte, and in cirrhosis this may be reflected in the impaired protein synthesis, although the catabolic rate remains unchanged.

Transferrin is also glycosylated in hepatocytes. The amino acids are assembled in the rough endoplasmic reticulum on ribosomes. N-glycosylation occurs via a lipid intermediate precursor (dolichol-pyrophosphoryl-oligosaccharide) and the sugars (N-acetyl glucosamine, galactose and sialic acid) are added sequentially. This complex is transferred to the polypeptide using a highly specific oligosaccharyltransferase and the complex then passes to the smooth endoplasmic reticulum, and finally to the Golgi, a process where terminal glycosylation occurs. The oligosaccharide chains are elongated with the addition of sialic acid and other carbohydrates regulated by glycosyltransferases (N-acetylglucosaminyl-transferase I, II and IV, galactosyl- and sialyltransferase) (Schachter, 1986). It is at this stage that molecular heterogeneity of transferrin, with respect to carbohydrate content and structure, can occur.

There are two complex oligosaccharide chains with microheterogeneity of the branching and terminal carbohydrates. This may be because the synthesis does not occur according to a predefined structural requirement but from an interaction between several glycosyl transferases (de Jong and van Eijk, 1988). Following synthesis and glycosylation the transferrin is secreted from the hepatocytes by exocytosis from the Golgi (Baraona and Lieber, 1982).

3.4 Catabolism

The average half-life of transferrin is eight days with a turn-over of approximately 1g of transferrin per day. Desialylated transferrin is still able to carry iron, but the altered transferrin has an increased affinity for the hepatocyte transferrin receptor (Regoeczi et al. 1984). There are two antigenically distinct membrane receptors that transferrin binds to: the transferrin receptor and the asialoglycoprotein receptor. Hepatocytes have ten times as many asialoglycoprotein receptors as transferrin receptors and it has been shown that there is greater deposition of iron by asialotransferrin than sialylated transferrin (Regoeczi et al. 1984).

The process of tissue injury itself may start desialylation: there is lysosomal release of intracellular sialidase, released from lysosomes from broken cells, which causes desialylation (van Eijk et al. 1987). The desialylated transferrin is then transported to the liver. 'Ageing' of circulating glycoproteins results in the loss of carbohydrates, and these are then detected by specific hepatocyte receptors and removed from the circulation. Transferrin is catabolised in the liver. Desialylation has been shown to take place in liver endothelial cells (Irie et al. 1988).

Transferrin lacking terminal sialic acid results in an exposed galactose, which triggers the asialoglycoprotein receptor endocytosis mechanism (Schachter, 1984). The galactose binds to the asialoglycoprotein and transferrin receptors simultaneously and the receptor transferrin complex is endocytosed by the hepatocyte and enters a lysosome (Young and Aisen, 1981). There are asialoglycoprotein receptors not only on hepatocyte surfaces, but also on fibroblasts, lymphocytes and reticulo-endothelial cells. The hepatic receptors are located on the hepatocyte sinusoidal and lateral membranes. Regoeczi *et al* have suggested that this dual receptor binding results in a preferential increase in hepatic parenchymal iron in alcohol misusers, as opposed to the more physiological delivery via transferrin receptors to reticulo-endothelial cells (Regoeczi et al. 1984).

Normally after internalisation via the transferrin receptor the glycoprotein, transferrin, separates from the receptor and recirculates to the cell surface. However asialotransferrin taken up by the asialoglycoprotein receptor, does not recycle, but enters lysosomes where it is broken down and the iron is released into the hepatocyte. In chronic alcohol abuse with increased levels of serum

carbohydrate deficient transferrin, there is a consequent increase in hepatic iron (Regoeczi et al. 1984).

3.5 Microheterogeneity of transferrin

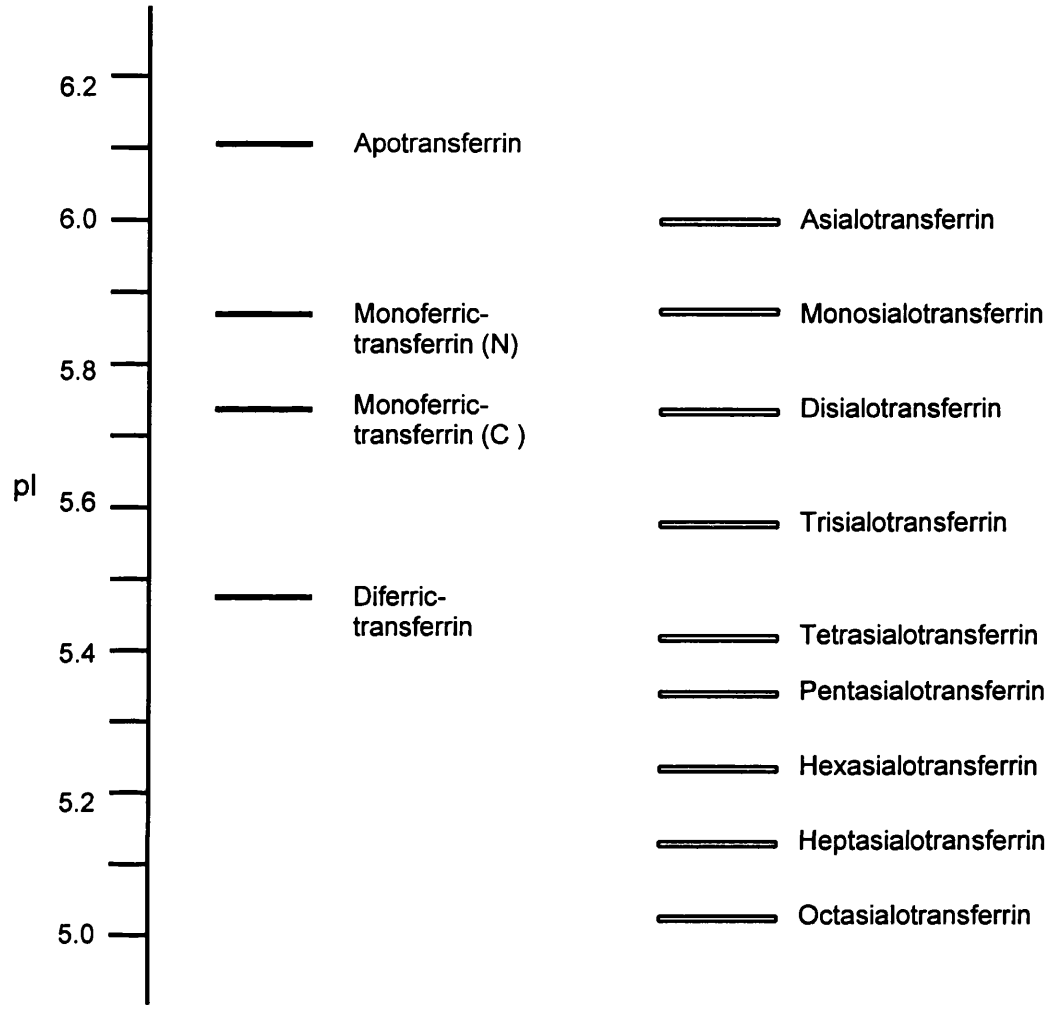
Traditionally transferrin variants have been detected principally by electrophoretic methods. There are three major determinants affecting the electrophoretic microheterogeneity of transferrin: genetic variations, iron content and the carbohydrate composition of transferrin (de Jong et al. 1990).

Of the genetic variations human transferrin C is the usual transferrin type found in more than 95% of European populations. Transferrin B (present in some Asians and Japanese) has a lower Isoelectric point (pI) and transferrin D (present in some American Blacks and Japanese) has a higher pI. These different variants of transferrin can be accounted for by substitutions in the amino acid sequence of transferrin.

Transferrin can exist in four different states with regard to iron content: no iron present (apotransferrin); monoferric iron with one iron present at either the N domain or at the C domain, and diferric transferrin with both iron binding sites occupied. Iron occupies the N domain more often than the C domain. Iron binding affects the affinity of transferrin for the transferrin receptor: diferric transferrin has the greater affinity, with apotransferrin having the least at physiological pH. This enables transferrin to recycle. Normally serum transferrin is 30% saturated with iron.

Nine different transferrin variants can exist, each with an isoelectric point (pI) between 5 and 6, resulting from variations in the level of glycosylation of the oligosaccharide chains (Figure 3.2). There can be variation in the branching of the glycan chains, but it is the number of sialic acid residues present that determines the electrophoretic differences, since sialic acid is the only charged sugar. Transferrin can have between 0 and 8 sialic acid residues present per molecule. In the latter case (octasialotransferrin), the pI is 5.0. With decreasing numbers of sialic acid residues present, the pI increases so that asialotransferrin (0 sialic acid residues) has a pI of 5.9. In the serum of healthy individuals there are predominantly four giving a pI of 5.4, but to a lesser degree also five (pI 5.3) and three (pI 5.5) sialic acid residues.

Figure 3.2: Diagrammatic representation of the pI of the different forms of transferrin



Adapted from van Eijk and de Jong, 1992, showing the different isoelectric points of the transferrin variants when fully iron saturated on the right, and effect of altered transferrin iron status on the left.

3.6 Effects of alcohol on transferrin metabolism

Martensson *et al* showed that, in the presence of chronic alcohol abuse of >1000 g ethanol per week, transferrin had significantly greater a-, mono- and disialotransferrin isoforms, but not trisialo or other higher isoforms (Martensson et al. 1997). So, if CDT is that portion of transferrin which alters with chronic alcohol consumption, then it contains only a-, mono- and disialotransferrin. However it has been suggested that part of the trisialotransferrin also becomes elevated with chronic alcohol exposure (Heggli et al. 1996).

Schellenberg *et al* found that serum CDT levels were proportional to ethanol intake in levels less than 80g/day (Schellenberg et al. 1991). However they have suggested a plateau effect as the serum CDT levels did not increase with heavy alcohol intakes near 700g/day.

3.6.1 MECHANISMS OF FORMATION OF CDT

There are two possible mechanisms for the formation of CDT: incomplete glycosylation in the formation of transferrin, or abnormal degradation with the increased loss of sialic acid from normal transferrin.

It has been shown in alcohol misusers that serum glycoproteins and membrane-bound glycoconjugates are altered. As these are representative of the late stages of glycosylation, it implies a defect arising from impairment of glycosylation. Regoeczi *et al* have suggested that sialic acid deficient transferrin is the likely result of incomplete glycosylation (Regoeczi et al. 1984). Ethanol affects membrane-bound enzymes, such as sialyl transferase which is found in the Golgi apparatus (Rubin and Rottenberg, 1982). Schellenberg *et al* found that ethanol intake correlated with membrane sialic acid content, suggesting a failure of glycosylation (Schellenberg et al. 1991).

It is known that these glycosyltransferases are themselves derived from the liver. Xin *et al* (Xin et al. 1995) have shown that, in the presence of ethanol, the activity of sialyltransferase involved in the synthesis of the carbohydrate side chain is reduced by 23%, but the activity of plasma membrane sialidase, involved in removal of the carbohydrates from the transferrin, was increased by 41%.

Stibler & Borg have also described a decrease in the activities of glycosylation enzymes, which they attributed to the effect of acetaldehyde (Stibler and Borg, 1991). The effect of acetaldehyde, rather than a direct effect of ethanol itself, on the inhibition of glycosylation in the Golgi, has also been shown by Kawahara *et al* (Kawahara et al. 1993). Acetaldehyde may also cause impairment of other membrane processes, such as exocytosis (Stibler and Borg, 1991).

Thus the defect in transferrin may result, not from an alteration in glycosyltransferases, causing a reduction in normal glyco-transferrin synthesis, but from an increase in glycosidase activity. However Stibler *et al* have shown there to be normal activity of these enzymes, sialidase and β-galactosidase, in alcoholic patients (Stibler et al. 1984).

It has been suggested that serum CDT may be elevated in alcohol misusers as a result of impaired uptake or clearance of sialic acid deficient transferrin. Petren has suggested that this may be due to membrane dysfunction (Petren and Vesterberg, 1988). Marshall *et al* have shown that, in severe liver disease, there is a reduction in the number of hepatic asialoglycoprotein receptors, and that these may also be reduced by alcohol (Marshall et al. 1974).

Further, it has been suggested that transferrin naturally loses sialic acid as an 'ageing' phenomenon (Petren et al. 1987). In cirrhosis there is a reduction in asialoglycoprotein receptors on the sinusoidal and lateral membranes of hepatocytes, but an increase on the canalicular surface (Burgess et al. 1992). This abnormal distribution may result in a reduced clearance of asialoglycoproteins. This is in contrast to the findings of Henriksen *et al* who found that the level of serum CDT in non-alcoholic liver cirrhosis was the same as that in healthy volunteers (Henriksen et al. 1997).

Petren & Vesterberg followed alcohol misusers through detoxification and showed that in abstinence the transferrin synthesised had a higher sialic acid content than that present during active drinking, but that the sialic acid deficient transferrin concentration remained constant (Petren and Vesterberg, 1988). They concluded that the sialic acid deficient transferrin resulted not from an impairment of sialylation but a reduction in clearance, probably as a result of impaired transferrin asialotransferrin receptor status.

In summary, there is evidence both for incomplete glycosylation and for abnormal degradation of transferrin as causes for the formation of carbohydrate deficient transferrin in response to alcohol misuse, but the precise nature is not yet fully understood.

3.7 Methods for assessing serum CDT

3.7.1 ISOELECTRIC FOCUSING

Detection of carbohydrate deficient transferrin is a two-fold process: first separation of transferrin from other proteins on the basis of charge, and then quantification of the transferrin. The first process in all methods of detection is iron saturation of the transferrin so that it is all present as diferric transferrin, mono- and apo-transferrin have higher isoelectric points than diferric transferrin (Figure 3.2).

Isoelectric focusing (IEF) was the original method used by Stibler *et al* (Stibler, 1978) and takes place on iron saturated polyacrylamide gels with a pH range to allow separation of transferrin isoforms according to isoelectric point. Quantification of the transferrin isoforms was by immunofixation (IF) (Stibler, 1979). This method was laborious, time-consuming and technically demanding. The IEF was modified to use an agarose gel with quantification by zone immunoelectrophoresis assay (ZIA) (Petren and Vesterberg, 1984). Later, although IEF has continued to be used, the quantitation stage has been by western blotting (WB) (Xin et al. 1991). Bean *et al* have used laser densitometry (LD) for quantitation. After IEF the gels undergo immunoblotting (IB) and then each visible band of transferrin is measured by a laser scanner which measures the optical density, which is proportional to the transferrin.

3.7.2 ION EXCHANGE CHROMATOGRAPHY

An alternative method of separation has been the ion-exchange chromatography, whereby transferrin isoforms were eluted from serum using a gel column with an ion-exchange resin to which they adhere. The isoforms were then released by washing with a pH gradient and then underwent chromatofocusing (Storey et al. 1985). More frequently the separation has been on disposable minicolumns with quantitation by radioimmunoassay (RIA) (Stibler et al. 1986). Schellenberg *et al* developed a rate nephelometric method for quantitating the transferrin isoforms following separation by anion exchange chromatography (Schellenberg et al. 1996). In this method antibodies are added to the transferrin isoforms and a light scatter reaction is produced which is measured.

3.7.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) (Jeppsson et al. 1993) and Fast Protein Liquid Chromatography (FPLC) (Sillanaukee et al. 1994) are two other methods based on anion-exchange chromatography whereby the transferrin isoforms are eluted against a salt gradient. The collected fractions can then be quantitated either by analysis of the chromatograms obtained (Heggli et al. 1996), or RIA has been used (Sillanaukee et al. 1994).

3.8 Serum CDT as a marker for alcohol misuse

3.8.1 RESEARCH TECHNIQUES

Helena Stibler originally used isoelectric focusing (IEF) to detect serum CDT and in 98 alcohol misusers sensitivities which ranged from 64-81% (Stibler et al. 1979). This early study has been subsequently validated by other workers with sensitivities ranging from 31% (Bisson and Milford-Ward, 1994) to 100% (Vesterberg et al. 1984). The low sensitivity of 31% was in a series of 62 male soldiers aged under 30 years and was attributed to exaggerated self-reporting of alcohol consumption. However it is possible that age is an important factor in serum CDT estimations. Apart from these two reports, others based on IEF to detect CDT isoforms show broadly consistent results with high sensitivities and this method has been used world-wide in first world countries. Recent more accurate data may be due, in part, to the development of ultra-thin gels to provide higher resolution.

In contrast column chromatographic techniques, in particular Fast Protein Liquid Chromatography (FPLC) and High Performance Liquid Chromatography (HPLC), either alone, or in conjunction with electrophoresis, seem to give slightly lower sensitivities (Sillanaukee et al. 1994; Storey et al. 1985).

The specificity for these 'research' techniques reported over the last twenty years has been between 90 and 100%, the majority greater than 95%, regardless of the technique used (Table 3.1).

Thus, research techniques for the detection of serum CDT appeared to provide good sensitivity (81-100%) and specificity (97-99%) as a marker of alcohol misuse (Stibler et al. 1979; Storey

et al. 1987; Storey et al. 1985). However the techniques used in these series were too time-consuming and laborious to be applicable for routine analysis.

3.8.2 COMMERCIAL ASSAYS

In an attempt to simplify the detection of CDT Helena Stibler modified the original method (Stibler et al. 1986) and based on this Pharmacia and Upjohn (Uppsala, Sweden) derived a commercial kit, CDTect. The assay involves preliminary separation of CDT from serum by anion-exchange chromatography on disposable microcolumns with subsequent detection and quantification of CDT isoforms using radioisotopic (^{125}I) or, more recently, enzyme-immunoassay. It detects a-, mono- and disialotransferrin isoforms collectively, and the results are expressed in units of CDT/l: the upper limit (defined by the manufacturers Pharmacia & Upjohn) for men is 20 U/l, for women 26 U/l. There has been some improvement in time needed to analyse samples, but the method is still time-consuming and is not fully automated. Costs vary from country to country, averaging £37 per test in the UK, although it is now no longer available.

AXIS (AXIS Biochemicals ASA, Oslo, Norway) have developed an alternative method which again involves a preliminary separation of serum CDT by microcolumn anion exchange chromatography. Initially quantitative estimates of CDT were by radio-immunoassay, but this has now been replaced by an immunoturbidimetric technique. Although the original AXIS %CDT kit detected, like CDTect, a-, mono-, and disialotransferrin, the immunoturbidity kit detects half of the trisialotransferrin. The CDT present in the sample is expressed as a ratio of CDT to total transferrin (see below) and the cost is about £15 per test. The upper limit for both men and women is 2.5% and the test (with Boehringer) is shortly to become fully automated.

CDTect and AXIS %CDT are recent developments but despite advantages there has been some decrease in overall specificity (63-100%). The change from 'research' techniques to commercial disposable methods has resulted in some loss of sensitivity with considerable variation in results, in some cases as low as 30% (Yamauchi et al. 1993). The initial studies performed in the late 1980's and early 1990's, using either CDTect or AXIS %CDT showed sensitivities in the range of 80-90%. These used anion exchange chromatography and radio-immunoassay. Given these results with a commercial kit, however, there has been considerable interest in the assay and since 1993 a large number of excellent studies have been done. The majority used CDTect-RIA, although recent studies use CDTect EIA (enzyme immunoassay). More recently still, the AXIS %CDT assay has been evaluated, initially with radio immunoassay, but now using the

immunoturbidimetric method (AXIS %CDT TIA) for quantification of the CDT. This method, like CDTect, again has shown variable sensitivities, from 36.6% (Yamauchi et al. 1993) to 88% (Radosavljevic et al. 1995). As with the 'research' techniques, the specificities have almost all been in the range of 90-100%, with only a few exceptions (Lieber et al. 1993; Meregalli et al. 1995; Radosavljevic et al. 1995; Sillanaukee et al. 1994) (Table 3.2).

The use of the commercial assays has allowed studies of larger populations, including a range of sub-groups, although still focusing on male subjects. The focus has been on individuals from developed countries.

One criticism of the majority of the studies is that the time from the last drink is often not given. This has implications given the serum half-life of transferrin (eight days) and CDT (14-17 days). Cut-off levels that define sensitivity and specificity have usually been those suggested by the manufacturers', 20 U/l for men, and 26 U/l for women (CDTect) and 2.5% (AXIS %CDT RIA) or 6.0% (AXIS %CDT TIA), although some studies have used a higher level, chosen by the authors for reasons that remain unclear (Lof et al. 1994; Werle et al. 1997). This has increased the specificity of the test but decreased the sensitivity.

3.8.2.1 Comparison of commercial techniques

There have been several studies that have evaluated the relative performance of CDTect and AXIS %CDT in the same population (Yamauchi et al. 1993; Bell et al. 1994a; Tsutsumi et al. 1994; Sorvajarvi et al. 1996; Huseby et al. 1997a; Nilssen and Huseby, 1992a) (Table 3.3). Radioimmunoassay has been used for quantification of CDT in both methods. These studies have shown broadly comparable results, with higher specificities than sensitivities, other than the result from Tsutsumi *et al* (Tsutsumi et al. 1994) which showed a slightly higher sensitivity using AXIS %CDT. However the cut-off at 2% was lower than that normally used, which would account for this result.

3.8.2.2 Expression of serum CDT

CDT may be expressed in absolute units (as for CDTect) or as a ratio of CDT to total transferrin (as for AXIS %CDT) since transferrin levels may be affected by liver disease. In support of the CDT ratio method Caldwell *et al* (Caldwell et al. 1995) found that there was no significant difference in CDT in alcohol misusers with or without liver disease using the CDT ratio.

Schellenberg *et al* (Meregalli et al. 1995) also found that there were no false positive values in non-alcoholic liver disease using the CDT ratio. Jeppsson *et al* (Helander and Carlsson, 1996b) used a commercial HPLC to detect and quantify CDT, and then expressed this as a ratio to total transferrin. Using this method there was a close to 100% sensitivity in detecting >70g alcohol intake per day. However Sorvajarvi *et al* (Sorvajarvi et al. 1996) compared CDT expressed in absolute terms as CDTect and in relative terms as AXIS %CDT in the same population. The sensitivity dropped from 59% to 34% by expressing the result in relative terms, but the specificity increased from 81% to 100%.

There have also been several studies looking at both research and commercial techniques in the same populations. These have almost all shown a higher sensitivity and specificity with the research (predominantly isoelectric focusing) rather than with the commercial technique (Xin et al. 1992; Lieber et al. 1993; Lof et al. 1993; Stibler, 1993; Spies et al. 1995; Werle et al. 1997).

3.8.2.3 Gender differences

Stibler *et al* (Stibler et al. 1988) studied 100 healthy volunteers and showed that women have higher serum CDT values than men (Table 3.4). This is reflected in the higher cut-off value for CDT, as determined by CDTect, for women than for men. Although one of the reasons suggested for this is the higher oestrogen level in women, Groenbaek *et al* (Gronbaek et al. 1995) found no difference between pre and post menopausal women. Despite adjusting for this there is still a lower sensitivity and specificity for women than for men using either of the commercial assays. The only series to give a higher sensitivity and specificity for women is that by La Grange *et al* in 1994 (La Grange et al. 1994) which examined a total of 148 college students: 44 male and 104 female. The women had a sensitivity and specificity of 50% and 99% respectively, while the men had 20% and 89% respectively. However there were only two alcohol abusing women and five alcohol-abusing men in the population. Nystrom *et al* (Nystrom et al. 1992) found serum CDT to be an unreliable marker in women, with none of the seven heavy drinkers testing positive. However in the male group containing 23 heavy drinker the sensitivity was only 21.7%. One of the criticisms in the majority of these studies is the relatively small number of alcohol-abusing women as compared to the men.

3.8.2.4 Age differences

The poor performance of tests for CDT in the female populations seen above may also be attributed to the fact that they were recruited from young populations (La Grange et al. 1994;

Nystrom et al. 1992; Niemela et al. 1995; Huseby et al. 1997a). This effect can also be seen with other markers of alcohol-abuse: gamma glutamyl transpeptidase (GGT) and aspartate transaminase (AST) as well as serum CDT all gave inadequate sensitivity and specificity in a study by Chan *et al* (Borg et al. 1994). This was attributed to either the relatively short duration of alcohol exposure (an average of 5.6 years) and that young alcohol-misusers may be able to reverse the biochemical effects of alcohol more quickly. Yersin *et al* (Yersin et al. 1995) compared CDT with GGT and erythrocyte mean corpuscular volume (MCV) and found that serum CDT had the best specificity in young males, but GGT was superior in males over 60 years. Meregalli *et al* (Meregalli et al. 1995) found a reduction in specificity with age, with a low false positive rate amongst non-alcoholic liver disease in the young and a much higher rate in those over 60 years. They attributed this, at least in part, to an underestimate of alcohol intake in the elderly.

In young people the use of questionnaires may be the best indicator of alcohol-abuse. In young male soldiers Bisson (Bisson and Milford-Ward, 1994) showed a higher sensitivity with the Michigan Alcohol Screening Test (MAST) of 100% and for the CAGE of 93%, as compared to 31% for serum CDT. However La Grange (La Grange et al. 1994) showed, in a small number of alcohol-abusing students, that serum CDT and GGT were better.

3.8.2.5 Ethnic variations

False negative values are seen in the rare transferrin B variant, found in Indians. 30% of Japanese patients (Tsutsumi et al. 1994) and 1% of American Blacks (Murawaki et al. 1997) carried the D variant resulting in a low specificity due to the false positive rate. Behrens *et al* (Yersin et al. 1995) showed that Puerto Ricans had a higher CDT than either a Black or Caucasian population. These ethnic differences have implications for a cosmopolitan population, as seen in most large first-world cities today. Despite this, the ethnic mix has been rarely documented in many of these studies.

3.8.2.6 CDT compared to other markers

Conventional detection of alcohol-abuse and alcoholic liver disease has used the serum markers GGT and AST, and erythrocyte MCV, but their specificity has often been limited by the high false positives in non alcoholic liver disease. Serum CDT has been compared with these and GGT is probably the best alternative. Serum CDT has been shown to have a higher specificity (Behrens et

al. 1988a; Kwoh-Gain et al. 1990; Fletcher et al. 1991; Bell et al. 1993; Sillanaukee et al. 1993; Bell et al. 1994b; Jaakkola et al. 1994; Radosavljevic et al. 1995; Stauber et al. 1995; Yersin et al. 1995), while the sensitivity is similar to that of GGT. The sensitivity of CDT is higher than GGT in some series (Behrens et al. 1988a; Gjerde et al. 1988; Spies et al. 1995; Lesch et al. 1996b; Fletcher et al. 1991) and lower in others (Bell et al. 1993; Sillanaukee et al. 1993; Bell et al. 1994b; Lof et al. 1994; Wickramasinghe et al. 1994; Meregalli et al. 1995; Niemela et al. 1995; Stauber et al. 1995; Yersin et al. 1995; Helander et al. 1996a; Helander and Tabakoff, 1997). Both serum CDT and GGT have been shown to be independently related to alcohol-abuse (Helander and Tabakoff, 1997) and so the combination of markers is logical. Anton and Moak (Anton and Moak, 1994) showed that, in combination, the sensitivity almost doubled in women from 44% to 72%, and in men there was an increase from 79% for serum CDT and 65% for GGT, to 95% when used together (Table 3.5).

In the presence of mild liver disease Meregalli *et al* (Meregalli et al. 1995) and Niemela *et al* (Niemela et al. 1995) showed that serum CDT had the highest specificity and has is most valuable, particularly in differentiating alcoholic from non-alcoholic liver disease, in mild to moderate rather than severe disease.

There are some alternative markers of alcohol-abuse. The ratio of mitochondrial AST to total AST (mAST:tAST) has been shown to have a higher sensitivity but lower specificity than serum CDT in two series (Fletcher et al. 1991; Kwoh-Gain et al. 1990). β-Hexosaminidase (Hultberg et al. 1995) has a similar sensitivity and specificity to serum CDT.

3.8.3 CURRENT USE OF CDT

3.8.3.1 Screening

The development of commercial assays has enabled large studies to examine population screening. While it is obviously highly desirable for a single marker to provide the sensitivity and specificity for population screening it is unlikely that this will occur in reality. Certainly serum CDT is unable to meet this need in unselected populations. Nilssen *et al* (Nilssen et al. 1992b) described a sensitivity < 26% and a specificity of >90%, in unselected screening. Fagerberg *et al* (Fagerberg et al. 1994) showed a false positive rate of 87% when screening 439 hypertensive male patients.

As has been illustrated, the use of serum CDT in young people, for screening, would not justify the cost of the test with the low specificities (Nystrom et al. 1992; Gronbaek et al. 1995).

While the use of serum CDT for screening women is limited, in middle aged men it may be more useful. Gronbaek *et al* (Gronbaek et al. 1995) looked at drinking at a harmful level, more than 35 beverages per week, and above the recommended level (above 21 and 14 per week for men and women respectively). Overall serum CDT had the best performance for both levels of drinking in men, but was not particularly helpful in women (Table 3.6).

3.8.3.2 Monitoring

Serum CDT has a half-life of seven to twenty days and elevated serum CDT has been shown to revert to normal levels following ten to fourteen days of abstinence (Stibler et al. 1978). Serum CDT has been compared to serum AST, ALT and erythrocyte MCV within ten days of detoxification and then two weeks later. Serum CDT gave the best indication, in terms of sensitivity and specificity, of recent heavy drinking and monitoring for a relapse (Gjerde et al. 1988). Helander (Helander and Carlsson, 1996b) showed that serum GGT took longer (three weeks) to normalise than serum CDT.

In a number of studies looking at serum CDT monitoring for alcohol abuse during and after detoxification CDT has often been a helpful adjunct, but cannot be relied on as the sole marker in all individuals. However, it has been reported by several authors (Behrens et al. 1988b; Carlsson et al. 1993; Helander et al. 1996a) that serum CDT and GGT are complimentary markers and should be used in combination initially. Then, as they are both independent markers of alcohol-abuse, the marker that is initially raised in the individual should be used for monitoring and the other test can be stopped. In this way expense is minimised. Serum CDT, although expensive, should not be measured more than once per week, in view of the half-life.

Using the manufacturer's cut-off may not make the most use of serum CDT for monitoring an individual. Borg *et al* (Borg et al. 1995) showed in a study of alcohol-misusers undergoing detoxification, which used a cut-off of three times the minimum level for the individual, that 79% relapses were detected, supporting the suggestion that serum CDT should probably be used with an individualised reference range.

In abstinence erythrocyte MCV often remains elevated due to it's long half-life of 90 days, and serum AST and GGT will often be elevated as a reflection of liver disease. Bell *et al* (Bell et al. 1993) showed that serum CDT was a better indicator of abstinence than MCV, AST or GGT.

Although serum CDT has usually been shown to be more useful in men than women, Helander (Helander and Carlsson, 1996b) found that over three to five weeks, serum CDT was more useful in women and GGT in men (Table 3.7).

3.8.4 CONCLUSION

With alcohol misuse assuming ever increasing importance, both medically and economically, there is likely to an increasing demand for reliable and accurate markers of alcohol-abuse. CDTect has been shown to perform well in some studies but not so well in others (Allen et al. 1994). There remain a number of important questions to be evaluated in order to determine the optimum role for CDT (Potter, 1994).

Table 3.1: Detection of serum CDT using 'research' techniques

Author	Subjects	Technique	Sensitivity (%)	Specificity (%)
Stibler et al. 1979	98 alcoholics, 22 NALD, 100 controls	IEF/IF	81	99
Vesterberg et al. 1984	20 alcoholics 26 controls	IEF/ZIA	95	92
Storey et al. 1985	20 alcoholics 14 NALD 3 healthy volunteers alcohol loaded 14 controls	Ion exchange column / chromatofocusing	85	100
Kanitz et al. 1994	50 alcoholics 25 controls	IEF/IF	82	100
Storey et al. 1987	20 alcoholics 14 NALD 10 Diabetics 13 healthy volunteers	Ion exchange column / RIA	90	100
Chan et al. 1989	35 alcoholics (aged 20-28) 39 controls (students 18-29 years)	IEF/ZIA	15	96
Kapur et al. 1989	Alcohol misusers: 22 >80g/day, 68 < 50g/day 47 NALD 38 General out-patients	IEF/IF	90	99
Poupon et al. 1989	173 consecutive attendees at family doctor, including 20 alcohol misusers	IEF/IF	45	89
Xin et al. 1991	58 alcohol misusers 7 abstinent alcoholics 7 NALD 16 healthy volunteers	IEF/WB	78	100
Bean and Peter, 1993	48 alcohol misusers 48controls	IEF/IB/LD	67	83
Jeppsson et al. 1993	60 active alcohol misusers 50 abstinent alcohol misusers 284 men with a raised GGT 45 occasional drinkers 11 teetotal	HPLC	100	100
Lof et al. 1993	20 alcohol misusers 20 healthy volunteers	IEF	100	95
Anton and Bean, 1994	59 alcohol misusers 61 matched controls	IEF/WB	(M) 85 (F) 33	(M) 93 (F) 95
Bisson and Milford-Ward, 1994	62 male alcohol abuse soldiers 51 controls (army volunteers)	IEF	31	94
Sillanaukee et al. 1994	28 alcohol misusers 16 weekend drinkers 26 healthy controls	FPLC/RIA IEF/ IF	59 89	92 88
Tsutsumi et al. 1994	130 alcohol misusers 54 NALD as controls	IEF	63	82

Godsell et al. 1995	38 alcohol dependants 58 hazardous drinkers 38 controls	IEF/ nephelometry	67	95
Spies et al. 1995	97 ICU trauma patients: 53 alcohol misusers 44 non-alcoholics	MAEC/TIA	83	100
Heggli et al. 1996	17 heavy drinkers 25 social drinkers 9 abstinents	HPLC including trisialotransferrin.	82 (100 inc trisialoTf)	100
Schellenberg et al. 1996	74 alcohol misusers 90 abstains	AEC/ nephelometry	73	90
Bean et al. 1997	32 alcohol misusers 33 social drinkers 8 total abstinents 7 pregnant women	AXIS HPLC IEF/IB/LD	87 83	100 94
Martensson et al. 1997	42 alcohol misusers 73 non-alcoholics from screening	FPLC	(M) 75 (F) 86	(M) 92 (F) 93
Werle et al. 1997	51 alcohol misusers 20 NALD 30 healthy volunteers	HPLC	80	97

Table 3.2: Detection using commercial techniques

Author	Subjects	Technique	Sensitivity (%)	Specificity (%)
Stibler et al. 1986	77 alcohol misusers 187 NALD /hospital patients 33 abstinents 80 healthy volunteers	MAEC/RIA	91	100
Stibler and Hultcrantz, 1987	15 alcoholic liver disease 87 NALD	AEC/RIA	87	100
Behrens et al. 1988a	107 alcohol misusers	MAEC/RIA	81	91
Gjerde et al. 1988	34 alcohol misusers	AEC/RIA	68	100
Stibler et al. 1988	58 alcohol dependants 62 healthy volunteers	MAEC/RIA	83	100
Schellenberg et al. 1989	160 alcohol misusers 23 NALD 50 healthy volunteers	MAEC/RIA	76	90
Kwoh-Gain et al. 1990	26 alcoholics	MAEC (Pharmacia) RIA	81	97
Fletcher et al. 1991	26 alcohol misusers 21 NALD 19 NASH 16 healthy volunteers	AEC/RIA	81	98
Stibler et al. 1991	78 alcohol misusers 20 abstinent alcohol misusers 55 NALD 27 CADG syndrome 71 normal consumers	MAEC/RIA	94	98
Xin et al. 1992	77 alcohol misusers 8 abstinent alcohol misusers 7 NALD 16 healthy volunteers	MAEC/RIA : CDTect	60	100
Bell et al. 1993	102 drinking alcohol misusers 66 abstinent alcohol misusers 70 NALD 100 healthy blood donors 82 healthy employees	MAEC: RIA	61	92
Lof et al. 1993	20 alcohol misusers	AEC/RIA	95	90
Sillanaukee et al. 1993	96 alcohol misusers 77 heavy drinkers 122 social drinkers	CDTect RIA	79	92
Yamauchi et al. 1993	87 alcoholics with liver disease 25 alcoholics, no liver disease 27 viral liver disease 37 healthy volunteers	1. CDTect RIA 2. AXIS %CDT RIA	1. 29 2. 37	1. 92 2. 85
Anton and Bean, 1994	59 alcohol misusers 61 matched employees	CDTect RIA	(M) 66, (F) 44	(M) 98, (F) 100
Anton and Moak, 1994	59 alcohol misusers 61 matched employees	CDTect RIA	(M) 79 (F) 44	(M) 93 (F) 90

Jaakkola et al. 1994	86 patients with acute pancreatitis: 42 alcoholic 24 possible alcohol misusers 20 non-alcoholic	CDTect RIA	75	100
Kanitz et al. 1994	101 alcohol dependants 30 NALD 31 healthy volunteers	CDTect RIA	61	97
Lof et al. 1994	62 alcohol misusers 57 heavy drinkers 135 social drinkers 13 teetotals 36 pregnant women	CDTect RIA	56	85
Sillanaukee et al. 1994	28 alcohol misusers 16 weekend drinkers 26 healthy controls	CDTect RIA	89	85
Tsutsumi et al. 1994	130 alcohol misusers 54 NALD as controls	1. CDTect RIA 2. AXIS %CDT	1. 58 2. 60	1. 61 2. 54
Wickramasinghe et al. 1994	49 alcohol misusers 15 healthy volunteers	CDTect RIA	71	92
Caldwell et al. 1995	32 alcoholics with liver disease 14 alcoholics without liver disease	AEC/RIA	80	92
Hultberg et al. 1995	42 alcohol misusers 11 abstinent alcohol misusers	CDTect-RIA	83	91
Meregalli et al. 1995	68 alcohol misusers 72 NALD/abstinent alcoholics 51 controls	CDTect RIA	67	82
Niemela et al. 1995	373 alcohol misusers 47 healthy volunteers	CDTect RIA	36	100
Radosavljevic et al. 1995	26 alcohol misusers 170 GI non-misusers	AXIS %CDT RIA	88%	82%
Spies et al. 1995	97 trauma patients in ICU: 53: alcohol misusers 44: non alcohol-misusers	CDTect RIA	74	95
Sorvajarvi et al. 1996	83 alcohol misusers 89 controls	1.CDTect RIA 2 AXIS %CDT RIA	1. 59 2. 34	1. 81 2. 100
Bean et al. 1997	32 alcohol misusers 33 social drinkers	AXIS %CDT TIA	87	98
Helander and Tabakoff, 1997	23 alcohol dependants 25 heavy drinkers 26 light/moderate drinkers 21 non-drinkers	CDTect RIA	33	94
Huseby et al. 1997a	137 alcohol dependants 202 general surgical patients	1.CDTect RIA 2.AXIS % CDT RIA	76 77	86 92
Huseby et al. 1997b	202 acute surgical inpatients: 57 alcohol dependants 145 drinking <60g/day	AXIS %CDT RIA	71	73
Martensson et al. 1997	42 alcohol misusers	CDTect RIA	86	92

Stowell et al. 1997a	1. Older males: 19 alcohol misusers, 3 heavy drinkers, 19 moderate drinkers, 15 non-drinkers 2. Young adults: 30 heavy drinkers, 81 moderate drinkers, 42, light drinkers, 59 non-drinkers	1. CDTect RIA 2. AXIS %CDT RIA	Older: 1. 83 2. 78 Younger: 1. 37 2. 43	Older: 1. 88 2. 94 Younger: 1. 93 2. 88
Stowell et al. 1997b	18 alcohol misusers 20 moderate drinkers 4 heavy non - alcoholics 15 non-drinkers	CDTect: MAEC RIA	83%	83%
Werle et al. 1997	51 alcohol dependants 20 NALD 30 healthy volunteers	CDTect RIA	47	>95

Table 3.3: Comparison of commercial CDT techniques

Author	Subjects	CDT technique (1)	Sens (%)	Spec (%)	CDT technique (2)	Sens (%)	Spec (%)
Yamauchi et al. 1993	112 alcohol misusers 27 viral liver disease 37 healthy volunteers	CDTect RIA	29	92	AXIS %CDT RIA	37	85
Bell et al. 1994a	26 alcohol misusers 476 non-abusing medical patients	CDTect RIA	69	92	AXIS % CDT RIA 1. 1 step elution 2. 2 step elution	1. 65 2. 50	1. 76 2. 90
Tsutsumi et al. 1994	130 alcohol misusers 54 NALD as controls	CDTect RIA	58	61	AXIS %CDT	60	54
Sorvajarvi et al. 1996	83 alcohol misusers 89 controls	CDTect RIA expressed absolutely & relatively	absolute 59 relative 45	absolute 81 relative 99	AXIS %CDT RIA	34	100
Huseby et al. 1997a	137 alcohol dependants 202 general surgical controls	CDTect RIA	76	86	AXIS %CDT RIA	77	92
Stowell et al. 1997a	1. Older males: 19 alcohol misusers 3 heavy drinkers 19 moderate drinkers 15 non-drinkers 2. Young adults: 30 heavy drinkers 81 moderate drinkers 42 light drinkers 59 non-drinkers	CDTect RIA	Older 83 Younger 37	Older 88 Younger 93	AXIS %CDT RIA	Older 78 Younger 43	Older 94 Younger 88

Table 3.4: Gender differences

Author	CDT method	Male Subjects	Sensitivity	Specificity	Female Subjects	Sensitivity	Specificity
Stibler et al. 1991	MAEC/ RIA	43 healthy volunteers 58 alcohol misusers	97	98	28 healthy volunteers 20 alcohol misusers	85	98
Nystrom et al. 1992	CDT RIA (Pharmacia)	102 students at health screening	22	96	187 students at health screening	0	96
Anton and Bean, 1994	1.CDTect RIA 2.IEF/IB/LD	41 alcohol misusers 40 controls	CDTect:66 IEF/IB/LD 85	CDTect 98 IEF/IB/LD 93	18 alcohol misusers 21 controls	CDTect 44 IEF: 33	CDTect 100 IEF 95
Anton and Moak, 1994	CDTect RIA	41 alcohol misusers 40 controls	79	93	18 alcohol misusers 21 controls	44	90
La Grange et al. 1994	CDT RIA (Pharmacia)	All students: 5 heavy drinkers 39 controls	20	89	All students: 2 heavy drinkers 102 controls	50	99
Gronbaek et al. 1995	CDT-RIA	180 subjects from heart study	Harmful: 82 Rec levels: 61	Harmful: 77 Rec levels: 80	220 subjects from heart study	Harmful 0 Rec levels 12	Harmful 93 Rec levels: 93
Niemela et al. 1995	CDTect: MAEC/ RIA	294 alcohol misusers 27 healthy volunteers	36	100	79 alcohol misusers 15 healthy volunteers	27	100
Huseby et al. 1997a	1. CDTect RIA 2. AXIS % CDT RIA	103 alcohol dependants 32 surgical patients	1. 75 2. 70	1. 81 2. 89	34 alcohol dependants 70 surgical patients	1. 48 2. 52	1. 86 2. 92
Huseby et al. 1997b	AXIS %CDT RIA	132 surgical inpatients	49	85	70 surgical inpatients	67	85
Martensson et al. 1997	CDTect FPLC abs & relative to transferrin	28 alcohol misusers Non-alcoholic 40-45 yr olds for screening: 28 high intake, 29low intake	1. 86 2. 75, as %TTf: 79	1. 92 2. 92 as %TTf: 93	14 alcohol misusers Non-alcoholic 40-45 yr olds for screening: 14 high intake 15 low	1. 86 2. 86, as %TTf:93	1. 93 2. 93 as %TTf: 93
Stowell et al. 1997a	1. CDTect (RIA) 2. AXIS %CDT (RIA)	56 older drinkers Young 129: heavy 25 moderate 63 light 14 abstinents 27	Older: 1. 83 2. 78 Younger 1. 40 2. 44	Older: 1. 88 2. 94 Younger: 1. 88 2. 83	Older drinkers: 0 Young 83: heavy 5 moderate 18 light 28 abstinents 32	Older: no females Younger: 1. 20 2. 40	Older: no females Younger: 1. 97 2. 92
Werle et al. 1997	1. CDTect: MAEC/ RIA 2. HPLC	32 alcohol misusers 11 NALD 12 healthy volunteers	1. 50 2. 88	1. >95 2. 97	19 alcohol misusers 9 NALD 18 healthy volunteers	1. 53 2. 68	1. >95 2. 97

Table 3.5: CDT compared to other markers of alcohol misuse

Author	Subjects	CDT Technique	Sens (%)	Spec (%)	Other markers	Sens (%)	Spec (%)
Schellenberg and Weill, 1987	50 alcohol misusers 25 controls	IEF/ IF	82	100	GGT	86	76
Behrens et al. 1988a	107 alcohol misusers 59 abstinent alcohol misusers 64 NALD 15 healthy volunteers	MAEC/ RIA	81 (56 as transferrin ratio	91 (97 as transferrin ratio)	GGT MCV	59 25	50 95
Gjerde et al. 1988	34 alcohol misusers 35 controls	AEC/ RIA	68	100	MCV GGT ALT AST	65 50 50 26	89 86 86 92
Stibler et al. 1988	58 alcohol misusers 62 healthy volunteers	MAEC/ RIA	83	100	GGT AST ALT MCV	59 50 47 91	Not given
Chan et al. 1989	35 alcohol misusers 39 controls	IEF/ ZIF	15	96	mAST:tAST GGT	0 16	97 95
Kapur et al. 1989	22 alcohol misusers 68 abstinent alcoholic liver disease 47 NALD 38 hospital out-patients	IEF/ IF	90.5	98.8	MCV GGT AST/ALT	65 90 19	93 41 99
Poupon et al. 1989	20 alcohol misusers 153 consecutive primary care patients 58 teetotal	IEF/ IF	45	89	GGT MCV	52 32	80 91
Schellenberg et al. 1989	160 alcohol misusers 23 NALD 50 healthy volunteers	CDTect IEF	76 81	90 92	GGT GDH	88 81	90 92
Kwoh-Gain et al. 1990	26 alcohol misusers 21 NALD 16 healthy volunteers	MAEC/ RIA	81	97	GGT MCV AST ALT mAST: tAST	69 73 69 58 69	59 76 68 57 46
Fletcher et al. 1991	26 alcohol misusers 19 non alcoholic steato hepatitis 21 NALD 16 healthy volunteers	AEC/ RIA	81	98	GGT MCV AST ALT mAST:tAST	69 73 69 58 92	55 79 66 50 50
Bell et al. 1993	102 alcohol misusers 66 abstinent alcoholic liver disease 70 NALD 100 blood donors 82 employees	MAEC/ RIA	61 (72 as CDT/ Tf)	92 (83 as CDT/ Tf)	MCV GGT AST ALT	70 85 75 55	66 18 38 46
Sillanaukee et al. 1993	122 Social drinkers 77 heavy drinkers 96 alcohol misusers	CDTect RIA	79	92	MCV GGT ALT AST	47 65 47 57	93 87 87 98

Anton and Moak, 1994	59 alcohol misusers	CDTect RIA	(m) 79 (f) 44	(m) 93 (f) 90	GGT	(m) 65 (f) 44	(m)100 (f) 100
Bell et al. 1994b	502 consec medical patients (26 >50g/day alcohol)	CDTect: MAEC/ RIA	69 (65 as CDT/tf)	92 (93 as CDT/tf)	GGT AST ALT MCV	73 50 35 52	75 82 86 85
Bisson and Milford-Ward, 1994	62 alcohol misusers (soldiers)	IEF	26	94	MAST CAGE MCV GGT AST:ALT	100 93 18 12 4	86 86 94 100 94
Jaakkola et al. 1994	86 patients with acute pancreatitis (42 alcoholic, 24 possible alcohol misusers)	CDTect RIA	75	100	GGT MCV Lipase /amylase	? >70 >70	? <50 <50
La Grange et al. 1994	148 students	CDT RIA	20 (m) 50 (f)	89 (m) 99 (f)	GGT	57 (m) 50 (f)	68 (m) 70 (f)
Lof et al. 1994	57 heavy drinkers 62 alcohol misusers	CDTect RIA	56	85	MCV GGT ALT AST CDT&GGT	63 61 40 56 85	77 100 85 92 92
Wickramasinghe et al. 1994	49 alcohol misusers 15 healthy volunteers	MAEC/ RIA: CDTect	71	91.8	MCV GGT AST ALT CDT & GGT	38 79 67 35 87	
Godsell et al. 1995	38 'alcohol dependants' 58 'hazardous drinkers'	IEF (lab) (Tf by immunonephelometry)	Alcohol dep. 67 hazardous 62	Alcohol dep. & hazardous 95	GGT	Alcohol dep. 70 hazardous 19	Alcohol dep. & hazardous 95
Gronbaek et al. 1995	400 randomly selected inhabitants participating in a heart study	CDT-RIA	(m) 82 (f) 0	(m) 77 (f) 93	AST sMAST	AST (m) 27 (f) 100 sMAST (m) 91 (f) 50	AST (m) 96 (f) 97 sMAST (m) 95 (f) 98
Hultberg et al. 1995	42 alcohol misusers 11 abstinent alcohol misusers	CDTect-RIA	83	91	β-Hex	91	100
Meregalli et al. 1995	68 alcohol misusers 72 NALD 51 controls	CDTect: MAEC/ RIA	67	82	GGT MCV	81 87	60 63
Niemela et al. 1995	173 alcohol misusers with liver disease 200 alcohol misusers without liver disease 42 healthy controls	CDTect: MAEC/RIA	34	100	GGT MCV	47 34	97 94
Radosavljevic et al. 1995	From 196 consecutive GI patients 26 alcohol misusers 170 non-misusers	AXIS %CDT RIA	88	82	GGT MCV MCV & GGT	88 46 42	42 88 92

Spies et al. 1995	97 male traumatised ICU patients 53 alcohol misusers 44 non alcohol misusers with multiple trauma	1.MAEC/ TIA (lab) 2. CDTect RIA	1. 83 2. 74	1. 100 2. 95	MCV GGT ALT AST	15 36 63 85	98 95 62 36
Stauber et al. 1995	106 in patients with GGT >50u/l 93 liver out-patients	CDTect RIA	70	84	MCV GGT	45 83	85 16
Yersin et al. 1995	552 emergencies patients 650 in primary care	MAEC/RIA: CDTect	58	62	GGT MCV	69 27	65 91
Helander et al. 1996a	114 alcohol misusers	CDTect: RIA	(m)58 (f) 35		GGT GGT & CDT	(m) 67 (f) 59 (m) 83 (f) 70	
Lesch et al. 1996b	101 consecutive in-patients	AXIS % CDT	70	98	GGT	65	83
Helander and Tabakoff, 1997	120 divided into abstinents, light drinker; heavy; alcohol dependants	CDTect RIA	33	94	AST GGT	40 35	87 85
Huseby et al. 1997a	137 alcohol misusers 202 general surgical patients	1. CDTect RIA 2. AXIS % CDT RIA	1. 69 2. 67	1. 51 2. 44	GGT	63	87
Huseby et al. 1997b	202 acute surgical inpatients (57 alcohol misusers)	AXIS %CDT RIA	71	73	GGT GGT/CDT	58 87	59 45
Stowell et al. 1997b	18 alcohol misusers 20 moderate drinkers 4 heavy non - alcoholics 15 non-drinkers	CDTect: MAEC RIA	83	83	MCV GGT ALT AST B-Hex	53 50 67 50 94	91 91 94 89 91

Table 3.6: Screening using CDT

Author	Subjects	Technique	Sensitivity	Specificity
Poupon et al. 1989	173 consecutive from family doctor: 20 excessive drinkers (>80 g/day) 58 teetotal	IEF & immunofixation: transferrin index	45	89
Nilssen et al. 1992b	481 from general population screening	CDTect RIA	At 95th centile (52g/day): 13 (m) 0 (f)	At 95th centile (52g/day): 95 (m) 94 (f)
Nystrom et al. 1992	289 students for health screening: Heavy drinking: > 10kg/yr Social <10kg/yr Teetotal	CDT RIA	(m) 22 (f) 0	(m) 96 (f) 96
Jeppsson et al. 1993	284 men with a raised GGT 60 alcohol misusers 50 abstinent patients following detox 11 Teetotallers 45 occasional drinkers	HPLC	55>40g/day 100>70g/day	100
Sillanaukee et al. 1993	197 consecutive attendees for health screening: 122 social drinkers 77 non-alcoholic heavy drinkers 96 chronic alcoholics (>1000 g/wk)	CDTect RIA	79.2 (29 heavy drinkers)	92
Bell et al. 1994a	502 consecutive medical patients, of which 26 >50g/day alcohol	1. CDTect RIA 2. AXIS % CDT RIA (1 step elution) 3. AXIS % CDT RIA (2 step elution)	1. 69 2. 65 3. 50	1. 92 2. 76 3. 90
Bell et al. 1994b	502 consecutive medical patients of which 26 >50g/day alcohol	CDTect: MAEC/RIA, expressed absolutely & relative to total Tf	69 absolute 65 relatively	92 absolute 93 relatively
Fagerberg et al. 1994	439 treated hypertensive men, of which 32 alcohol misusers. Questionnaire: used 24 g/day as cut-off for heavy drinking: rec level Sweden	CDTect RIA	44	87
La Grange et al. 1994	148 students	CDTect RIA	20 (m) 50 (f)	89 (m) 99 (f)
Lof et al. 1994	62 alcohol misusers From screening programme: 57 heavy drinkers 13 teetotallers, 135 social, 57 heavy drinkers, 36 pregnant women	CDTect RIA	56	85
Gronbaek et al. 1995	400 randomly selected inhabitants participating in a heart study	CDT-RIA	(m) 81 (f) 0	(m) 77 (f) 93
La Grange et al. 1995	49 students: 11 abstinents 15 light (<0.7kg/yr) 23 moderate (<14 kg/year)	MAEC/RIA	N/A	96
Yersin et al. 1995	1202 consecutive patients: 552 emergencys 650 primary care	MAEC/RIA: CDTect	58	62

Lesch et al. 1996b	101 hospital consecutive in-pts: 59 <60g/day (no liver disease) 21<60g/day (liver disease) 5 >60g/day (no liver disease) 16>60g/day (liver disease)	AXIS % CDT	70	98
Helander and Tabakoff, 1997	98 men: 21 abstinents 28 light drinkers: <30 g/day 26 heavy drinkers: >30 g/day, not dependant 23 alcohol dependants	CDTect RIA	33	94
Huseby et al. 1997a	202 general surgical patients, of which 57 alcohol dependant	1. CDTect RIA 2. AXIS % CDT RIA	1. 51 2. 44	1. 86 2. 92
Huseby et al. 1997b	202 acute surgical inpatients	AXIS %CDT RIA	71 (>60g/day) 47 (all patients)	73 (all drinking <60g/day)

Table 3.7: Monitoring using CDT

Author	Number/ Type of Subjects	Sample frequency	Self-report/ Method to verify drinking	Technique CDT
Stibler et al. 1978	16 alcohol misusers: at intoxication and after 10-14 days abstinence.	Days 1 and 14	In-patients	IEF
Behrens et al. 1988b	72 alcohol misusers during detoxification	Every 6-9 days, for 4 weeks, follow-up for 6 months.	Breathalyser, bloods, family, friends	AEC/RIA
Carlsson et al. 1993	15 alcohol dependants for detoxification	1 x week CDT	Daily urine: 5 HTOL, 5 HIAA, ethanol	AEC/RIA
Borg et al. 1995	22 alcohol misusers for detoxification 8 healthy volunteers	Over 6 months. In pt 1-2 weeks, then seen 3 x wk as OP. Samples 1 x wk	Urine 5-HTOL daily	CDTect: MAEC/RIA
Rosman et al. 1995	86 male alcoholics participating in a hepatitis vaccination programme	1 x month for 9 months, then at 12 months	Self-report and collateral	CDTect RIA
Anton et al. 1996	35 male alcoholics for detox, part of naltrexone/ placebo trial	Baseline, than weeks 4, 8, 12, 26	Collateral/self-reports.	CDTect RIA
Helander and Carlsson, 1996b	16 in-patients detox with disulfiram	Studied over 3-5 weeks	Self-report/ breath alcohol	CDTect RIA
Helander et al. 1996a	10 male out-patients for detox	Followed over 6 months	Self-report 3 x week Daily 5HTOL:5HIAA	CDTect: RIA
Lesch et al. 1996a	92 alcohol dependants for detox.	Days 0, 3, 7, 14, 21, 28	Daily breathalyser	AXIS %CDT RIA
Mitchell et al. 1997	53 alcohol dependants	Every 2 weeks for 6 months	Self-report/ breathalyser	CDTect: MAEC/RIA

4. A CLINICAL STUDY OF CARBOHYDRATE DEFICIENT TRANSFERRIN AS A MARKER OF ALCOHOL MISUSE: SENSITIVITY AND SPECIFICITY DETERMINATIONS

4.1 Introduction

Serum CDT has been shown to be a promising marker for alcohol misuse. There has, however, been little published assessment into its use within UK populations. Published studies of non-UK populations have described results obtained when comparing CDTect and AXIS %CDT: Bell *et al* (Bell et al. 1994a) in a large study concerned with screening over 500 Norwegian in-patients found a sensitivity of 69% using CDTect. However AXIS %CDT, with a one step elution, resulted in a sensitivity of 65% and for the two step elution, 50%. Similarly, the respective specificities were 92%, 76% and 90%. Sorvajarvi *et al* (Sorvajarvi et al. 1996) also compared CDTect and AXIS %CDT in a population from Finland, but further expressed CDTect in both absolute terms (units /litre) and also levels of units/litre relative to the concentration of total serum transferrin (CDTect/transferrin). Sensitivity using CDTect was 59% in absolute terms and 45% relative to total transferrin. The sensitivity was 35% using AXIS %CDT. The corresponding specificities were 81% for CDTect (units/litre), 99% (CDTect/transferrin) and 100% using AXIS %CDT.

The differences in performance may result from methodological differences. Whilst CDTect quantifies the a-, mono- and di-sialotransferrin isoforms, AXIS %CDT detects not only these but also the trisialotransferrin isoform. Heggli *et al* (Heggli et al. 1996) showed that the inclusion of trisialotransferrin in the calculation of CDT levels increased the sensitivity and specificity. However Martensson *et al* (Martensson et al. 1997) showed that there was no increase in the trisialotransferrin isoforms in either heavy drinkers or alcohol misusers. Differences may also arise as a result of the expressing serum CDT in either absolute forms or relative to total transferrin concentrations. Several previous studies have indicated that there is no advantage in assay performance by expressing CDTect adjusted for total transferrin (Bell et al. 1994b; Martensson et al. 1997; Sorvajarvi et al. 1996; Behrens et al. 1988) although performance has been enhanced when total transferrin has been taken into account (Bell et al. 1993; Lesch et al. 1996). Further differences in performance may be ascribed to the demographics of the target population.

Aim: to determine the sensitivity and specificity of serum CDT as a marker of alcohol misuse. The means by which this was done was to use two commercial kits to assay CDT levels in serum derived from two UK population groups.

Purpose: to compare assay techniques to define assay performance by expressing CDT in absolute terms and also adjusted for serum total transferrin levels.

4.2 Materials and Methods

4.2.1 Measurement of CDT: General considerations

The separation of the sialic acid deficient transferrin isoforms from normal serum transferrin occurs using anion exchange chromatography. The resin in the chromatography column contains a rigid matrix which carries a net positive charge and acts as an anion exchange site where the substitution of one ionic species for another can occur. Serum placed on the column contains both normal transferrin and the more positively charged sialic acid deficient transferrin (cationic). The resin attracts and retains normal transferrin because it is more negatively charged than the sialic acid deficient transferrin. The eluate from the column therefore contains the carbohydrate-deficient as opposed to the normal transferrin and be quantified by immunological-based techniques.

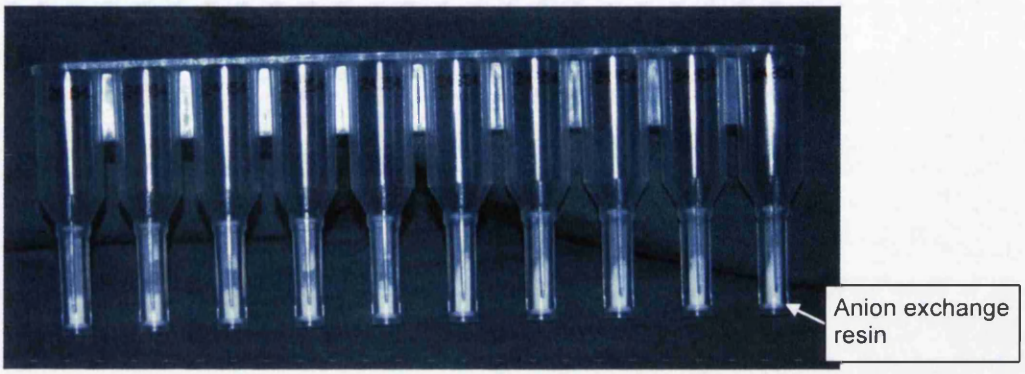
4.2.2 CDT quantitation by CDTect

CDTect was measured using CDTect RIA in the first part of the study and then using CDTect EIA in the second. The assay technique was a two stage process. First the a-, mono- and di-sialotransferrin isoforms were separated from the rest of the transferrin in the serum sample. Then the separated transferrin was quantitated, either by a radioimmuno assay or by an enzyme immuno assay.

The CDTect assay was performed as follows (Stibler et al. 1986). The dry microcolumns were tapped on the bench surface so that the resin in each column settled and was uniformly packed. Each column was hydrated with elution buffer for 15 minutes in an equilibration box provided by the manufacturers. A further 2 ml of elution buffer was then added to the columns. The

elution buffer was allowed to pass through the columns for at least 1½ hours until the top of each column looked dry.

Figure 4.1: CDTect minicolumns



1 strip of 10 CDTect minicolumns

A 1 ml serum aliquot was thawed at room temperature. Serum samples were then iron saturated so that each transferrin molecule theoretically contained two iron atoms. 200 µl of ferric citrate was added to 50 µl of serum sample followed by 1 ml of elution buffer was added, giving a total volume of 2.25 mls of sample-buffer mixture.

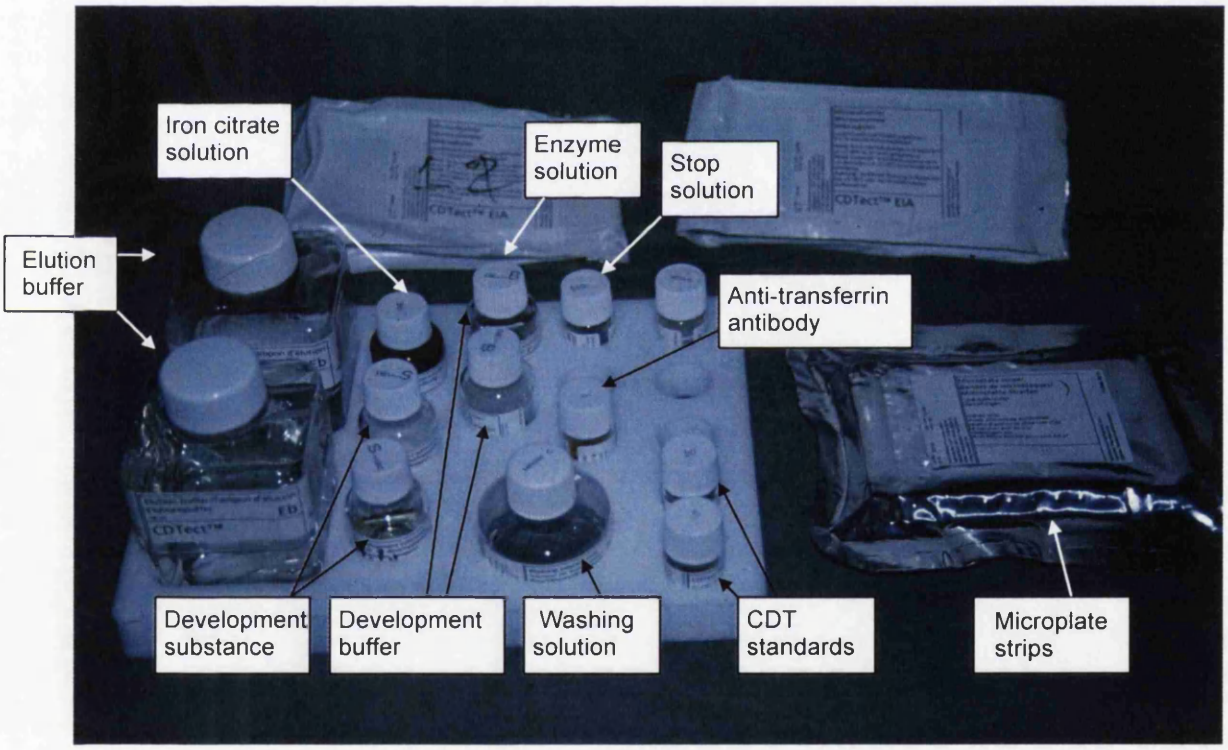
An equilibrated microcolumn was placed over an elution tube and 500 µl of the sample-buffer mixture was pipetted directly onto the microcolumn. After approximately 30 minutes, when all the sample-buffer mixture had passed through the tube and the top filter looked dry, the microcolumns were tapped over the elution tubes so that the last drops of eluate were collected.

Radioimmuno assay. The eluate was quantitated for transferrin using a radioimmuno assay (RIA). ^{125}I-labelled transferrin was added to the eluate as a tracer, and then the antibody (rabbit anti-human transferrin) was added. Then a double antibody suspension of sheep antibodies against the rabbit IgG was added in excess. The incubation mixture was centrifuged and then the radioactivity was measured. A standard curve was produced using purified human serum transferrin, provided in the kit, and the sample radioactivity was then used to indicate the transferrin concentration.

Competitive enzyme immunoassay. Quantitation of eluted transferrin isoforms was performed using a competitive enzyme immunoassay. The assay involves competing an unknown sample (eluate containing CDT) with a standard transferrin-enzyme conjugate for the

binding to a mouse monoclonal antibody (Mab) with specificity for human transferrin. The more transferrin in the unknown sample then the less of the standard transferrin enzyme conjugate that will bind to the antibody. Transferrin/anti-transferrin complexes are captured and retained in the wells by the use of a polyclonal antibody, immobilised to the wells, with specificity for mouse IgG. Therefore once all of the unused reagents have been washed away, the enzyme on the standard transferrin that has been captured is used to catalyse a colour reaction that can be measured spectrophotometrically. The more colour there is present indicates that more of the enzyme-transferrin conjugate has been immobilised and that there is little CDT in the sample. A standard curve is constructed where known standards containing exact amounts of CDT are substituted for the samples in the competitive binding assay.

Figure 4.2: CDTect EIA kit (Pharmacia & Upjohn)



The contents of the CDTect kit are shown. The microplate strips were packaged in a blister pack and the microcolumns in two aluminium packs. The development solution was constituted by adding development substance to development buffer.

Finally the absorbance (optical density) was measured at 405 nm. With each run a standard curve was constructed, using the CDT standards supplied with the kit, substituting them for the sample. A dose/response curve was constructed using log concentrations of CDT standards versus optical density. Interpolation from the curve gave the unknown CDT concentrations.

The stated dynamic range from the manufacturer for the kit was 5 – 200 U/L, where 1 unit is approximately 1 mg/L.

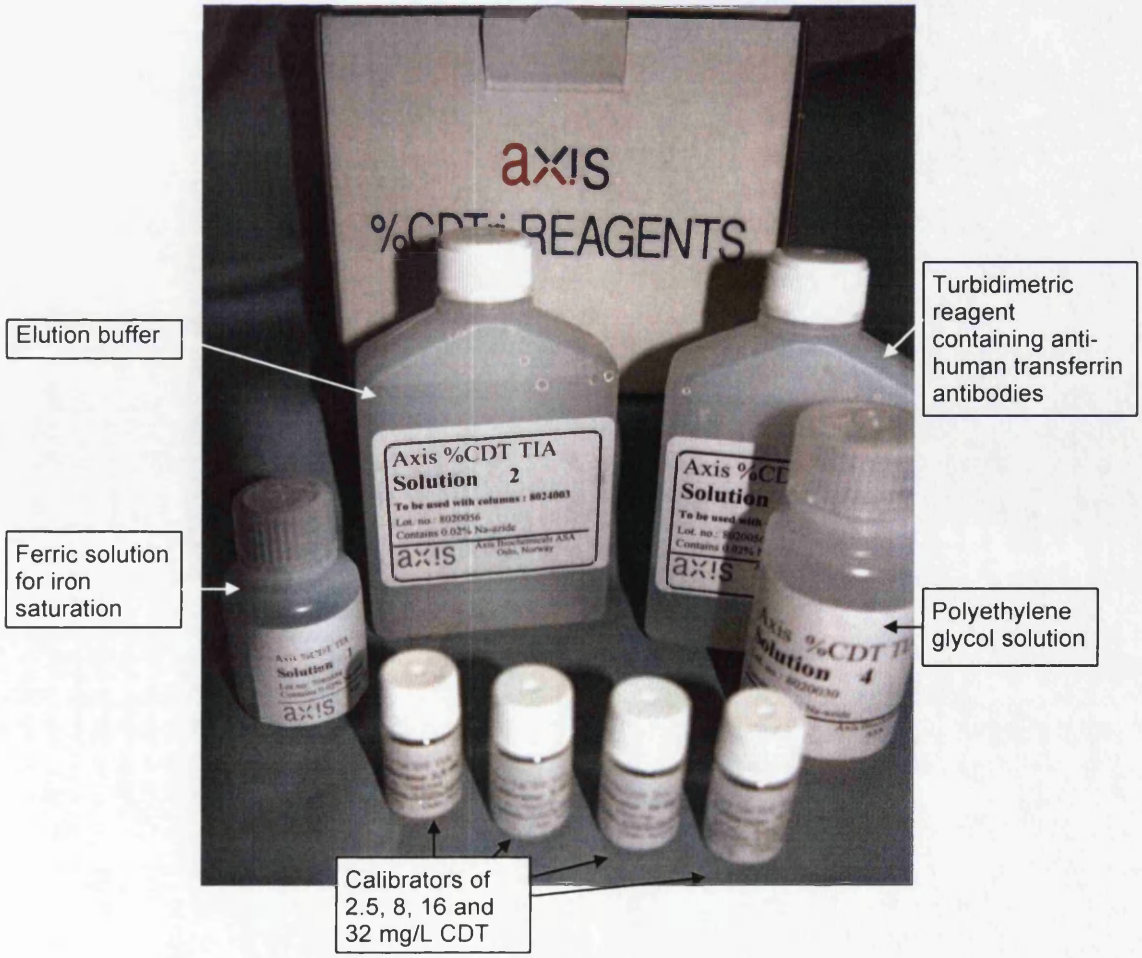
The kit upper reference limit was 20 U/l for men and 26 U/l for women.

4.2.3 CDT QUANTITATION BY AXIS %CDT

This kit measures not only the a-, mono-, disialo and half of the trisialotransferrin, but also the total transferrin. The carbohydrate deficient transferrin portion is then expressed as a ratio to the total transferrin.

The columns were prepared by removing first the top and then the bottom stopper from each column (figure 4.4). Each column was placed in a rack and the transport buffer was allowed to fully elute until the column looked dry.

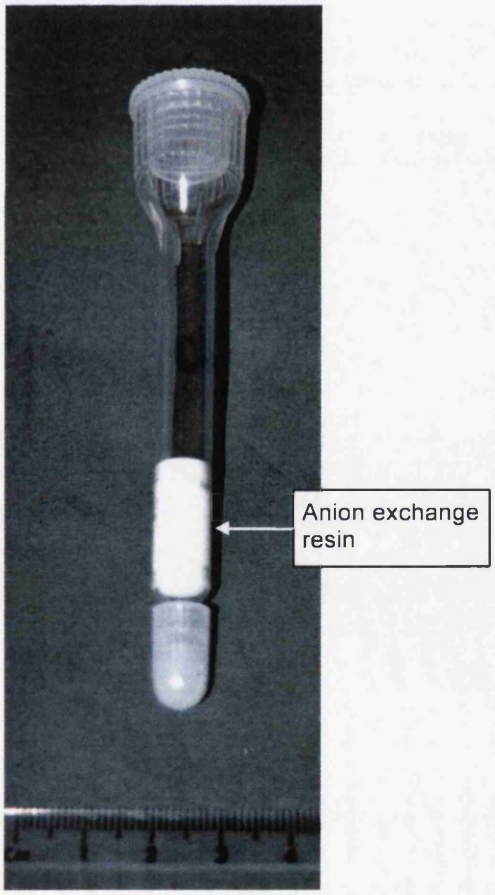
Figure 4.3: Contents of AXIS %CDT kit (AXIS Biochemicals ASA)



Each test serum sample was allowed to thaw at room temperature. 100 µl of each sample was pipetted into a test tube and was iron saturated by the addition of 500 µl buffered iron (solution 1) followed by vortexing. They were left to incubate for about 15 minutes at room temperature. The contents of each tube, referred to as the incubation solution, were subsequently used for anion-exchange chromatography of CDT isoforms and for the assessment of total transferrin.

Anion-exchange chromatography: 500 µl of the incubation solution was added to a column, by pipetting directly onto the top filter. Once this had been allowed to absorb onto the column 1.0 ml of solution 2 (elution solution) was then added to each of the columns. This eluate was discarded. The eluate following the addition of a further 2.0 mls of solution 2 was collected and retained for quantification of CDT.

Figure 4.4: AXIS %CDT minicolumn

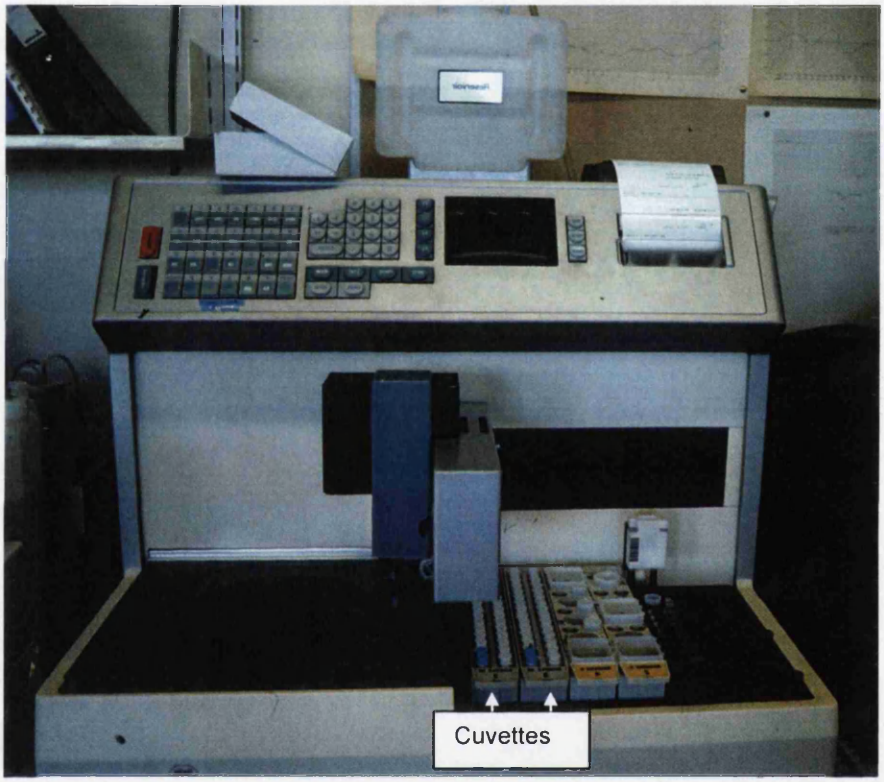


Internal controls were added for each run, utilising the low, medium and high CDT calibrator standards provided by AXIS. Each of the lyophilised standards was reconstituted with 1000 µl distilled water and aliquots were analysed in an identical manner to each of the serum samples.

For the measurement of the total transferrin, 50 µl of the incubation solution from each sample prior to chromatography was mixed with 2.0 mls of solution 2, the elution buffer.

For the quantification of both CDT and total transferrin an automatic procedure using a sophisticated electro-mechanical machine, the Cobas Mira, was used (Figure 4.5).

Figure 4.5: The Cobas Mira machine, used for immunoturbidimetry



This machine (marketed and supported by Hoffman LaRoche) can be programmed to perform multiple steps of dispensing accurate volumes of reagents to a large number of samples in a precisely controlled series of steps and under rigorously controlled conditions. For this particular assay it had been programmed for immunoturbidimetry.

Immunoturbidimetry is an immunological method for quantifying biomolecules. When an antibody is added to antigen under pre-determined optimum immune conditions complexes are formed. The complexes had a maximum absorbance of light at 405 nm. A blank absorbance (optical density) of 405 nm was taken. The blank comprised 200 µl of calibrator, eluate from the column or total transferrin test solution, added to each Mira cuvette, and constituted the background absorbance. The absorbance was measured at 405 nm. For immunoturbidimetric

analysis of test samples, to each cuvette 100 µl of solution 3 was added, the turbidimetric reagent containing the anti-transferrin antibodies. The mixture was left to incubate for 15 minutes at room temperature. The absorbance was then re-measured at 405 nm. The background absorbance was subtracted from the turbidimetric values. A calibration curve was established using CDT concentrations of the calibrators versus absorbance at 405 nm. The CDT and total transferrin levels were measured using separate samples but using the same programme and instrument settings on the Cobas Mira.

The % CDT was calculated using the following formula:

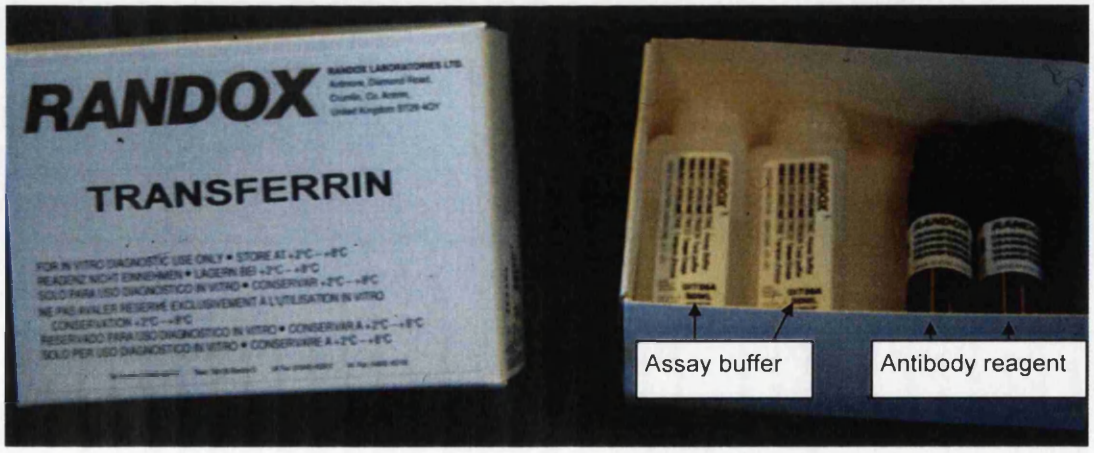
$$\%CDT = 7.7 \times \frac{\text{CDT concentration}}{\text{total transferrin concentration}} - 0.2$$

The figures 7.7 and 0.2 are constants that were derived from standardising the Cobas Mira during the programme sequence for immunoturbidimetry.

4.2.4 Measurement of Total Transferrin

A commercial assay (Randox) based on an immunoturbidimetric assay for quantification of total transferrin was used.

Figure 4.6: Transferrin immunoturbidimetry assay kit (Randox)



The kit contained (Figure 4.6):

- 3 x 50 mls of assay buffer, containing 6% Polyethylene glycol, 20 mmol/l Tris/HCl buffer (pH 7.4) and 150 mmol/l sodium chloride.

- 2 x 8 ml antibody reagent, containing anti (human) transferrin in 20 mmol/l, Tris/HCl (pH 7.4) and 150 mmol/l sodium chloride

Also required were 3 x 1.0 ml specific transferrin standards to calibrate the assay.

The coded serum samples for analysis were thawed at room temperature. The assay was established using the Cobas Mira (Figure 4.5) in a manner similar to that used for the AXIS %CDT method. 25 µl of sample was pipetted into a cuvette containing 975 µl of 0.9% sodium chloride giving a final serum dilution of 1:40. 50 µl of this diluted sample was pipetted into a Cobas Mira cuvette and 1.0 ml of the assay buffer was added. They were left to incubate at room temperature for 10 minutes. The absorbance was read at 340 nm (absorbance 1). Following this 100 µl of antibody reagent was added to each cuvette and after 30 minutes incubation at room temperature the absorbance was again read at 340 nm (absorbance 2). The calibrators were assayed in an identical manner.

A standard curve was plotted of Log (absorbance 1 - absorbance 2) versus transferrin, and the transferrin concentrations in each sample obtained by interpolation.

The normal range of human serum transferrin is 200-400 mg/dL (2.0-4.0 g/L) using this particular method. There were several samples with readings well below this range and low standards were specifically requested from Randox so that accurate estimations could be made.

4.2.5 DEFINITIONS

4.2.5.1 Sensitivity

This is the true positive rate, or the proportion of alcohol misusers who had a positive (higher than the cut-off CDT value) test result, out of all the alcohol misusers:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{All actively drinking alcohol misusers}}$$

The false negative rate, or those actively drinking alcohol misusers with a CDT level in the normal range, will adversely affect the sensitivity.

4.2.5.2 Specificity

This is the true negative rate, or those individuals who are not actively drinking and with a test result within the normal range, as a proportion of all non-actively drinking individuals:

$$\text{Specificity} = \frac{\text{True negative individuals}}{\text{All non-actively drinking individuals}}$$

The false positive rate, or those individuals who are not actively drinking but with a positive (higher than manufacturers' cut-off) test result, will adversely affect the specificity.

4.3 Retrospective analysis

The sensitivity and specificity was determined in a large population of individuals using previously archived sera. The population comprised sub-groups of drinking and abstinent alcohol misusers, healthy volunteers and individuals with non-alcoholic liver disease.

4.3.1 Patients and Methods

590 sera were retrieved from previously archived material that had been collected and stored at -20^0C for up to 5 years. The sera were derived from a total of 298 men and 292 women who were categorised into four sub-groups (Table 4.1).

1. **Healthy volunteers**, n=94 (39 men and 55 women) were recruited from among laboratory personnel and relatives of patients. None had a history or clinical evidence of alcohol misuse or chronic liver disease and none consumed alcohol in excess of 20g/day. Eighty eight had normal liver function tests results. Five individuals with Gilbert's syndrome had elevated serum bilirubin concentrations of between 20 - 31 μmol/L (reference range 5-17 μmol/L), one further individual had an unexplained and consistent elevation of his serum aspartate aminotransferase (AST) activity to 45 U/L (5-40 U/L). Twenty-one women were taking the oral contraceptive pill, otherwise none was on regular medication.

Table 4.1: Demographic details of the study population

Study subgroup	Number	Sex ratio ♂:♀	Median (range) age (years)
Healthy volunteers	94	39:55	32 (18-78)
Hospital inpatients	88	42:46	67 (23-92)
Non-alcoholic liver disease (total)	197	82:115	52 (15-84)
Non-cirrhotic	*101*	*44:57*	*50 (15-84)*
Cirrhotic	*96*	*38:58*	*54 (21-82)*
Alcohol misusers (total)	211	135:76	51 (21-77)
Non-cirrhotic	*88*	*61:27*	*47 (21-77)*
Cirrhosis	*123*	*74:49*	*53 (33-77)*

2. **Hospital inpatients,** n=88 (42 men and 46 women) were recruited from the general medical wards. None had a history or clinical evidence of alcohol misuse or chronic liver disease and none consumed alcohol in excess of 20g/day. The majority were receiving long-term medication for a variety of underlying conditions including ischaemic heart disease, diabetes mellitus and peptic ulceration, or else they were prescribed medication for their current complaint. Liver function tests results were abnormal in thirty two individuals (36.4%), but in each instance the abnormalities could be explained either by the underlying clinical condition or the prescribed medication. Twelve patients (13.6%) had elevated serum AST activity; seventeen (19.3%) had elevated serum gamma glutamyl transpeptidase (GGT) activity and one an elevated erythrocyte mean corpuscular volume (MCV).

3. **Non-alcoholic liver disease,** n=197 (82 men and 115 women) were recruited from both the in-patients and out-patients services of the University Department of Medicine at the Royal

Free Hospital. The diagnosis of non-alcoholic liver disease was based on retrospective analysis, clinical, laboratory, radiological and histological findings. Patients were further categorised as having 'non-cirrhotic' or 'cirrhotic' liver disease. There were 101 non-cirrhotics, of whom 44 were men and 57 women. There were 96 cirrhotics, of whom 38 were men and 58 women. None gave a history of alcohol misuse or consumed alcohol in excess of 20g/day. The majority were receiving medication, mainly vitamin supplements, diuretics, H_2 receptor antagonists and non-absorbable disaccharides.

4. **Alcohol misusers**, n=211 (135 men and 76 women) were recruited from both the in-patients and out-patient services of the University Department of Medicine at the Royal Free Hospital. The diagnosis of alcohol misuse was based on a history of alcohol consumption in excess of 60g/day for at least one year prior to the study. The degree of liver injury was categorised following histological examination of needle biopsy material as non-cirrhotic or cirrhotic. 88 individuals had non-cirrhotic (61 men and 27 women) and 123 with cirrhosis (74 men and 49 women). At the time of the study, 140 individuals (66.4%) had been abstinent from alcohol for a median (range) of 10.5 (0.3 - 420) months. In each instance the last alcoholic beverage had been consumed ≥168 hours previously; this group was referred to as 'abstinent' (Table 4.2).

Table 4.2: Details of the patients with a history of chronic alcohol misusers who were abstinent at the time of the study

Population subgroup (n)	Sex ratio ♂:♀	†Age (years)	†Drinking history (years)	†Alcohol intake (g/day)	†Abstinence (months)
Non-cirrhotic (48)	34:14	49 (27-70)	19 (2-45)	130 (60-624)	4 (0.3-96)
Cirrhotic (92)	56:36	54 (33-77)	18 (5-60)	127 (60-380)	16 (0.3-420)
Total abstinent alcohol misusers (140)	**90:50**	**53 (27-77)**	**18 (2-60)**	**129 (60-624)**	**11 (0.3-420)**

†Data expressed as median (range)

The remaining 71 (33.6%) individuals had been misusing alcohol for a median of 18 (4 - 50) years consuming a median of 120 (60 - 552) g of alcohol daily to within 7 days of the study, that is ≤ 168 hours previously; this group was referred to as 'drinking' (Table 4.3).

Table 4.3: Details of the patients with a history of alcohol misuse who were actively drinking at the time of the study

Population subgroup (n)	Sex ratio ♂:♀	†Age (years)	†Drinking history (years)	†Alcohol intake (g/day)	†Last drink (hours)
Non-cirrhotic (40)	27:13	48 (21-71)	18 (4-49)	130 (64-552)	18 (1-168)
Cirrhotic (31)	18:13	50 (35-73)	18 (5-50)	120 (60-320)	21 (2-168)
Total drinking alcohol misusers (71)	**45:26**	**49 (21-73)**	**18 (4-50)**	**120 (60-552)**	**18 (1-168)**

†Data expressed as median (range)

Sera used in this study had been collected between three and five years previously and stored frozen at -20^{0}C. It is known that they had thawed out at least once during this time. They were all retrieved, re-catalogued and re-coded as a complete collection following reassessment of the clinical data. The coded sera were transported, frozen on dry-ice at -70^{0}C, to the relevant laboratories for CDT levels determined using the commercial kits CDTect RIA and AXIS %CDT TIA, total transferrin levels measured using an immunoturbidimetric assay, as described previously. All sera were tested blind and in duplicate. The results were analysed and collated with the clinical data.

CDTect was expressed as absolute values (U/L) and also in relation to total transferrin (%).

4.3.2 STATISTICAL ANALYSIS

Statistical advice was received pre-hoc. The *p* value taken to indicate a level of significance was 0.05 for all the statistical analyses performed.

Each population was tested for normal distribution using the Shapiro-Wilk W test and not found to be normally distributed. Non-parametric tests were used throughout. Box-Whisker plots were used to graphically show the central location and scatter/dispersion of the results obtained for the sub-groups within the population. Vertical box-plots for each for each sub-

group are shown side by side. Differences between the sub-groups were assessed using the Mann-Whitney U analysis, a non-parametric test to assess differences between groups of data.

The relationship between serum CDT values and total serum transferrin levels was assessed statistically using the Spearman Rank correlation. Differences between the sub-groups were examined using the Kruskall-Wallis 1-way ANOVA, a non-parametric test to detect differences between the groups. If this was positive then the Chi-Square test was used to assess the differences in the sub-groups. Assay performance was determined by calculation of sensitivities (the percentage true positive rate) and specificities (the percentage of true negative rate) in the entire study population and in the various population subgroups using the method of Galen and Gambino. Receiver Operating Characteristic (ROC) analysis was performed to determine the relationships between the true positive rate (sensitivity) and the false positive rate (1-specificity). The area under the ROC curve was used as a measure of overall test accuracy. The Shapiro-Wilk analysis, Box-Whisker plots, Spearman Rank correlations, Chi-square, Galen/Gambino and ROC analyses were performed using *Astute: Statistics Add-in for Microsoft™ Excel.*

4.3.3 RESULTS

4.3.3.1 CDT (CDTect and AXIS %CDT) and total transferrin levels

Table 4.4 shows the results for the CDTect, CDTect/total transferrin and AXIS %CDT values for each sub-group.

Table 4.4: CDTect, AXIS %CDT and total transferrin values for each of the subgroups

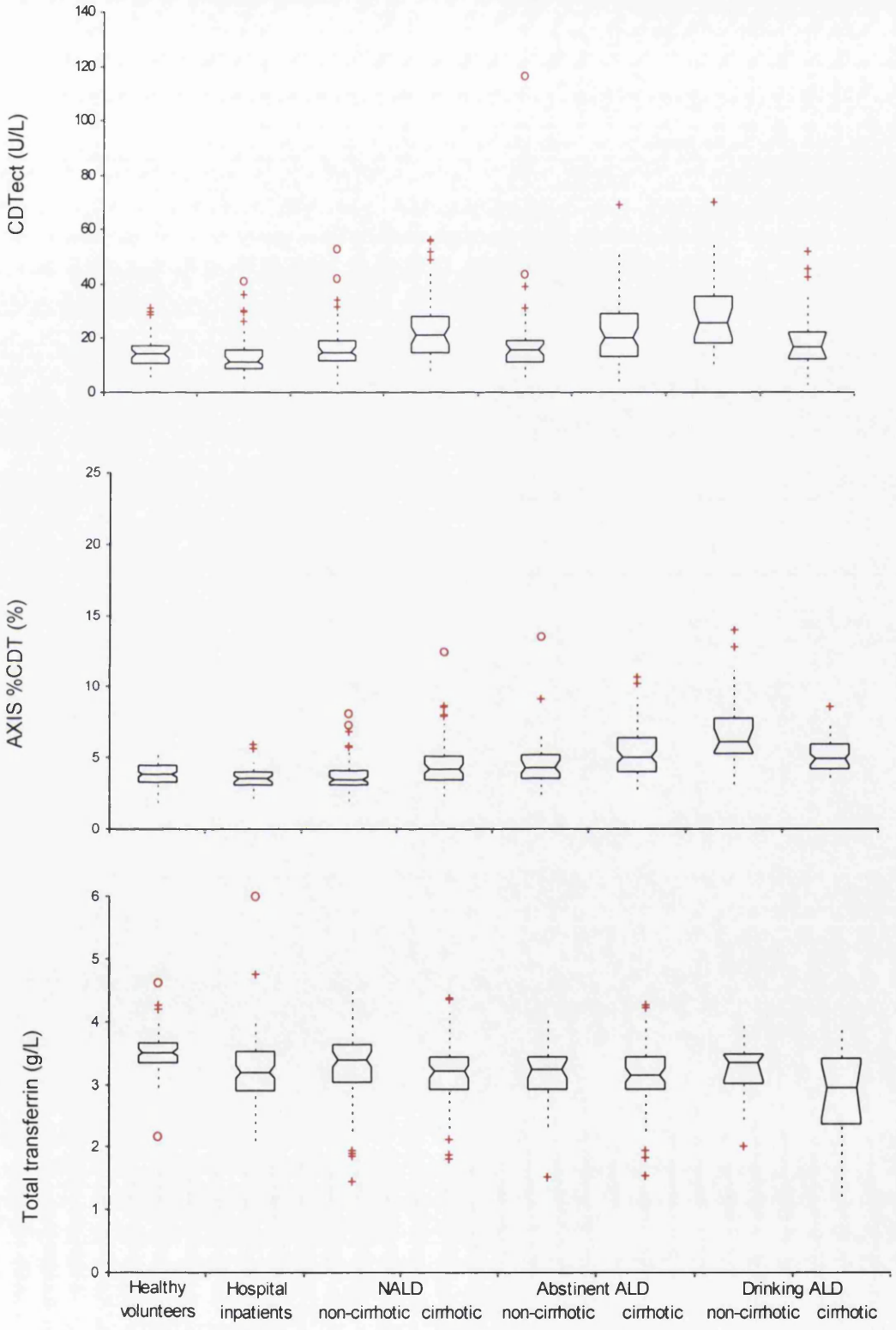
Study subgroup	CDTect (U/L)			AXIS %CDT (%)			Total transferrin (g/L)		
	Total	♂	♀	Total	♂	♀	Total	♂	♀
Healthy volunteers (n=94)	14.1 (5.4-31.1)	11.4 (5.4-25.2)	16.1 (6.4-31.1)	3.8 (1.8-5.60	3.8 (1.8-5.6)	3.8 (2.5-5.5)	3.5 (2.2-4.6)	3.5 (2.2-4.0)	3.5 (2.9-4.6)
Hospital inpatients (n=88)	11.3 (5.1-40.9)	9.9 (5.1-24.7)	12.5 (5.4-40.9)	3.5 (2.1-6.0)	3.3 (2.1-4.6)	3.6 (2.6-6.0)	3.2 (2.1-6.0)	3.1 (2.1-3.9)	3.2 (2.3-6.0)
Non-alcoholic liver disease (n=197)	17.7 (3.2-56.3)	17.1 (3.2-52.7)	18.2 (5.7-56.3)	3.7 (1.9-12.4)	3.8 (2.1-8.7)	3.7 (1.9-12.4)	3.3 (1.5-4.5)	3.2 (1.9-4.3)	3.3 (1.5-4.5)
Non-cirrhotic (n=101)	14.9 (3.2-52.7)	14.5 (3.2-52.7)	15.3 (5.7-52.6)	3.4 (2.1-8.1)	3.4 (2.1-81)	3.5 (1.9-6.9)	3.4 (1.5-4.5)	3.3 (1.9-4.2)	3.5 (1.5-4.5)
Cirrhotic (n=96)	21.2 (8.1-56.3)	19.0 (8.1-44.6)	22.1 (9.4-56.3)	4.2 (2.3-12.4)	4.5 (2.3-8.7)	4.0 (2.8-12.4)	3.2 (1.8-4.4)	3.1 (1.9-4.3)	3.2 (1.8-4.4)
Abstinent alcohol misusers (n=140)	18.1 (3.7-116.4)	17.2 (5.5-116.4)	18.5 (3.7-50.7)	4.7 (2.4-13.5)	4.7 (2.8-13.5)	4.9 (2.4-9.2)	3.2 (1.5-4.3)	3.2 (1.5-4.3)	3.2 (1.6-4.1)
Non-cirrhotic (n=48)	15.8 (5.5-116.4)	15.3 (5.5-116.4)	17.0 (8.6-38.7)	4.3 (2.8-13.5)	4.2 (2.8-13.5)	4.6 (2.4-9.2)	3.2 (1.5-4.1)	3.2 (1.5-4.1)	3.3 (2.4-3.8)
Cirrhotic (n=92)	20.1 (3.7-68.9)	19.7 (6.8-68.9)	20.7 (3.7-50.7)	5.0 (2.7-9.2)	5.1 (3.6-8.6)	5.0 (2.7-9.2)	3.1 (1.5-4.3)	3.2 (1.5-4.3)	3.2 (1.6-4.1)
Drinking alcohol misusers (n=71)	20.7 (2.6-70.0)	20.7 (7.9-55.7)	19.5 (2.6-70.0)	5.5 (3.1-14.0)	5.8 (3.1-14.0)	5.1 (3.1-11.1)	3.2 (1.3-3.9)	3.2 (2.0-3.9)	3.0 (1.2-3.9)
Non-cirrhotic (n=40)	25.7 (10.5-70.0)	27.2 (10.5-55.7)	25.6 (11.2-70.0)	6.1 (3.1-14.0)	6.6 (3.1-14.0)	5.9 (3.1-11.1)	3.3 (2.0-3.9)	3.3 (2.4-3.9)	3.4 (2.0-3.7)
Cirrhotic (n=31)	17.0 (2.6-51.7)	18.6 (7.9-51.7)	15.7 (2.6-45.5)	4.9 (3.6-8.6)	5.1 (3.6-8.6)	4.4 (3.6-7.5)	2.9 (1.2-3.9)	3.1 (2.0-3.9)	2.6 (1.2-3.9)

Total = ♂+ ♀, data expressed as median (range)

The results from the two methods for measuring CDT and also for the total transferrin levels in the sera were collected into the respective sub-groups and expressed graphically using Box-Whisker plots. In the Box-Whisker plots (Figure 4.7) the notched box shows the median, lower and upper quartiles, and confidence interval around the median. The dotted-line connects the nearest observations within 1.5 IQRs (inter-quartile ranges) of the lower and upper quartiles. Red crosses (+) and circles (o) indicate possible outliers - observations more than 1.5 IQRs (near outliers) and 3.0 IQRs (far outliers) from the quartiles. These show the values for CDTect, AXIS %CDT and total transferrin for each sub-group, with the results from the men and women pooled together.

The population subgroups were analysed initially using the Kruskall-Wallis test and then population sub-groups were compared using the Mann-Whitney U.

Figure 4.7: CDT and transferrin values for the population sub-groups



CDTect

The values for each group are shown in Table 4.4 and with the men and women together, are shown in Figure 4.7. The healthy volunteers and hospital patients had the lowest CDTect values, while the non-cirrhotic drinking alcohol misusers had, as expected, the highest.

While the non-cirrhotic non-alcoholic liver disease had a median (range) values of 14.9 (3.2-52.7) U/L and non-cirrhotic abstinent alcohol misusers 15.8 (5.5-116.4) U/L, these values were not different from the healthy volunteers 14.1 (5.4-31.1) U/L. However the cirrhotic populations were significantly ($p<0.05$) higher: 21.2 (8.1-56.3), 20.1 (3.7-68.9) and 17.0 (2.6-51.7) for the non-alcoholic liver disease, abstinent alcohol misusers and alcohol misusers respectively.

In the drinking alcohol misusers the non-cirrhotics had significantly higher ($p=0.002$) CDTect values; 25.7 (10.5-70.0) U/L, median (range), than the cirrhotics; 17.0 (2.6-51.7) U/L, median (range). As a group these are significantly higher than the non-alcoholic liver disease non-cirrhotics; 14.9 (3.2-52.7) U/L, median (range) and the abstinent non-cirrhotic alcohol misusers 15.8 (5.5-116.4) U/L, median (range) ($p<0.0001$). The drinking cirrhotic alcohol misusers had values which were not dissimilar from the abstinent alcohol misusers; 17.0 (2.6-51.7) and 18.1 (3.7-116.4) U/L respectively.

AXIS %CDT

Using AXIS %CDT there was a significant difference ($p<0.05$) between the subgroups, excepting between the hospital inpatients and the non-cirrhotic non-alcoholic liver disease, and within the non-alcoholic liver disease groups. As with CDTect, the cirrhotic drinking alcohol misusers were no different to the cirrhotic abstinent alcohol misusers. Again, as with CDTect, the non-cirrhotic drinking alcohol misusers had the highest levels (median 6.1, range 3.1-14.0%)

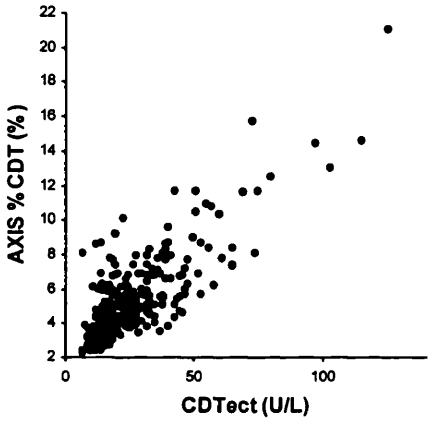
Total transferrin

The healthy volunteers had the highest levels of total transferrin at 3.5 (2.2-4.6) g/L, median (range). This was significantly higher than all the other subgroups ($p<0.05$). The lowest values were found in the actively drinking cirrhotic alcohol misusers; 2.9 (1.2-3.9) g/L, which was significantly lower than the other subgroups excepting the abstinent alcohol misusers; 3.2 (1.5-4.3) g/L median (range).

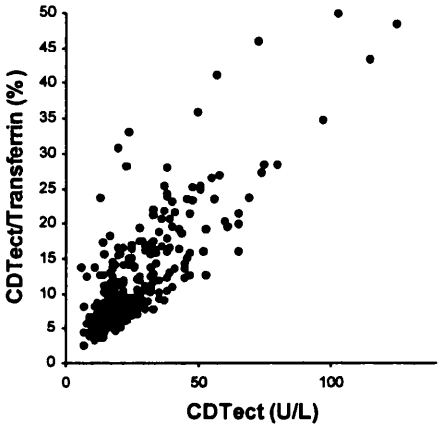
4.3.3.2 Assay correlations

The assay methods were compared using Spearman Rank correlation (Figure 4.8). Significant correlations were observed between both absolute and relative serum CDT values obtained using the two assay methods. The correlation coefficient is r squared (rs) where 1.0 denotes a perfect correlation. The highest correlation was between CDTect and CDTect/transferrin, 0.94.

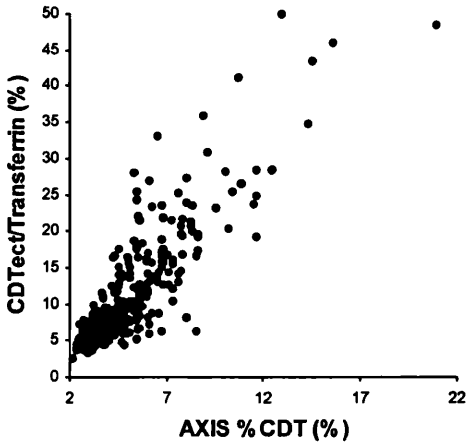
Figure 4.8: Spearman rank correlations of relationships between assay methods



rs=0.66
p<0.0001



rs = 0.94
p<0.0001

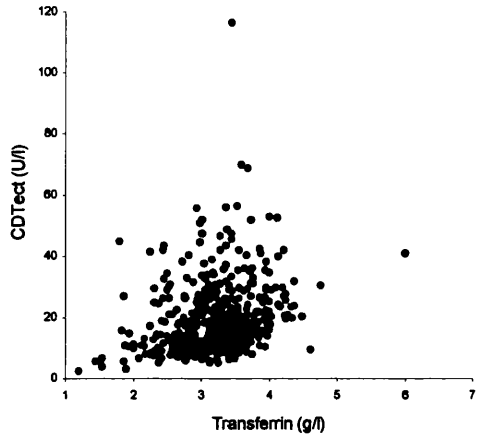


rs = 0.75
p<0.0001

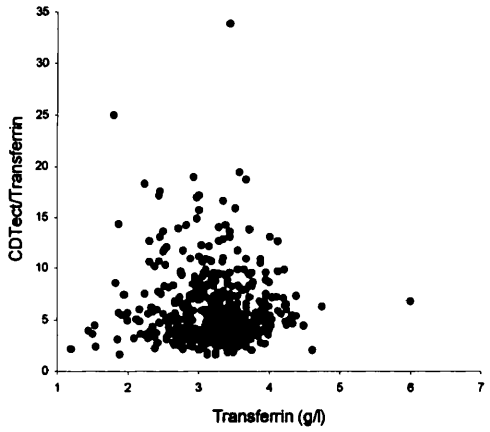
The correlation between the assay methods and total transferrin was also examined using Spearman Rank correlation (Figure 4.9). While values for both CDTect and AXIS %CDT correlated with total transferrin concentrations (p<0.0001) values for CDTect/Transferrin did not. The correlation coefficients were low, indicating a poor degree of correlation, and in the

case of both methods expressed relative to total transferrin, CDTect and AXIS %CDT, the correlation was inverse.

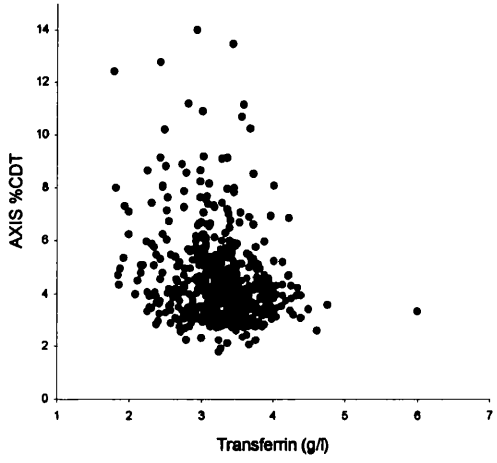
Figure 4.9: Relationship between expression of CDT and total transferrin concentrations



rs=0.27
p<0.0001



rs= -0.01
p=0.80



rs= -0.18
p<0.0001

4.3.4 ASSAY SENSITIVITY AND SPECIFICITY

The manufacturers' cut-offs were derived from Scandanavian populations. In order to make the cut-offs relevant to the more ethnically diverse UK population, the mean + two standard deviations of the values found in the healthy volunteers group was used to calculate the cut-offs. This method has been used in other published work (Bell et al. 1993; Yamauchi et al. 1993; Sillanaukee et al. 1994; Wickramasinghe et al. 1994; Werle et al. 1997). The data was analysed using a cut-off calculated from the mean + two standard deviations of the healthy volunteers (Table 4.5). The cut-offs were calculated for the men and women separately.

The differences in sensitivity and specificity between the sub-groups for each assay, between assays and gender differences were assessed using the Chi-square test.

Table 4.5: Assay cut-offs using the mean + 2 SD of the healthy volunteers

Assay	Men	Women
CDTect (U/l)	21.0	26.3
CDTect/Tf (%)	6.2	7.2
AXIS %CDT (%)	5.7	5.4

4.3.4.1 Sensitivity

The sensitivity, or true positive rate, was calculated for the actively drinking alcohol misusers and then for the non-cirrhotics and cirrhotic sub-groups (Table 4.6).

Table 4.6: Sensitivities for the alcohol misuse population

Population (n)	CDTect (%)			CDTect/Transferrin (%)			AXIS %CDT (%)		
	Total	♂	♀	Total	♂	♀	Total	♂	♀
Non cirrhotic (40)	*50.0*	*59.3*	*30.8*	*61.5*	*63.0*	*58.3*	*62.5*	*59.3*	*69.2*
Cirrhotic (31)	*29.0*	*33.3*	*23.1*	*32.3*	*38.9*	*23.1*	*30.0*	*38.9*	*16.7*
Drinking alcohol misusers (71)	**40.9**	**48.9**	**26.9**	**48.6**	**53.3**	**40.0**	**48.6**	**51.1**	**44.0**

Total = ♂ + ♀

Amongst the drinking alcohol misusers the sensitivities were similar (p=0.451): 40.9% using CDTect and 48.6% using CDTect/Transferrin or AXIS %CDT.

The male subjects appeared to have a higher sensitivity than the female subjects, both overall and within either of the histological sub-groups. However, regardless of the assay used, these differences were not significant.

The performance of the non-cirrhotic sub-group of the population appeared to be higher than the cirrhotic. However these differences were not significant using CDTect, either for the men, women or the total population. There was a significant difference (p=0.028) using CDTect/transferrin: non-cirrhotics 61.5% and cirrhotics 32.3%, but this disappeared when gender was taken into account. Using AXIS %CDT the non-cirrhotics had a higher sensitivity (62.5%) than the cirrhotics (30.0%), p=0.014, repeated in the female (p=0.025) but not the male (p=0.03) population.

4.3.4.2 Specificity

The specificity was calculated for:

(i) the total population overall from all those who were not actively misusing alcohol

(ii) for each of the individual sub-groups.

The values were calculated for the men and women separately and collectively (Table 4.7).

Table 4.7: Summary of specificities for the study populations, using the mean + 2 SD of the healthy volunteers to define cut-off

Population (n)	CDTect (%)			CDTect/Transferrin (%)			AXIS %CDT (%)		
	Total	♂	♀	Total	♂	♀	Total	♂	♀
Healthy volunteers (94)	95.7	97.4	94.5	96.8	97.4	96.4	98.9	100	98.1
Hospital inpatients (88)	92.1	92.9	91.3	95.4	95.1	95.7	97.7	100	95.7
Non-alcoholic liver disease (197)	76.1	72.0	79.1	68.7	64.2	71.9	90.3	91.4	89.5
Non cirrhotic (101)	*89.1*	*86.4*	*91.2*	*83.8*	*76.7*	*89.3*	*95.0*	*93.0*	*96.4*
Cirrhotic (96)	*62.1*	*55.3*	*67.2*	*53.1*	*50.0*	*55.2*	*85.4*	*89.7*	*82.8*
Alcohol misusers (140)	67.1	62.2	76.0	61.2	59.6	64.0	68.8	70.8	65.3
Non cirrhotic (48)	*83.3*	*79.4*	*92.9*	*76.6*	*75.8*	*78.6*	*83.0*	*81.8*	*85.7*
Cirrhotic (92)	*58.7*	*51.8*	*69.4*	*53.3*	*50.0*	*58.3*	*61.1*	*63.6*	*57.1*
Total abstinent population (519)	**80.0**	**75.9**	**83.8**	**76.3**	**72.8**	**79.6**	**87.3**	**86.5**	**87.9**

Total = ♂ + ♀

Total population

In the total population there was no significant difference in the specificities that had been derived from either of the two CDTect methods or from AXIS %CDT: CDTect 80.0%, CDTect/total transferrin 76.3%, AXIS %CDT 87.3%.

Although the women appeared to have a higher specificity than the men, the difference was only significant for CDTect (p=0.03) for the population overall, and there were no significant differences within the population subgroups.

Healthy volunteers and non-alcoholic patient sub-groups

The specificity amongst the healthy volunteers was >95%, by definition, as they were used to define the cut-offs. This was not significantly higher than the results for the hospital controls for any of the assays, but the hospital controls were significantly higher than the non-alcoholic liver disease groups using all assay methods (p<0.05). Although the non-alcoholic liver disease

appeared higher than the alcoholic liver disease groups, this was only significant for AXIS %CDT ($p<0.0001$).

Alcohol misusers

Within the non-alcoholic liver disease and the alcoholic liver disease groups the non-cirrhotics had a higher specificity than the cirrhotics, regardless of the assay method ($p<0.05$).

The performance of the different assays was comparable within each of the histological sub-groups other than within the non-alcoholic liver disease group. AXIS had a significantly higher specificity, 90.3%, than both CDTect, 76.1%, ($p=0.0003$) and CDTect/Transferrin, 68.7%, ($p<0.0001$). Within the non-cirrhotic sub-group the AXIS %CDT assay was significantly higher, 95.0 %, than CDTect/transferrin, 83.8 %, ($p=0.02$), but there was no difference from CDTect, 89.1 %. However the AXIS %CDT assay was higher than the CDTect ($p=0.0006$) and CDTect/transferrin ($p<0.0001$) within the cirrhotic group; 85.4, 62.1 and 53.1 % respectively.

4.3.5 SUMMARY

Overall both assays performed poorly, particularly in terms of sensitivity which overall was less than 50%, but also in terms of specificity. These values were considerably less than other published series (Caldwell et al. 1995; Hultberg et al. 1995; Spies et al. 1995; Bean et al. 1997; Martensson et al. 1997).

Poor performance of CDT levels in determining alcohol misuse may be accounted for, in part, by using a cut-off of only seven days to define abstinence. However, when the cut-off period used to define active drinking was changed from 7 days to 14 days a total of 14 individuals were reclassified as actively drinking rather than as abstinent. However this made little, if any, difference to overall assay performance (Table 4.8).

Table 4.8: Serum CDT assay performance in individuals with a history of alcohol misuse in relation to the period used to define abstinence

Assay Procedure	7-day cut-off		14-day cut-off	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
CDTect	40.8	67.1	39.1	66.9
CDTect/Tf	48.6	61.2	48.8	62.6
AXIS %CDT	48.6	68.6	49.4	71.3

4.3.6 RECALCULATION OF SENSITIVITY AND SPECIFICITY USING THE MANUFACTURERS' CUT-OFFS

While some series have used the mean + two standard deviations of the healthy volunteers to define the cut-offs (Bean et al. 1997; Caldwell et al. 1995) other, particularly in more recent series, have used the manufacturers' cut-offs (Hultberg et al. 1995; Spies et al. 1995). It was therefore decided to recalculate the sensitivity and specificity using the manufacturers' cut-offs to ascertain the effect that this would have on performance.

The upper limit of the reference ranges for CDTect and AXIS %CDT were taken from the manufactures' kit instructions. CDTect was also expressed relative to total transferrin using the manufacturers' cut-offs for total transferrin, and the cut-off was calculated separately for men and women (Table 4.9). Values above these cut-off points were considered 'positive'.

Table 4.9: Upper limits of the assay methods, using manufacturers' cut-offs

Assay	Men	Women
CDTect (U/l)	20.0	26.0
CDTect/Tf (%)	5.0	6.5
AXIS %CDT (%)	6.0	6.0

4.3.6.1 Sensitivity using manufacturers' cut-offs

The sensitivity for the alcohol misusing population, overall, was the same for CDTect and AXIS %CDT at 47.9% (Table 4.10). Expressing CDTect as a ratio to total transferrin significantly increased the sensitivity (p=0.03) to 66.2%. In the non-cirrhotic population, the

sensitivity was 56.1% for CDTect, increasing by 23.9% (p=0.03) to 80.0% if it was expressed as a ratio to total transferrin. Overall, the non-cirrhotic population had a higher sensitivity than the cirrhotic population for CDTect/transferrin and AXIS %CDT (p<0.05), but not for CDTect. Although the males of each population appeared to have a higher sensitivity than the females, other than amongst the non-cirrhotic population using the AXIS %CDT test, the differences were non-significant.

Table 4.10: Sensitivities for the alcohol misuse population

Population (n)	CDTect (%)			CDTect/Transferrin (%)			AXIS %CDT (%)		
	Total	♂	♀	**Total**	♂	♀	**Total**	♂	♀
Non cirrhotic (40)	*56.1*	*66.7*	*38.5*	*80.0*	*85.2*	*69.2*	*62.5*	*59.3*	*69.2*
Cirrhotic (31)	*36.7*	*44.4*	*23.1*	*48.4*	*61.1*	*30.8*	*29.0*	*38.9*	*15.4*
Alcohol misuser (71)	**47.9**	**57.8**	**30.8**	**66.2**	**75.6**	**50.0**	**47.9**	**51.1**	**42.3**

Total = ♂ + ♀

Summary

In this population, using either cut-off, the sensitivity was poor. For both CDTect and AXIS %CDT in the total alcohol misusing group, the sensitivity was less than 50%. The total transferrin was lowest in the actively drinking alcoholic cirrhotics (Table 4.4 and Figure 4.7). When CDTect was expressed as a proportion of the total transferrin there was no significant in crease in the sensitivity when the mean + two standard deviations of the healthy volunteers was used to define the cut-off, but there was when the manufacturers' cut-offs were used. However when AXIS %CDT was used there was no significant difference from CDTect, with a sensitivity less than 50%. For both cut-offs the non-cirrhotics had a higher sensitivity than the cirrhotics using CDTect/transferrin and AXIS %CDT, methods where the CDT was expressed as a proportion of the total transferrin, and so the low total transferrin in the cirrhotic group is unlikely to be the reason for the low CDT values and poor sensitivity.

Regardless of the cut-off used, there was no gender difference.

4.3.6.2 Specificity using manufacturers' cut-offs

Within the total abstinent population, the AXIS %CDT assay gave a higher specificity, 90.3% than the CDTect, 78.4%, which in turn was higher than the CDTect/transferrin, 67.2% ($p<0.0001$).

Table 4.11: Specificities of the study population using the manufacturers' cut-off

Population (n)	CDTect (%)			CDTect/Transferrin (%)			AXIS %CDT (%)		
	Total	♂	♀	**Total**	♂	♀	**Total**	♂	♀
Healthy volunteers (94)	94.7	94.9	94.5	88.3	82.1	92.7	100	100	100
Hospital inpatients (88)	90.9	92.9	89.1	90.8	90.2	91.3	100	100	100
Non-alcoholic liver disease (197)	73.6	68.3	77.4	59.5	49.4	66.7	92.3	91.5	93.0
Non cirrhotic (101)	*86.1*	*81.8*	*89.5*	*75.8*	*62.8*	*85.7*	*96.0*	*93.2*	*98.2*
Cirrhotic (96)	*60.4*	*52.6*	*65.5*	*42.7*	*34.2*	*48.3*	*88.5*	*89.5*	*87.9*
Alcohol misusers (140)	66.4	62.2	74.0	48.9	43.8	58.0	74.5	72.7	77.6
Non cirrhotic (48)	*81.3*	*79.4*	*85.7*	*61.7*	*54.5*	*78.6*	*89.4*	*87.9*	*92.9*
Cirrhotic (92)	*58.7*	*51.8*	*69.4*	*42.4*	*37.5*	*50.0*	*66.7*	*63.6*	*71.4*
Total abstinent population (519)	**78.4**	**74.3**	**82.3**	**67.2**	**59.2**	**55.7**	**90.3**	**87.0**	**76.0**

Total = ♂ + ♀

Throughout the abstinent population the specificity was higher using AXIS %CDT than CDTect/transferrin, regardless of the sub-group ($p<0.05$). Amongst the non-alcoholic population AXIS %CDT specificity was higher than CDTect ($p<0.05$) and CDTect/transferrin ($p<0.0001$).

As with the previous calculations of specificity there appeared to be a gender difference, but this was only significant using CDTect/transferrin where the women had a higher specificity

than the men (p=0.016) in the non cirrhotic sub-group of the non-alcoholic liver disease population.

The specificity of the healthy volunteers was not significantly higher than that of the hospital inpatients, regardless of the assay used. However the hospital inpatients had a significantly higher specificity than those with non-alcoholic liver disease using CDTect (p=0.016) and CDTect/transferrin (p<0.0001). Although the abstinent alcohol misusers appeared to have a lower specificity than those with non-alcoholic liver disease this was only significant using AXIS %CDT (p<0.0001).

In both the non-alcoholic liver disease and the abstinent alcohol misusers groups the non-cirrhotic sub-groups had a higher specificity than the cirrhotic groups, regardless of the assay used (p<0.05), other than the non-alcoholic liver disease group using AXIS %CDT.

Summary

Using either of the cut-off methods there were similar results, with the specificity higher than the sensitivity. When the mean + two standard deviations of the healthy volunteers was used there was no difference overall between the assay methods, when the manufacturers' cut-offs were used the AXIS %CDT gave a specificity than the CDTect which was higher than CDTect/transferrin.

Using either cut-off the healthy volunteers had a specificity >90%, regardless of the assay method, and there was decreasing specificity with increasing liver injury. The non-cirrhotic part of the non-alcoholic liver disease group and the alcohol misusers both had a higher specificity than the cirrhotic part. As with the sensitivity, although the total transferrin was lower in the cirrhotics, the assay methods expressing CDT as a proportion of the total transferrin (CDTect/transferrin and AXIS %CDT) had a lower specificity in the cirrhotic part of the group.

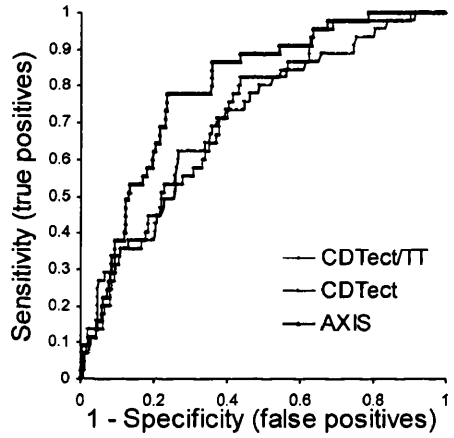
4.3.6.3 ROC curves

ROC (Receiver operator characteristics) curves were used to evaluate the performance of the markers. They can be used to identify the optimum test cut-off values and to allow the efficacy of different tests to be compared. They are generated by plotting the true positive rate

(sensitivity) against the false positive rate (1-specificity) at various cut-offs for the test. The area under the curve of an ideal test is 1.0 (a line at a right angle), and of a worthless test is 0.5 (a line at 45^0). Thus the area under the curve is a measure of the discriminating ability of a test. The optimum cut-off values are the best combined value for the highest sensitivity when traded against specificity. The cut-off value is generally taken as the top left point on the curve.

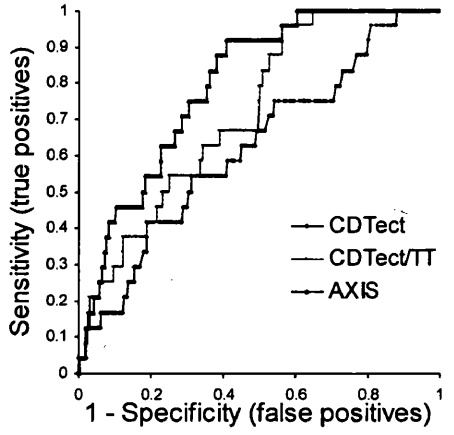
Figure 4.10: Receiver Operating Characteristic (ROC) curves for CDTect, CDTect/transferrin and AXIS %CDT for men and women

Men



	Area	*p*
CDTect	0.721	<0.0001
CDTect/transferrin	0.709	<0.0001
AXIS %CDT	0.796	<0.0001

Women



	Area	*p*
CDTect	0.622	0.02
CDTect/transferrin	0.719	<0.0001
AXIS %CDT	0.795	<0.0001

ROC curves (Figure 4.10) of the assays confirm, by visual inspection, the overall better performance of AXIS %CDT assay for both the men and the women. This test results in the largest area under the curve for each. Each ROC curve was significantly different from a straight line, indicating a performance that was better than a random test.

The performance of each ROC curve was compared to assess the assay differences and AXIS %CDT was significantly better than CDTect and CDTect/transferrin for both sexes, but there was no difference between CDTect and CDTect/transferrin.

From the ROC curves the optimal cut-offs for CDTect, CDTect/transferrin and AXIS %CDT were calculated for men and women separately (Table 4.12).

Table 4.12: Cut-offs calculated from the ROC curves, with corresponding sensitivity and specificity

	Cut-off	Sensitivity (%)	Specificity (%)
CDTect (men)	16.9 U/L	73	61
CDTect (women)	12.6 U/L	77	28
CDTect/transferrin (men)	4.2 %	82	45
CDTect/transferrin (women)	4.3 %	96	36
AXIS %CDT (men)	4.5 %	78	64
AXIS %CDT (women)	3.7 %	92	44

The cut-offs determined from the ROC curves were all lower than those derived from the mean + two standard deviations of the healthy volunteer population. The cut-offs from the manufacturers' and the mean + two standard deviations of the healthy volunteers all had a lower value for the male population than the female, however here the cut-offs for CDTect and AXIS %CDT were both higher for the men. These cut-offs resulted in higher sensitivity but lower specificity than those derived from the mean + two standard deviations of the healthy volunteers, which would be expected from a lower cut-off value.

4.3.7 Discussion

The absolute values (Table 4.4 and Figure 4.7) showed lower values for the healthy volunteers, hospital inpatients and non-cirrhotic non-alcoholic liver disease groups using both CDTect and AXIS %CDT assays. The cirrhotics, whether from the non-alcoholic or the abstinent alcohol misusers had higher results and therefore false positive rates. This was reflected in the poorer specificity in these groups. For both CDTect and AXIS %CDT the non-cirrhotic drinkers had higher CDT results, but the cirrhotics were lower resulting in higher false negative rates and a lower sensitivity.

The total transferrin results span a greater range and have a lower median in cirrhotics, as expected. This is probably a reflection of the poorer synthetic function of the cirrhotic liver.

The AXIS %CDT assay and CDTect/transferrin should account for these variations in total transferrin by expressing the abnormal part of transferrin as a ratio, while the ROC curve (Figure 4.10) for CDTect/transferrin has a higher area than CDTect, the AXIS %CDT is marginally higher still. The ROC curves also show that, regardless of the cut-off, the overall performance is poor. They enable a cut-off to be chosen that will give the desired sensitivity or specificity, or to chose the best compromise. When this was done the cut-offs were all lower than those determined from the mean + two standard deviations of the healthy volunteers, and the results showed higher sensitivity but lower specificity. For the female populations the specificity was particularly poor, in particular for CDTect at 28%. The ROC curves did not show plots with an obvious top left hand corner and there could be considerable variation in the most desirable cut-off point.

If a cut-off is raised this increases the number of individuals testing negative and decreases the number testing positive. Thus the specificity increases and sensitivity decreases. Using the manufacturers' cut-offs rather than the mean + two standard deviations this is lower for CDTect, either relatively or absolutely, but higher for AXIS %CDT. It is not surprising therefore, that, in general, using the manufacturers' cut-offs there is a small decrease in specificity for CDTect, but increase for AXIS %CDT, and a small increase in sensitivity for CDTect and decrease for AXIS %CDT.

Regardless of the assay technique, cut-off or histological sub-group, there was little gender difference and so the total populations are considered together.

Using the mean + two standard deviations, by definition the healthy volunteers have a specificity greater than 95%. However this cut-off gives an unacceptably poor sensitivity, regardless of assay technique, of less than 50%. High false positive rates resulting in a low specificity are seen with increasing levels of significant liver injury, for both the non-alcoholic and alcoholic liver disease groups.

These results are poor, particularly in terms of sensitivity, regardless of either cut-off or assay method compared to other studies done (Stibler, 1991). This may for a number of reasons. The

populations previously studied of this size have often been Scandanavian, and our London population was of diverse ethnic origin. However, when we used the mean + two standard deviations of the healthy volunteers to define the cut-offs the results were little different from using the manufacturers' cut-offs. Our population derived from a liver unit providing a tertiary referral service. We had a large alcoholic liver disease group with a large proportion of histologically advanced liver disease and non-alcoholic liver disease, both of which proved to have poorer performance in terms of sensitivity and specificity. However, within the alcoholic liver disease group the results for the non-cirrhotics were still poor.

Within the non-cirrhotic alcohol misusers, whether drinking or abstinent, were a small number of individuals with alcoholic hepatitis. This is a significant liver injury and so may have compromised the sensitivity and specificity of this group. However the results in the non-cirrhotic group were higher than those in the cirrhotic group.

Within the alcoholic liver disease group the definition of drinking and abstinent was taken at a cut-off of seven days. Although there was little change in terms of sensitivity and specificity (Table 4.8) using a fourteen day cut-off alternative results may have been achieved using more defined drinking and abstinent criteria for alcohol misusers. The drinking habits of a London population may well be different and more diffuse than those of Scandanavian populations, but a careful history had been taken on each individual patient and only those with a strong history of misusers were included.

In addition the sera used had been stored for some time at -20^0C, and this may have affected in some way the composition of the samples. The assay techniques could have, in some way, been inferior to those used in other studies, although all assays were performed at the relevant UK reference laboratories.

4.4 Contemporary serum collection

4.4.1 INTRODUCTION

The results from the archived collection were disappointing, particularly in terms of sensitivity, compared to previously published series (Table 3.2), the reasons for this have been discussed. It was therefore decided to repeat the study using a complete new collection of sera, in particular with more stringent criteria for 'drinking' and 'abstinent' so that the two groups were clearly defined.

As in the previous study, the aim of this second study was to assess the sensitivity and specificity of both commercial assays used to detect CDT in the setting of a cosmopolitan UK population.

4.4.2 PATIENTS AND METHODS

A total of 363 subjects were studied, 204 men and 159 women (Table 4.13). The individual was assessed fully with regard to the medical history and a detailed alcohol history was elucidated. Investigations conducted as appropriate to establish the individuals' 'disease' status.

1. **Healthy volunteers**. For the reference population 49 healthy volunteers were recruited from hospital staff and from those who answered advertisements placed in the local press. They had no past history, clinical signs or laboratory markers suggestive of alcohol misuse or liver disease, no current illnesses nor were taking any medication other than the oral contraceptive pill. Two individuals with Gilberts syndrome were included. None drank alcohol in excess of 20g of alcohol per day.

2. **Hospital patients**. 50 general hospital patients were recruited from the general medical wards of The Royal Free Hospital. None had a past history, clinical signs or laboratory markers suggestive of either alcohol misuse or chronic liver disease. None drank alcohol in excess of 20g per day. Any abnormalities of standard liver function tests were explicable by the underlying disease process. All prescribed medication was documented.

Table 4.13: Demographic details of the 'contemporary' study population

Study subgroup	Number	Ratio ♂:♀	Median (range) age (years)
Healthy volunteers	49	17:32	38.1 (19-79)
Hospital inpatients	50	25:25	69.1 (25-90)
Non-alcoholic liver disease	74	38:36	53.0 (18-78)
Non-cirrhotic	*35*	*18:17*	*52.6 (18-74)*
Cirrhotic	*39*	*20:19*	*53.9 (21-78)*
Abstinent alcohol misusers	95	59:36	53.0 (28-78)
Non-cirrhotic	*22*	*15:7*	*52.4 (34-78)*
Cirrhotic	*73*	*44:29*	*53.1 (28-72)*
Drinking alcohol misusers	95	65:30	46.8 (19-72)
Non-cirrhotic	*69*	*49:20*	*46.8 (19-67)*
Cirrhotic	*26*	*16:10*	*48.4 (35-72)*

3. **Non-alcoholic liver disease.** 74 were recruited from in-patient and out-patient departments at The Royal Free Hospital. The diagnosis of their liver disease was confirmed by clinical, laboratory, radiological and histological variables and the patients were subdivided into non-cirrhotic and cirrhotic categories. None had a history of past or present alcohol misuse and their current alcohol intake did not exceed 20g/day. All medication were documented.

4. **Alcohol Misusers.** 190 were recruited from in-patients and out-patient populations of the Royal free Hospital. These individuals had a history of alcohol misuse of at least 60g per day for a minimum of one year. Individuals were subdivided, based on liver histology obtained by needle biopsy, into non-cirrhotic and cirrhotic liver disease. There were 95 'drinkers' who had actively misused alcohol to within five days of the study (Table 4.14).

Table 4.14: Details of the patients with a history of alcohol misuse who were actively drinking at the time of the study

Population subgroup (n)	†Alcohol intake (g/day)	†Last drink (hours)
Non-cirrhotic (69)	164 (64-680)	21.0 (4-52)
Cirrhotic (26)	120 (64-360)	25.5 (0-120)
Total drinking alcohol misusers (95)	**160 (64-680)**	**23.0 (0-120)**

†Data expressed as median (range)

Of the 95 abstinents, there were 59 men and 36 women who had all been abstinent for a minimum of six weeks (Table 4.15). Abstinence was assessed by direct questioning, corroborated from close relatives where possible and by using breathalyser and serum ethanol measurements.

Table 4.15: Details of the patients with a history of chronic alcohol misuse who were abstinent at the time of the study

Population subgroup (n)	†Abstinence (months)
Non-cirrhotic (22)	2.8 (1.4-316)
Cirrhotic (73)	11.6 (1.6-210)
Total abstinent alcohol misusers (95)	**7.3 (1.4-316)**

†Data expressed as median (range)

4.4.2.1 Assessment

45 mls of venous blood was taken from each individual. The blood was collected into the following tubes:

- one 2.7 ml tube, containing 1.6 mg EDTA, for full blood count analysis;

- one 3ml tube, containing 0.3 mls citrate solution, for a clotting screen;
- three 4.7 ml plain tubes, containing beads/gel as clot activator, for serum; one for electrolyte and liver function tests and two for serum storage;
- one 9 ml tube containing beads/ clot activator for a full iron profile

All samples were dispatched immediately to the haematology and biochemistry laboratories within the Royal Free Hospital and analyses were performed within the routine laboratory analyses. The following laboratory test results were obtained:

- Full blood count (FBC) in haematology for:
  - haemoglobin (Hb) (normal range 11.5-15.5 g/dl)
  - Mean corpuscular volume (MCV) (normal range 85-95 fl)
- Clotting study in haematology for prothrombin time (normal range 12-16 seconds)
- Liver function tests in Chemical Pathology for:
  - Albumin (normal range 35-50 g/l)
  - Bilirubin (normal range 5-17 μmol/l)
  - AST (normal range 5-40 U/l)
  - ALT (normal range 5-40 U/l)
  - Alkaline phosphatase (normal range 35-130 U/l)
  - γGT (normal range 10-48 U/l)
- Iron studies in haematology for:
  - Iron (normal range 11-36 μmol/l)
  - TIBC (normal range 53-85 μmol/l)
  - Saturation % (normal range 20-40 %)
  - Ferritin (normal range 39-300 μg/l)

The prothrombin time and liver function tests were used as part of the assessment of disease status.

Blood was allowed to clot at room temperature for a minimum of one hour in the two remaining plain 4.7 ml serum monovette tubes. The samples were then centrifuged at 3,500 rpm for 15 minutes. The serum was pipetted into four plain tubes as four approximately one ml aliquots and coded appropriately These were then frozen at -20^{0}C until they were required for analysis.

Each was thawed at room temperature when required for analysis. The following analyses were performed:

1. CDTect EIA
2. AXIS %CDT TIA
3. Total transferrin

The results were then collated and analysed in terms of sensitivity and specificity using the Galen and Gambino analysis.

4.4.3 STATISTICAL ANALYSIS

Normality was tested using the Shapiro-Wilk analysis. The groups were not normally distributed and so non-parametric statistical analyses were used. Box-Whisker plots were used to analyse the absolute value results for CDTect, AXIS %CDT and total transferrin for each of the sub-groups. The Mann-Whitney U test was used to determine differences in values between the values for the sub-groups. Spearman Rank correlations were used to determine the relationships between the assays and also the relationship to total transferrin. The inter-group differences were compared using the non-parametric methods of Kruskall Wallis and Chi-Square tests. The results were analysed in terms of sensitivity and specificity using the Galen and Gambino analysis and intergroup differences were compared using the Chi-square tests. Receiver Operating Characteristic (ROC) analysis was performed to determine the relationship between sensitivity and specificity at various cut-offs. All statistical analyses were performed using *Astute: Statistics Add-in for Microsoft™ Excel.*

4.4.4 RESULTS

4.4.4.1 Assay values

Table 4.16: Assay values for CDTect, AXIS %CDT and total transferrin

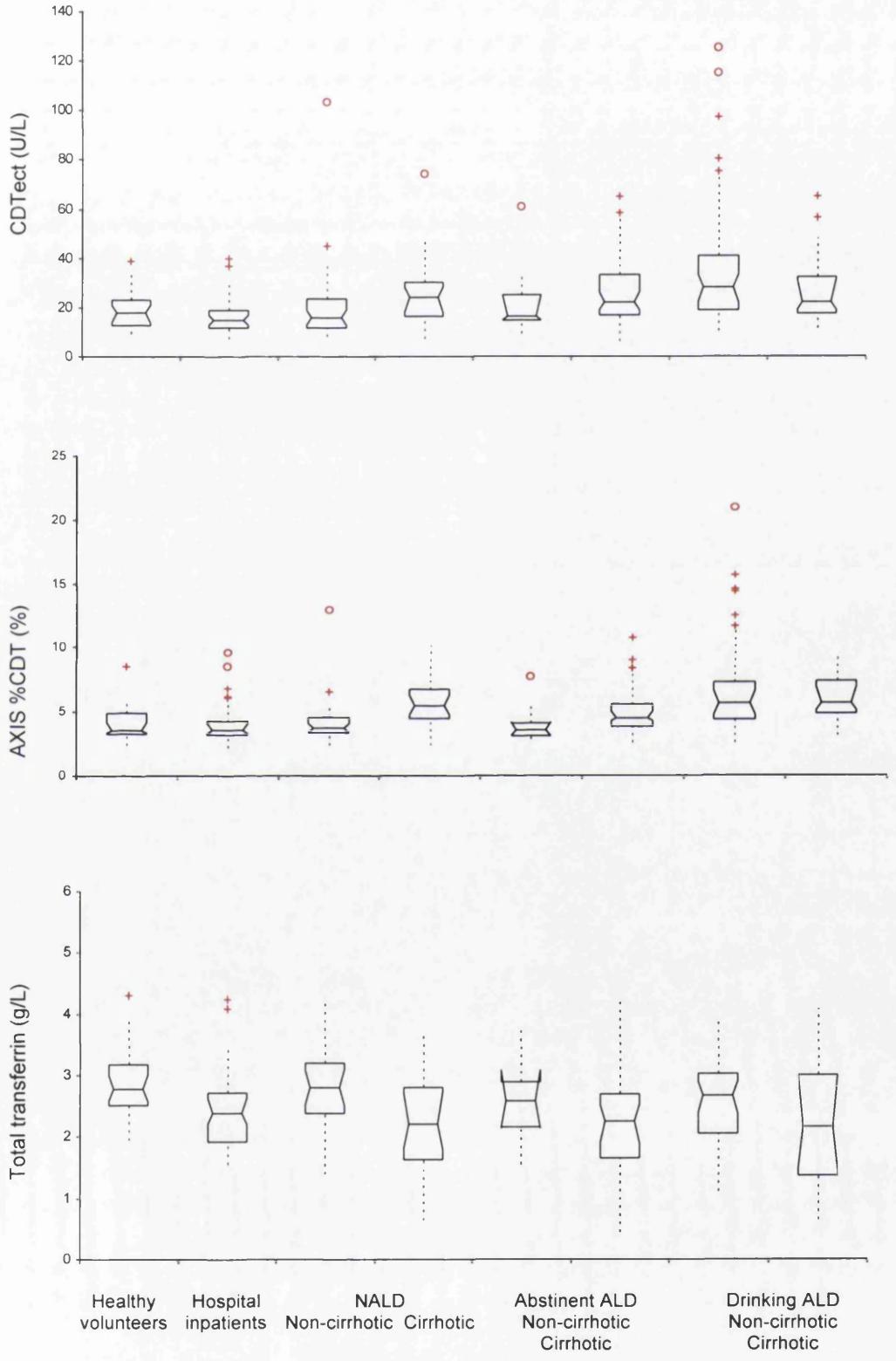
Study subgroup	CDTect (U/L)			AXIS %CDT (%)			Total transferrin (g/L)		
	Total	♂	♀	Total	♂	♀	Total	♂	♀
Healthy volunteers (n=49)	18 (9-39)	12 (9-21)	21 (13-39)	3.6 (2.4-8.6)	3.4 (2.4-5.6)	4.2 (2.7-8.6)	2.8 (1.9-4.3)	2.6 (1.9-3.0)	3.0 (2.2-4.3)
Hospital inpatients (n=50)	15 (7-40)	14 (7-40)	16 (10-29)	3.6 (2.2-9.6)	3.3 (2.3-4.9)	3.9 (2.2-9.6)	2.4 (1.5-4.2)	2.4 (1.6-4.1)	2.4 (1.5-4.2)
Non-alcoholic liver disease (n=74)	19 (7-103)	14 (8-28)	27 (7-103)	4.6 (2.4-13.0)	3.9 2.4-5.6)	5.1 (2.4-13.0)	2.5 (0.7-4.2)	2.5 (0.8-2.4)	2.8 (0.7-4.2)
Non-cirrhotic (n=35)	16 (8-103)	12.5 (8-17)	27 (12-103)	3.7 (2.4-13.0)	3.4 (2.4-4.8)	4.2 (2.4-13.0)	2.8 (1.4-4.2)	2.5 (1.4-3.4)	3.2 (2.1-4.2)
Cirrhotic (n=39)	24 (7-74)	22.5 (9-46)	26 (7-74)	5.5 (2.4-10.1)	5.3 (2.4-8.3)	5.5 (3.6-8.1)	2.2 (0.7-3.7)	2.3 (0.8-3.4)	2.2 (0.7-3.7)
Abstinent alcohol misusers (n=95)	20 (6-65)	19 (6-65)	25 (9-58)	4.4 (2.6-10.8)	4.4 (2.7-10.8)	4.4 (2.6-3.1)	2.3 (0.4-4.2)	2.4 (0.4-4.2)	2.3 (0.6-3.7)
Non-cirrhotic (n=22)	16 (9-61)	16 (13-61)	25 (9-33)	3.6 (2.8-7.8)	3.3 (2.8-7.8)	3.8 (3.1-5.6)	2.6 (1.5-3.7)	2.5 (1.6-3.7)	2.6 (1.5-3.4)
Cirrhotic (n=73)	22 (6-65)	21 (6-65)	25 (9-58)	4.5 (2.6-10.8)	4.4 (2.7-10.8)	4.6 (2.6-8.1)	2.3 (0.4-4.2)	2.3 (0.4-4.2)	2.2 (0.6-3.7)
Drinking alcohol misusers (n=95)	26 (10-125)	26 (11-125)	26 (10-65)	5.7 (2.6-21.0)	5.9 (2.6-21.0)	5.3 (2.8-8.3)	2.7 (0.7-4.1)	2.8 (0.7-3.9)	2.4 (0.9-4.1)
Non-cirrhotic (n=69)	28 (10-125)	32 (11-125)	26 (10-45)	5.7 (2.6-21.0)	5.8 (2.6-21.0)	4.7 (2.8-8.3)	2.7 (1.1-3.9)	2.8 (1.1-3.9)	2.5 (1.5-3.8)
Cirrhotic (n=26)	22 (11-65)	20.5 (11-56)	30 (16-65)	5.7 (3.1-9.2)	6.0 (3.1-9.2)	5.5 (3.9-7.7)	2.2 (0.7-4.1)	2.2 (0.7-3.6)	2.1 (0.9-4.1)

Total = ♂ + ♀, data expressed as median (range)

CDTect

The CDTect values for the sub-groups are shown in Table 4.16 and Figure 4.11. The healthy volunteers had median (range) levels, 18 (9-39) U/L that were significantly lower than these in the cirrhotic non-alcoholic liver disease, 24 (7-74) U/L (p=0.008), cirrhotic abstinent alcoholic liver disease, 22 (6-65) U/L, (p=0.0005) and all the drinking alcoholic liver disease sub-groups (p<0.001).

Figure 4.11: CDT and transferrin values for the population sub-groups



The hospital inpatients had CDT levels that were significantly lower; 15 (7-40) U/L, median (range), than other sub-groups ($p<0.05$) excepting the healthy volunteers. Amongst the non-cirrhotic population, the non-alcoholic liver disease and non-cirrhotic alcoholic liver disease groups were not significantly different. However the cirrhotic part of the non-alcoholic liver disease, alcoholic liver disease (both drinking and abstinent) had no different from each other, but were all significantly higher than the healthy volunteers, hospital patients and non-cirrhotic non-alcoholic liver disease groups ($p<0.01$).

Amongst the drinking alcohol misusers the non-cirrhotic group had CDT levels; 28 (10-125) U/L that were significantly higher ($p<0.05$) than all other groups except the cirrhotic alcohol misusers; 22 (11-65) U/L. The cirrhotics were significantly higher ($p<0.01$) than the healthy volunteers, hospital patients and non-cirrhotic non-alcoholic liver disease, but not the other cirrhotic groups.

AXIS %CDT

Within the abstinent groups there was no difference in the values between the healthy volunteers, hospital inpatients, non-cirrhotic non-alcoholic liver disease and non-cirrhotic abstinent alcohol misusers. However these were all significantly lower than the cirrhotic groups, both alcoholic and non-alcoholic, and the drinking population ($p<0.05$).

The drinking population, both non-cirrhotic; 5.7 (2.6-21.0) and cirrhotic; 5.7 (3.1-9.2), was significantly higher than the other groups ($p<0.05$), excepting the cirrhotic sub-group of, the non-alcoholic liver disease group; 5.5 (2.4-10.1).

Total transferrin

The normal range is 2.0-4.0 g/L. The majority of the healthy volunteers fell within this range; 2.8 (1.9-4.3) g/L and have significantly higher total transferrin ($p<0.05$) than other groups excepting the non-cirrhotic non-alcoholic liver disease group; 2.8 (1.4-4.2) g/L.

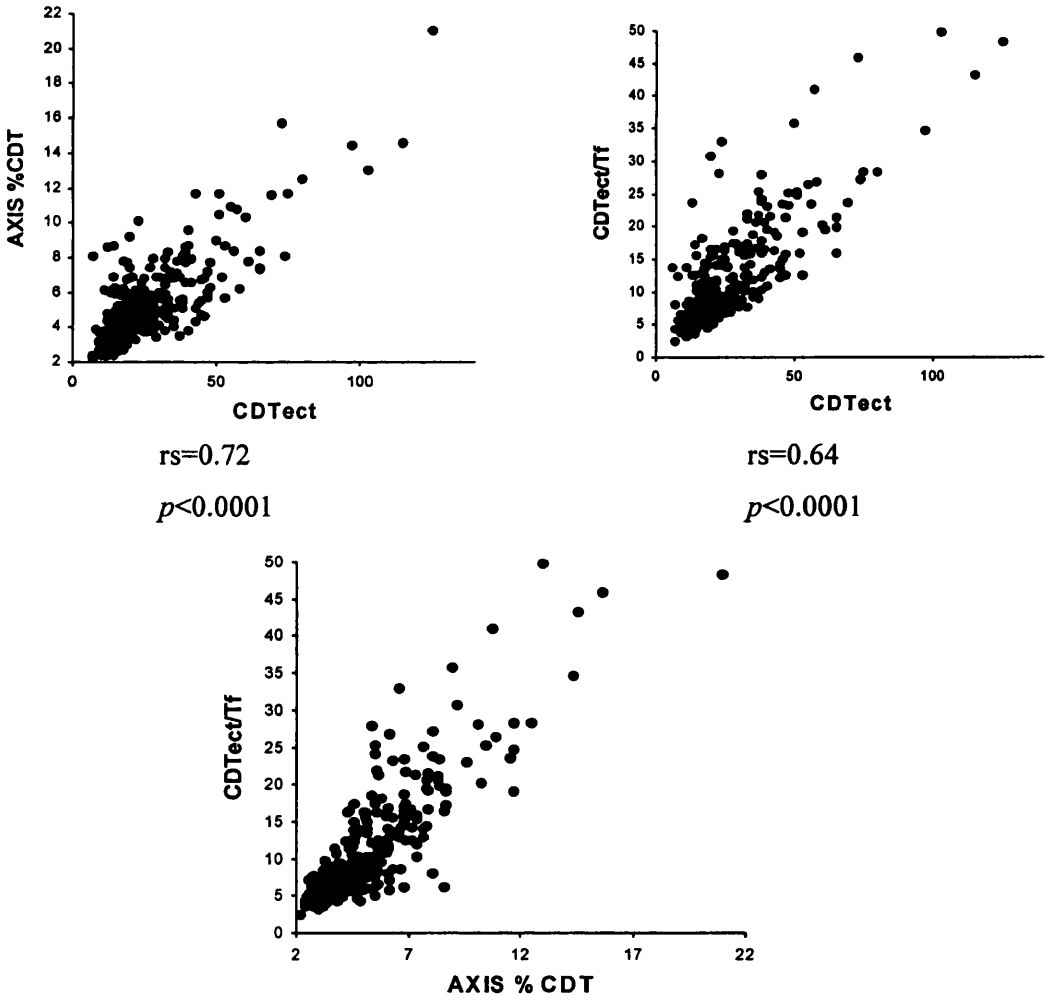
The cirrhotic groups and the hospital inpatients had similar total transferrin values which were significantly lower than the healthy volunteers and the non-cirrhotic non-alcoholic liver disease ($p<0.05$).

Within the alcohol misuse population there was no difference between the non-cirrhotic, whether drinking or abstinent, or cirrhotics sub-groups. The abstinent cirrhotics had transferrin levels, 2.3 (0.4-4.2) g/L that were significantly lower than the abstinent, 2.6 (1.5-3.7) g/L (p=0.049) and drinking, 2.7 (1.1-3.9) g/L non-cirrhotic populations (p=0.003).

4.4.4.2 Assay correlations

The assay correlations were compared using a Spearman Rank correlation (Figure 4.12). Significant correlations (p<0.0001) were observed between both absolute and relative serum CDT values obtained using both assay methods. The strongest correlation (rs value) was seen between AXIS %CDT and CDTect/transferrin (rs=0.83).

Figure 4.12: Relationship between assay methods and total transferrin



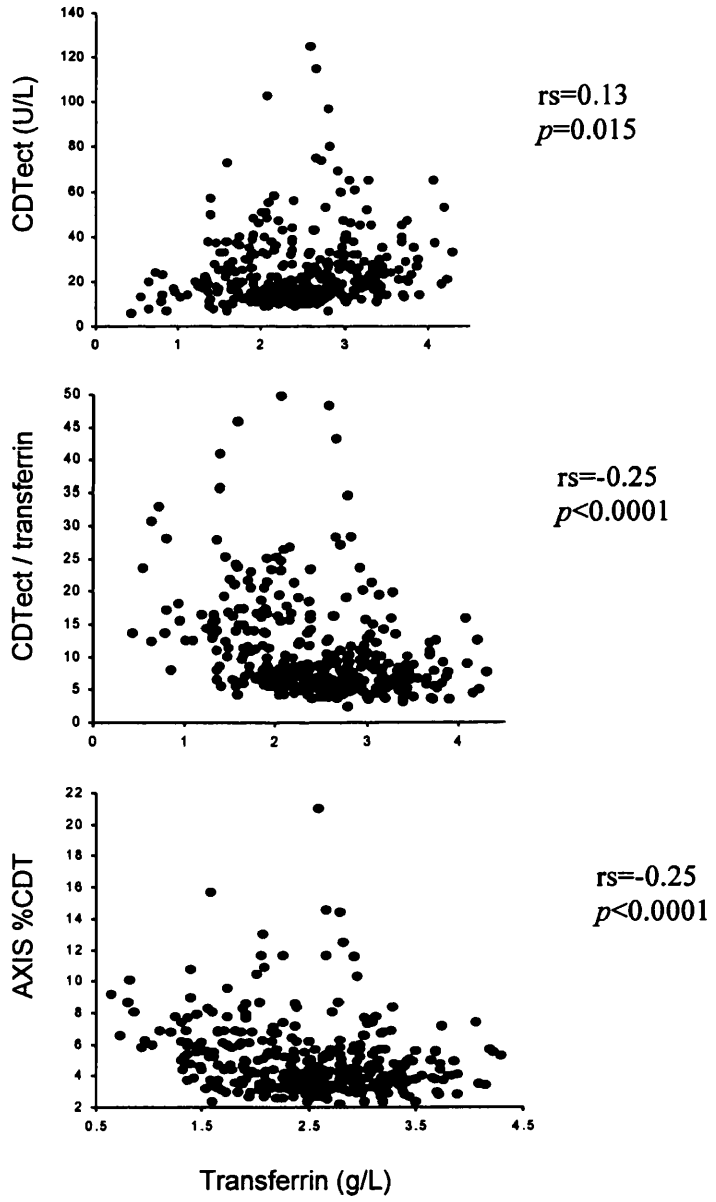
rs=0.72
p<0.0001

rs=0.64
p<0.0001

rs=0.83
p<0.001

The correlation between total transferrin and each assay method was also compared using the Spearman rank method (Figure 4.13). These show that, while there is a significant correlation between total transferrin and both assay methods, the correlation is not remarkable, indicated by the rs values ranging from –0.25 for both CDTect/transferrin and AXIS %CDT and 0.13 for CDTect.

Figure 4.13: Relationships between assay methods and total transferrin



4.4.5 ASSAY SENSITIVITY AND SPECIFICITY

Because better performance was established, in the previous study, using the manufacturers' cut-of values, this system was used here. The cut-offs used were the manufacturers' suggested values (Table 4.17). For the CDTect expressed relatively the cut-off was calculated for men and women separately.

Table 4.17: Manufacturers' cut-offs used in the contemporary collection

Assay	Men	Women
CDTect (U/L)	20.0	26.0
CDTect/Transferrin (%)	5.0	6.5
AXIS %CDT (%)	6.0	6.0

4.4.5.1 Sensitivity

The sensitivity in the total alcohol misusing population was higher using CDTect/transferrin (93.7%) than CDTect (58.9%), $p<0.0001$ which itself was higher than using AXIS %CDT (40.4%), $p=0.016$. In the non-cirrhotic and cirrhotic sub-groups CDTect/transferrin was significantly higher than either CDTect or AXIS %CDT ($p<0.0001$).

The performance in the non-cirrhotic was not significantly different from the cirrhotic population regardless of the method that was used to determine CDT levels. There were no gender differences.

Table 4.18: Sensitivities for the alcohol misusers in the 'contemporary' collection

Population (n)	CDTect (%)			CDTect/Transferrin (%)			AXIS %CDT (%)		
	Total	♂	♀	**Total**	♂	♀	**Total**	♂	♀
Non-cirrhotic (69)	*62.3*	*81.8*	*50.0*	*91.3*	*95.9*	*80.0*	*42.0*	*44.9*	*35.0*
Cirrhotic (26)	*50.0*	*50.0*	*50.0*	*100*	*100*	*100*	*34.6*	*37.5*	*30.0*
Alcohol misuser (95)	**58.9**	**63.1**	**50.0**	**93.7**	**96.9**	**86.7**	**40.4**	**43.8**	**33.3**

Total = ♂ + ♀

4.4.5.2 Specificity

As with the previous analysis the specificity was calculated both overall and for each individual sub-group. The analysis was performed/done for men and women separately and pooled (Table 4.19). The inter-group differences were examined using the Chi-square test.

Table 4.19: Specificities for the abstinent population, by sub-group

Population (n)	CDTect (%)			CDTect/Transferrin (%)			AXIS %CDT (%)		
	Total	♂	♀	**Total**	♂	♀	**Total**	♂	♀
Healthy volunteers (49)	87.8	94.1	84.4	42.9	47.1	40.6	98.0	100	96.9
Hospital inpatients (50)	92.0	88.0	96.0	42.0	28.0	56.0	88.0	96.0	80.0
Non-alcoholic liver disease (74)	59.5	68.4	50.0	27.0	36.8	16.7	74.3	78.9	69.4
Non-cirrhotic (35)	*74.3*	*100*	*47.1*	*40.0*	*50.0*	*29.4*	*94.3*	*100*	*88.2*
Cirrhotic (39)	*46.2*	*40.0*	*52.6*	*15.4*	*25.0*	*5.3*	*56.4*	*60.0*	*52.6*
Alcohol misusers (95)	58.9	57.6	61.1	8.4	3.4	16.7	82.1	78.0	88.9
Non-cirrhotic (22)	*81.8*	*80.0*	*85.7*	*13.6*	*6.7*	*28.6*	*95.5*	*93.3*	*100*
Cirrhotic (73)	*52.1*	*50.0*	*55.2*	*6.8*	*2.3*	*13.8*	*78.1*	*72.7*	*86.2*
Total abstinent population (268)	**70.5**	**70.5**	**70.5**	**26.1**	**22.3**	**30.2**	**84.6**	**84.8**	**84.4**

Total = ♂ + ♀

The specificity in the total abstinent population was significantly higher (p=0.0002) using AXIS %CDT (84.6 %) than CDTect (70.5 %). Expressing CDTect relative to total transferrin significantly reduced the specificity in the total population, 26.1 % (p<0.0001) and in all sub-groups (p<0.01). Using CDTect/transferrin was significantly lower (p<0.0005) in all sub-groups than using AXIS %CDT.

There were no gender differences, either overall in the total population or in any of the sub-groups, using either of the assays, other than using CDTect in the non-cirrhotic part of the non-alcoholic liver disease group (p=0.001).

Using CDTect the healthy volunteers and hospital patients had a significantly higher specificity, 87.8 % and 92.0 % respectively, than the non-alcoholic liver disease group, 59.5 %, (p<0.05) and the alcoholic liver disease group, 58.9 % (p<0.001), and of the cirrhotic part of both of these groups, 46.2 % and 52.1 % (p<0.0001). When CDTect was expressed relative to total transferrin the specificity was not significantly different between the healthy volunteers, the hospital patients, the non-alcoholic liver disease group or the non-cirrhotic part of the non-alcoholic liver disease group. However all these groups had CDT levels that were significantly higher than both the cirrhotic part of the non-alcoholic liver disease and the abstinent alcoholic liver disease groups (p<0.05). Using the AXIS %CDT assay the healthy volunteers had CDT levels, 98.0% that were significantly higher than the non-alcoholic liver disease, 74.3% (p=0.001), the cirrhotic non-alcoholic liver disease, 56.4% (p<0.0001), the alcoholic liver disease,82.1 % (p=0.025) and cirrhotic alcoholic liver disease, 78.1% (p=0.009) groups.

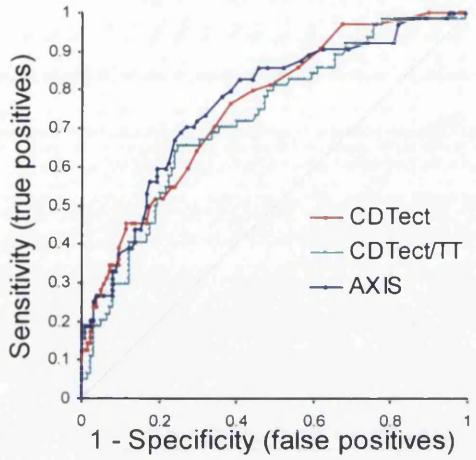
Using all three methods the non-cirrhotic part of the non-alcoholic liver disease population was significantly higher than the cirrhotic part (p<0.05). Within the alcoholic liver disease population, the non-cirrhotic group was higher than the cirrhotic group using CDTect (p=0.025) but not using either of the other two methods.

4.4.5.3 ROC curves

The overall performance of the assays were compared using ROC curves (Figure 4.14). These confirm the poor performance of CDT as a marker of alcohol misuse regardless of the assay technique or gender. The assays with the largest area under the curve and so 'best' performance in terms of sensitivity and specificity were those in the men. With the men the AXIS %CDT had the largest area under the curve but all three assays had very similar ROC curves. In the women the largest area under the curve was when CDTect was expressed as a ratio to total transferrin, then for AXIS %CDT.

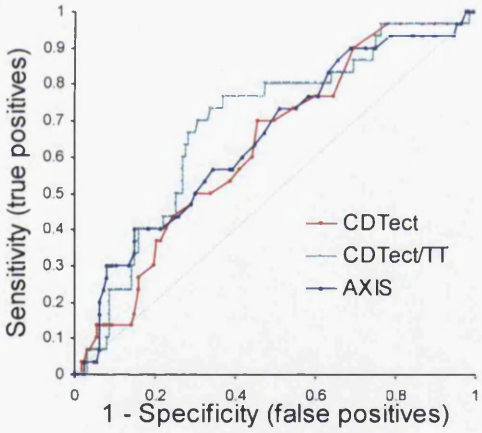
Figure 4.14: ROC curves of the assay methods for the contemporary collection

Men



	Area	*p*
CDTect	0.76	<0.0001
CDTect/transferrin	0.73	<0.0001
AXIS %CDT	0.77	<0.0001

Women



	Area	*p*
CDTect	0.63	0.007
CDTect/transferrin	0.69	0.0002
AXIS %CDT	0.65	0.004

The ROC curves did not show an obvious point for determining the best cut-off points in terms of sensitivity and specificity, however these have been estimated and are shown in Table 4.20. The cut-off values for the men were not higher than those for the women, in keeping with the manufacturers' cut-offs, and unlike those determined from the ROC curves for the retrospective analysis collection. The cut-offs for CDTect and AXIS %CDT were lower than those recommended by the manufacturers', and the resultant sensitivities were higher and specificities lower as a result. The reverse was true for CDTect/transferrin.

Table 4.20: Cut-off values, with sensitivities and specificities, determined from the ROC curves

	Cut-off	Sensitivity (%)	Specificity (%)
CDTect (men)	18 U/L	75.4	61.9
CDTect (women)	21 U/L	70.0	54.3
CDTect/transferrin (men)	6.4 %	81.2	47.1
CDTect/transferrin (women)	8.8 %	76.7	63.0
AXIS %CDT (men)	4.4 %	81.2	50.7
AXIS %CDT (women)	4.4%	73.3	48.8

4.4.6 COMPARISON OF SENSITIVITY AND SPECIFICITY BETWEEN THE RETROSPECTIVE ANALYSIS AND CONTEMPORARY COLLECTIONS

To compare the two populations the manufacturers' cut-offs were used for both. Differences between the retrospective analysis and the contemporary collection cannot be fairly compared as two different assays were used: CDTect RIA and CDTect EIA. There were no gender differences in either of the populations studied and so the men and women are considered together. Figure 4.15 shows the sensitivity and specificity for each sub-group for the two populations.

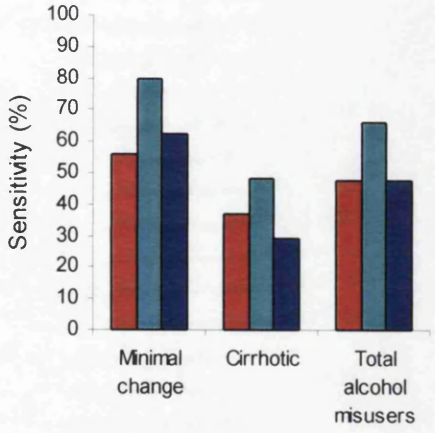
Sensitivity

When the two populations were compared for assay performance, the sensitivity of the CDTect/transferrin was higher in the contemporary collection than the retrospective analysis ($p<0.0001$). This was seen in the cirrhotic part of the groups ($p<0.0001$) but not the non-cirrhotic.

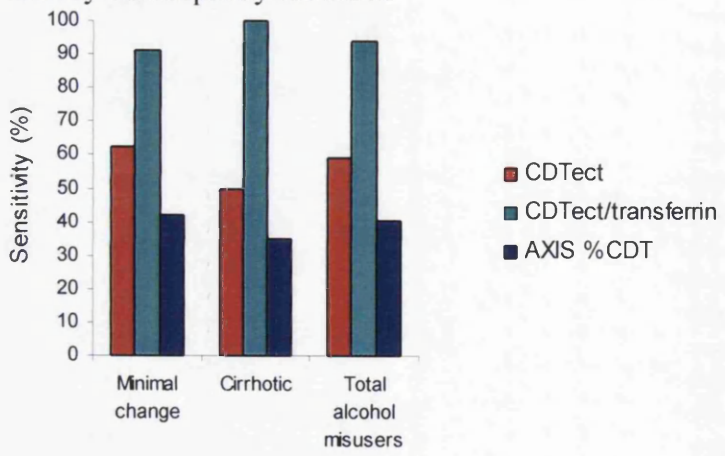
In the retrospective analysis collection the performance of CDTect was similar to AXIS %CDT, whereas in the contemporary collection the CDTect/transferrin was higher than CDTect which was higher than AXIS %CDT.

Figure 4.15: Sensitivities and specificities for the retrospective analysis and contemporary collections

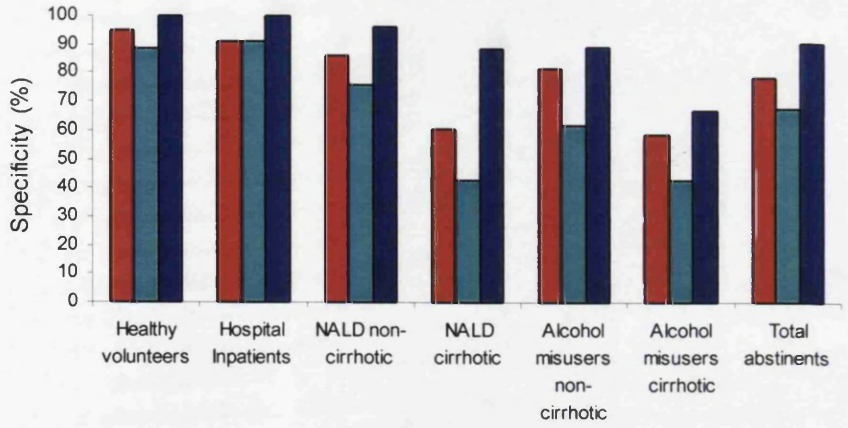
Sensitivity retrospective analysis collection



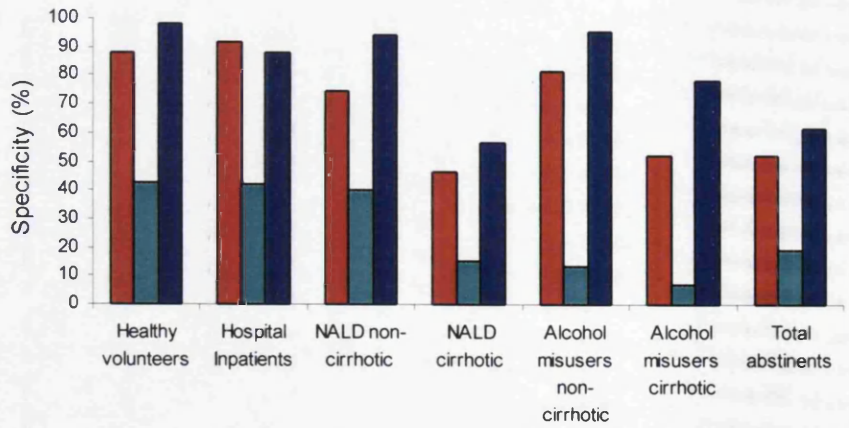
Sensitivity contemporary collection



Specificity retrospective analysis collection



Specificity contemporary collection



In both populations the non-cirrhotics had a higher sensitivity than the cirrhotics when the CDT was expressed as a proportion of the total transferrin (CDTect/transferrin and AXIS %CDT), but not in either case for CDTect. As previously discussed, the total transferrin was lower in both populations in the alcohol cirrhotics, and so if this was the reason for a lower level of the carbohydrate-deficient component than it would be expected that the sensitivity would be lower in the cirrhotics when the CDT was measured absolutely (CDTect) but similar to the non-cirrhotics when expressed as a proportion (CDTect/transferrin or AXIS %CDT). As the reverse was seen this is unlikely to be the explanation.

Specificity

The specificity in the total abstinent population was higher in the retrospective analysis collection than the contemporary, when assay methods were compared ($p<0.05$). In both populations the performance of AXIS %CDT was higher than CDTect which was higher than CDTect/transferrin.

When the assay performances were compared in the sub-groups, using CDTect/transferrin the specificity was higher in the retrospective analysis than the contemporary collection ($p<0.05$). When comparing CDTect, the specificity was higher in the non-alcoholic liver disease group in the retrospective analysis collection ($p=0.035$), other wise the performance was the same in both populations. AXIS %CDT had a higher specificity in the retrospective analysis collection in the hospital inpatients, non-alcoholic liver disease group, and the non-alcoholic cirrhotics sub-groups.

In both populations the specificity was higher in the non-cirrhotics than the cirrhotics.

The most obvious difference was the lower specificity and higher sensitivity for CDTect/transferrin in the contemporary collection than the retrospective analysis. The same cut-off was used in both cases, and both the CDTect and transferrin assays were performed by the same person using the same assays techniques, albeit separated in time. The total transferrin results were compared, using the Mann-Whitney U, analysis between the two populations and the contemporary collection had significantly lower transferrin results in all groups ($p<0.01$). The transferrin was lower, so that the ratio of CDTect to transferrin would have been higher, and therefore the sensitivity higher and specificity lower. When CDTect and

AXIS %CDT were compared between the two groups there were little differences, indicating the transferrin was the only major variant.

In summary in both populations the performance was poor in terms of sensitivity, regardless of the assay technique, while the specificity was higher. Cut-offs can be adjusted to in crease the sensitivity at the detriment of the specificity, and vice versa, while the ROC curves (Figures 4.10 and 4.14) show the overall performance of each assay. The performance was similar in both populations of each assay with no obvious single ideal cut-off, particularly for the contemporary collection.

4.4.7 DISCUSSION

The contemporary serum collection shows many similarities with the retrospective analysis collection. In both of these series there was no gender differences so that the groups were considered in total. This is in contrast to other published series for both CDTect and AXIS %CDT where the performance, particularly in terms of sensitivity, was particularly poor for the female part of the group (Anton and Bean, 1994; Gronbaek et al. 1995; Huseby et al. 1997).

Considering the values using the CDTect assay in the retrospective analysis versus the contemporary collection the values for CDTect in the total population have a similar median (range): 16 (3-116) U/L compared to 18.0 (6-103) U/L. In comparisons between the two groups it must be remembered that in the first analysis CDTect RIA was used and CDTect EIA was used in the second. Again the AXIS %CDT assay values were similar: 4.0 (1.8-14.0) % compared to 4.1 (2.2-13.0) %. However the results for the total transferrin were significantly lower in the contemporary collection than the retrospective analysis ($p<0.0001$), and there was a significant difference in each sub-group ($p<0.0001$). The assay kit methods used were the same for the measurement of transferrin in both series and were performed by the same person in the same laboratory, albeit at separate times. It may be that in storage the serum in the retrospective analysis deteriorated in some way and this resulted in a higher total transferrin measurement.

Expression of CDTect relative to total transferrin involves two assay methods and so the impression of two assay methods are involved rather than one. However identical cut-offs were used in the comparison between the two methods and it is the total transferrin that differs between the two series while the CDTect results were similar. The difference in the transferrin

results are likely to be, at least in part, the reason the results for CDTect expressed relatively are so much lower in terms of specificity and higher in terms of sensitivity than in the retrospective analysis collection.

In both series the performance has been better in terms of sensitivity and specificity for the healthy volunteers than for the hospital patients, which have been better than those with liver disease. However while in the retrospective analysis those with non-alcoholic liver disease had a higher specificity than those with alcoholic liver disease, in the contemporary collection the performances of the alcoholic and non-alcoholic liver disease groups was similar for both CDTect and AXIS %CDT.

The non-alcoholic liver disease group in the retrospective analysis series had a higher specificity ($p<0.05$) than in the contemporary series for all assay methods. The reasons for this are unlikely to be due to a difference in the disease status between the two groups as the populations both comprised approximately half cirrhotic and half non-cirrhotic individuals. However it has be shown that individuals with hepatitis C or primary biliary cirrhosis are likely to have an elevated serum CDT, and therefore test false positive, so reducing the specificity (Bean et al. 1995; Stauber et al. 1995; Behrens et al. 1988). While the proportion of those with primary biliary cirrhosis was similar in both series: 17.3% and 17.6%, the proportion of individuals with hepatitis C in the non-alcoholic liver disease group was higher in the contemporary series (40.5%), than in the retrospective analysis series (2.0%). It is possible that the increased proportion of hepatitis C, a reflection of the high incidence of hepatitis C disease in current hepatology practice, may account for the higher specificity in the retrospective analysis (73.6% using CDTect and 92.3% using AXIS %CDT) than in the contemporary series (59.5% using CDTect and 50.0% using AXIS %CDT).

The CDT levels have been noted to be higher in advanced non-alcoholic liver disease in other series (Radosavljevic et al. 1995; Tsutsumi et al. 1994). This results in a higher false positive rate and lower specificity. There is a reduced transferrin turnover in cirrhosis (Potter, 1994) and it is possible that the higher CDT levels may be due to reduced turnover and removal of the abnormal transferrin in liver disease. The levels of total transferrin were significantly lower in the cirrhotic subgroups than in the non-cirrhotic liver disease groups in both series. If the higher CDT levels were due to a reduction in the total transferrin production in liver disease, and thus a relative increase in the abnormal transferrin (CDT) then this should be reflected when CDT is expressed relative to total transferrin as CDTect/transferrin or AXIS %CDT.

However both the data series analysed here show that AXIS %CDT had higher CDT levels in the cirrhotic sub-groups than the non-cirrhotic and when CDTect was expressed relative to total transferrin there was still a higher false positive rate and lower specificity in the cirrhotic sub-groups (Figure 4.15). These results are in contrast to other published work which show that there is no reduction in specificity in liver disease (Bell et al. 1993; Stibler and Hultcrantz, 1987), so that CDT was recommended as a marker to differentiate alcoholic from non-alcoholic liver disease (Stibler et al. 1986; Kwoh-Gain et al. 1990; Stibler and Hultcrantz, 1987; Storey et al. 1985).

The sensitivity of the alcohol misusers in both series was poor, in comparison to other published work. In the retrospective analysis the sensitivity was 47.9% for both CDTect and AXIS %CDT, using the manufacturers' cut-offs. In the contemporary series the sensitivity was 58.9% for CDTect and 40.0% for AXIS %CDT. Other series have published sensitivities of greater than 90% for CDTect (Stibler et al. 1986; Stibler et al. 1991; Lof et al. 1993; Stibler, 1993) associated with specificities greater than 90%. However in these series the alcohol misusers had no clinical sign of liver disease and it is unlikely that they had large numbers of cirrhotics or those with advanced liver disease. The sensitivities of the non-cirrhotic group of the retrospective analysis was 56.1% for CDTect and 62.5% for AXIS %CDT, while for the contemporary series this was 62.3% for CDTect and 42.0% for AXIS %CDT.

Other published work has suggested that other groups have obtained results similar to these with sensitivity for CDTect (Yamauchi et al. 1993; Lof et al. 1994; Tsutsumi et al. 1994; Niemela et al. 1995; Sorvajarvi et al. 1996; Helander and Tabakoff, 1997; Werle et al. 1997) and for AXIS %CDT (Yamauchi et al. 1993; Tsutsumi et al. 1994; Sorvajarvi et al. 1996) less than 60%. These studies have all been using non-Scandanavian populations with the exception of two from Finland (Lof et al. 1994; Sorvajarvi et al. 1996). It may be that ethnically diverse populations with varied inherited forms of transferrin do not perform in the same manner as the Scandanavian populations in which the more impressive sensitivity have originated from. However, in an attempt to control for this the healthy volunteers were used to derive the cut-offs in the original analysis of the retrospective analysis series (Table 4.5). Even using these the sensitivity is poor: 40.9% for CDTect and 48.6% for CDTect/transferrin and AXIS %CDT (Table 4.6).

The overall performance in terms of sensitivity and specificity is shown by the ROC curves (Figures 4.10 and 4.14). These show that, regardless of the cut-off the performance of these

assays in our population, over two independent collections, is poor. The cut-offs can be raised to increase the number of individuals testing negative and will therefore increase the specificity but reduce the sensitivity. Alternatively the cut-offs can be reduced to increase the sensitivity at he expense of the specificity. Whatever the cut-off chosen it is impossible to obtain sensitivity and specificity of more than 90%. These two series demonstrate that this cannot be attributed to the performance of one individual assay, the means of expression, prolonged storage of serum, advanced liver disease, or to a more ethnically diverse population. It may however be a combination of these factors and has been seen in other published work.

In summary, the two assays performed comparably and that expressing serum CDT as a ratio to total transferrin has separate effects on sensitivity and specificity but no overall effect on assay performance. There was an unacceptably high false positive rate in individuals with non-alcoholic liver disease and in abstinent individuals with significant liver injury.

5. SERUM CARBOHYDRATE DEFICIENT TRANSFERRIN IN THREE CHRONIC DISEASES

5.1 Introduction

The results from the studies in Chapter 2 indicated that CDT determinations gave unacceptably high false positive rate for those with chronic liver disease. This was seen in individuals with both abstinent alcoholic and non-alcoholic liver disease when compared to healthy volunteers. It is not clear whether the false positive rate could be attributed to the liver disease *per se*, possible since transferrin is synthesised in the liver, or to the chronic inflammatory nature of the disease process.

Aim: to investigate CDT levels in three non-hepatological chronic diseases, to determine the false positive rates in the groups, and to compare them to the rates seen in non-alcoholic liver disease and healthy volunteers.

Purpose: to account for the CDT assay false positive results in chronic liver disease as a result of the chronic inflammatory process.

5.2 Patients and Methods

Patients with chronic rheumatoid disorders, inflammatory bowel disease and chronic renal failure were selected for study. They were all recruited from in and out-patients at the Royal Free Hospital. The control group derived from the healthy volunteers and the non-alcoholic liver disease from the contemporary collection, detailed in Chapter 4. All of the individuals studied had no past history, clinical signs or laboratory markers suggestive of either alcohol misuse or chronic liver disease. Their alcohol intake around the time of the study did not exceed 20g/day. Standard liver function tests were normal. Patients from the three disease groups were further assessed to confirm that the disease was both active and chronic using appropriate laboratory tests, including erythrocyte sedimentation rate (ESR).

The assessments used for the healthy volunteers and non-alcoholic liver disease patients have been previously described (chapter 4.4.3).

The demographic details of the present study group are shown in Table 5.1.

Table 5.1: Demographic details of the study groups

Population sub-group	Number	Sex ratio ♂:♀	Median (range) age Year
Healthy volunteers	49	17:32	38.1 (19-79)
Chronic non-alcoholic liver disease	74	38:36	53.0 (18-78)
Non-cirrhotic	*35*	*18:17*	*52.6 (18-74)*
Cirrhotic	*39*	*20:19*	*53.9 (21-78)*
Chronic rheumatoid disorders	30	8:22	52.8 (18-72)
Inflammatory bowel disease	30	14:16	44.2 (18-82)
Chronic renal failure	50	26:24	62.6 (20-83)

1. **Chronic rheumatoid disorders.** 30 individuals were assessed. The diagnoses were predominantly of rheumatoid arthritis (n=19) and scleroderma (n=8), one individual with Stills' disease, one with ANCA positive arthropathy and one with morphoea. The ESR median (range) was 16 (4-107) mm/hr.

2. **Inflammatory bowel disease.** 30 individuals were assessed. The diagnosis was predominantly of ulcerative colitis (n=15) and Crohn's disease (n=12), the remaining three had indeterminate distal colitis. The ESR median (range) was 34 (4-110) mm/hr.

3. **Chronic renal failure.** 50 patients were assessed. Patients were recruited with a creatinine clearance of less than 15 mls per minute and were not undergoing renal replacement therapy with dialysis. The aetiology of the chronic renal failure varied but was predominantly from hypertension, diabetes mellitus, polycystic disease and glomerulonephritis. The ESR median (range) was 40 (27-98) mm/hr.

4. **Healthy volunteers.** These were the same group of individuals detailed in Chapter 4.4.2.

5. **Non-alcoholic liver disease.** These were the same group of individuals detailed in Chapter 4.4.2.

5.2.1 SERUM ANALYSES

The sera were assayed for CDT using CDTect EIA and AXIS %CDT TIA, described previously. The cut-offs used were as recommended by the manufacturers'. For CDTect this was 20 U/l for men, 26 U/l for women and for AXIS %CDT, 6.0%. Assay results above the cut-off were considered false positive. Total transferrin levels were also measured using the method described in chapter 4.2.4.

5.2.2 STATISTICAL ANALYSIS

The three inflammatory disease groups were found not to be normally distributed, as had been found previously with the healthy volunteers and non-alcoholic alcohol misusers. All statistical tests used were non-parametric.

Non parametric analysis of variance was determined using the Kruskall-Wallis analysis and then inter-group comparisons were analysed by the Mann-Whitney U test. The false positive rates were calculated for each sub-group and then, as the numbers were relatively small, the inter-group differences were examined using Fisher's Exact test, or Chi-Square analysis for comparisons involving the non-alcoholic liver disease group where the numbers were greater than 30. The analyses were performed using *Astute: Statistics Add-in for Microsoft Excel.*

5.3 Results

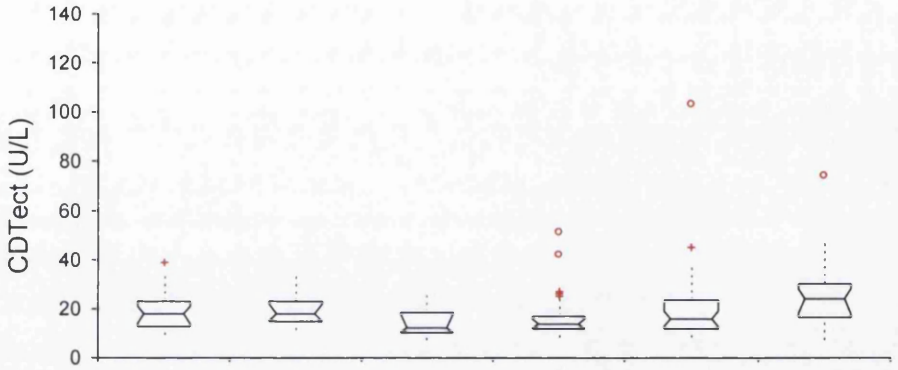
Results of serum CDT assays, both CDTect and AXIS %CDT, are illustrated in Figure 5.1, along with corresponding total transferrin levels. For CDTect the non-alcoholic cirrhotic group had significantly ($p<0.05$) higher CDT levels than all other groups. The inflammatory bowel disease and chronic renal failure groups were both significantly lower than the healthy volunteers and rheumatoid arthritis groups ($p<0.05$).

The AXIS %CDT analysis showed that the non-alcoholic liver disease cirrhotic group was significantly higher than all the other groups ($p<0.0001$). There were no other differences between the groups.

The total transferrin analysis indicated that the inflammatory bowel disease, chronic renal failure and non-alcoholic cirrhotic groups all had significantly lower ($p<0.01$) levels than the healthy volunteers, rheumatoid arthritis and non-cirrhotic non-alcoholic liver disease groups.

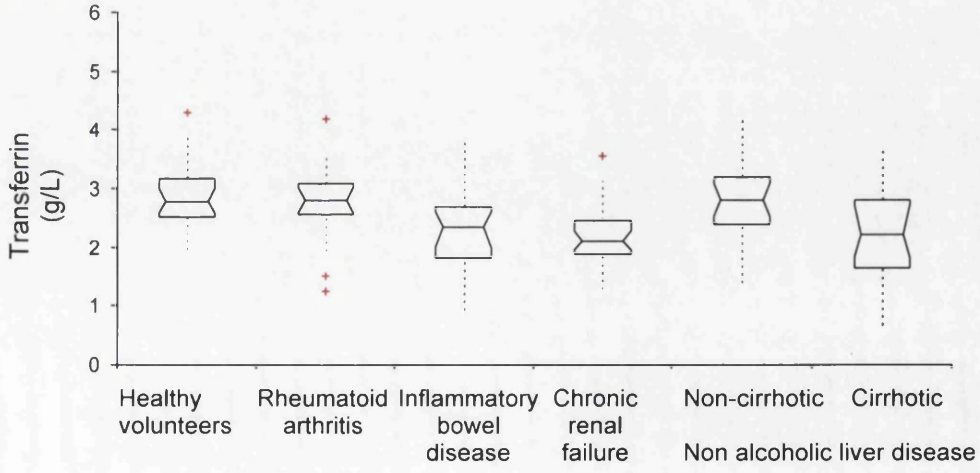
Figure 5.1: Serum analyses for CDT and transferrin in chronic disease

CDTect



AXIS %CDT

Total transferrin



Box-Whisker plots: the notched box shows the median, lower and upper quartiles, and confidence interval around the median. The dotted-line connects the nearest observations within 1.5 IQRs (inter-quartile ranges) of the lower and upper quartiles. Red crosses (+) and circles (o) indicate possible outliers - observations more than 1.5 IQRs (near outliers) and 3.0 IQRs (far outliers) from the quartiles.

False positive rates

The false positive rates for CDTect and AXIS %CDT were calculated for each of the sub-groups (Table 5.2). Differences between each group and assay comparisons were assessed using Fisher's Exact test where the numbers were less than 30, and the Chi-Square test for larger numbers.

Table 5.2: False positive rates for CDTect and AXIS %CDT

Population sub-group	Number	False positive rate	
		CDTect n (%)	AXIS %CDT n (%)
Healthy volunteers	49	6 (12.2 %)	1 (2.0 %)
Chronic non-alcoholic liver disease	74	30 (40.5 %)	19 (25.7 %)
Non-cirrhotic	*35*	*9 (25.7 %)*	*2 (5.7 %)*
Cirrhotic	*39*	*21 (53.8 %)*	*17 (43.6 %)*
Chronic rheumatoid disorders	30	1 (3.3 %)	0 (0 %)
Inflammatory bowel disease	30	3 (10.0 %)	0 (0 %)
Chronic renal failure	50	4 (8.0 %)	2 (4.0 %)

False positive rates for each of the three chronic disease groups were not significantly different from the healthy volunteers, regardless of the method used to determine serum CDT values.

Patients from the non-alcoholic liver disease group had a significantly higher false positive rate than the healthy volunteers or each of the three chronic disease groups, using CDTect ($p<0.01$) and AXIS %CDT ($p<0.005$). The non-cirrhotic component of the non-alcoholic liver disease group had a false positive rate similar to the healthy volunteers and three chronic disease groups using CDTect and AXIS %CDT.

However the patients with cirrhotic non-alcoholic liver disease had a significantly higher false positive rate than all of the other groups using CDTect ($p<0.0005$) or AXIS %CDT ($p<0.0001$).

5.3.1.1 Summary

Reduced levels of transferrin were seen in the inflammatory bowel disease, chronic renal failure and cirrhotic non-alcoholic liver disease groups.

CDT results, whether CDTect or AXIS %CDT, in the three chronic inflammatory groups were not significantly different from the results for the healthy volunteers. CDTect and AXIS %CDT results for the cirrhotic non-alcoholic liver disease group were significantly higher than those for the healthy volunteers.

False positive rates for the healthy volunteers, non-cirrhotic non-alcoholic liver disease and three inflammatory disease groups were not significantly different. The cirrhotic non-alcoholic liver disease group had higher false positive rates than all other groups.

5.4 Discussion

Serum CDT levels were similar in healthy volunteers and in patients with three chronic non-hepatological diseases. There was no difference between CDTect and AXIS %CDT. While total transferrin results may be altered in the chronic disease groups (Figure 5.1) as a consequence of malabsorption or maldigestion, this does not result in an increase in false positive rates. The level of transferrin was significantly lower in the inflammatory bowel disease and chronic renal failure groups. This may account for significantly lower CDTect concentrations in these groups, compared to healthy volunteers, if the abnormal transferrin (CDT) was present in the same amounts as the healthy volunteers, but the total measurable transferrin was reduced. When CDT was expressed as a ratio of the total transferrin (AXIS %CDT) the levels were not significantly different from the healthy volunteers.

Therefore at first sight it would appear that raised false positive levels were not associated with chronic disease *per se.* However, distortions in the underlying isoform profiles may mask underlying trends. Van Eijk *et al* found an increase in higher transferrin isoforms, penta- and hexa-sialotransferrin, in rheumatoid arthritis (van Eijk et al. 1987), but similar a-, mono- and di-sialotransferrin levels, compared to healthy volunteers.

The non-alcoholic liver disease group had a significantly higher false positive rate than the healthy volunteers and from each of the chronic disease groups, and this can be attributed to the

cirrhotic process rather than the non-cirrhotic. This finding has been described previously (Bell et al. 1993; Radosavljevic et al. 1995; Stauber et al. 1995) where the false positive rates were 45.5%, 31% and 22% respectively for the cirrhotic part of the non-alcoholic liver disease group. The present findings indicate that false positive rates in chronic liver disease are a consequence of the liver involvement, as opposed to any chronic inflammatory process. This is not unreasonable as transferrin is synthesised in the liver and in liver disease there may be subsequent pathology relating to transferrin synthesis and metabolism.

6. THE RELATIONSHIP BETWEEN CARBOHYDRATE DEFICIENT TRANSFERRRIN AND IRON STATUS

6.1 Introduction

Carbohydrate deficient transferrin has been shown to be affected by iron status in non-drinkers. It has been shown to be reduced in iron overload (Jensen et al. 1994), and shown to be increased in iron deficiency. Pre-menopausal women have higher serum CDT levels than post-menopausal women, although no direct relationship has been shown in this population (Stauber et al. 1996). Alcohol misusers may have altered iron status. They may be iron deficient, which is usually multifactorial in origin including poor dietary intake, bleeding from varices or chronic gastritis, and direct marrow toxicity, or they may be iron overloaded (Regoeczi et al. 1984).

In uncomplicated iron deficiency anaemia there is a reduction in serum iron, serum ferritin, erythrocyte mean corpuscular volume and haemoglobin. The total iron binding capacity (TIBC) is raised. Of these serum ferritin has been shown to accurately indicate the level of body iron stores, both in deficiency and in overload (Baynes, 1996). A grossly elevated level of serum ferritin has traditionally been used in the diagnosis of hereditary haemochromatosis (>1,000 μg/L). However, ferritin may also be elevated in other circumstances: infection, inflammation and cancer as part of the acute phase reaction. It is also elevated in alcohol misusers, and to a level higher than in non-alcoholic liver disease (Bell et al. 1994). The ferritin concentration may be raised under these circumstances, even if the individual is iron deficient, and so may be an unreliable indicator of iron status.

Recently it has been possible to measure soluble transferrin receptors, a means of assessing iron status. During development all human body cells are capable of expressing transferrin receptors. The highest concentration are present on those cells requiring iron so that about 75% of transferrin receptors are found on erythroid precursors in bone marrow. These increase when there is a decrease in functional iron available, and decrease in iron overload. The liver also expresses transferrin receptors, used for hepatocyte iron uptake (Chapter 3.4). The transferrin receptors are present on the cell surface and have maximal affinity for differic transferrin, moderate affinity for monoferric and almost none for apotransferrin. The differic transferrin is endocytosed by the receptor, the iron dissociated from the transferrin and the

apotransferrin re-cycles via endosomes to the cell surface. The apotransferrin/transferrin receptor complex separates leaving the receptor ready to bind further iron loaded transferrin. Placental transferrin receptors are the other significant site of transferrin receptors and are a major source of purified transferrin receptors used in transferrin receptor assays.

Soluble transferrin receptors are found in human serum. They result from the degenerative cleavage process of the cell transferrin receptors. The soluble transferrin receptors reflect iron status in that they are stable until iron stores are depleted, when they then increase (Skikne et al. 1990), and are down regulated in iron overload (Khumalo et al. 1998). These changes occur before the changes in transferrin saturation or mean red cell volume. The transferrin receptor : ferritin ratio should have an inverse linear relationship to iron status (Skikne et al. 1990).

This assessment should be of benefit in conditions where there is an elevated ferritin for reasons other than iron-deficiency, such as an chronic inflammatory diseases where the ferritin is raised as an acute-phase reactant (Baynes, 1996), but there may well be coexistent iron-deficiency (Mast et al. 1998). Transferrin receptors are also increased in other microcytic anaemias, such as the thalassaemias (Ahluwalia, 1998), but not in the anaemia of chronic disease, however here the ferritin concentration should be normal or high, in contrast to iron-deficiency where it is low (Kuiper-Kramer et al. 1997). They are also raised in haemolytic anaemia and macrocytosis. Currently the gold-standard for iron assessment is a bone-marrow examination whereby the iron stores can be directly assessed. However, this is invasive and has an appreciable morbidity, is painful, time-consuming and expensive, and soluble transferrin receptors have been suggested as an accurate non-invasive alternative (Skikne, 1998).

Carbohydrate deficient transferrin is elevated in alcohol misusers, but alcohol misuse in turn results in altered iron status. While ferritin may be an accurate determinant of iron status, this is not possible in alcohol misuse. At least in part, altered CDT status may be related to altered iron status independent of the effect of alcohol. This may in turn account for some of the false positive and false negative values seen using serum CDT. Soluble transferrin receptors have been shown to be a sensitive indicator of iron status and are not raised in chronic inflammatory conditions with the anaemia of chronic diseases seen in inflammation. The relationship between alcohol misusers and soluble transferrin receptors has yet to be determined.

Aim: to investigate the levels of soluble transferrin receptors in alcohol misusers and compare these with healthy individuals, hospital inpatients, those with non-alcoholic liver disease and those with three different chronic diseases: inflammatory bowel disease, chronic rheumatoid disorders and chronic renal failure.

Purpose: to determine the relationship between soluble transferrin receptors, iron status and serum CDT.

6.2 Patients and Methods

6.2.1 Patients

These were the same cohort of individuals described in Chapters 4.4.2, the contemporary collection, and 5.2, the three chronic disease groups. At the time of initial assessment iron status was investigated. A full iron profile was made as described in Chapter 4.4.2.1, which consisted of serum iron, total iron binding capacity, % saturation and ferritin. In all these individuals the serum CDTect and AXIS %CDT had already been assessed.

The soluble transferrin receptor assay was performed using serum samples that had been stored at -20^0C.

The details of the test population are shown in Table 6.1. The hospital patient and abstinent alcoholic cirrhotics have one and two fewer individuals respectively, than those described in Chapter 4.4.2. This was due to transferrin receptor data missing in these three individuals.

Table 6.1: Demographic details of the study population

Study subgroup	Number	Ratio ♂:♀	Median (range) age (years)
Healthy volunteers	49	17:32	38.1 (19-79)
Hospital inpatients	49	24:25	68.9 (25-90)
Non-alcoholic liver disease	74	38:36	53.0 (18-78)
Non-cirrhotic	*35*	*18:17*	*52.6 (18-74)*
Cirrhotic	*39*	*20:19*	*53.9 (21-78)*
Abstinent alcohol misusers	93	57:36	53.0 (28-78)
Non-cirrhotic	*22*	*15:7*	*52.4 (34-78)*
Cirrhotic	*71*	*42:29*	*53.1 (28-72)*
Chronic rheumatoid disorders	30	8:22	52.8 (18-72)
Inflammatory bowel disease	30	14:16	44.2 (18-82)
Chronic renal failure	50	26:24	62.6 (20-83)
Drinking alcohol misusers	95	65:30	46.8 (19-72)
Non-cirrhotic	*69*	*49:20*	*46.8 (19-67)*
Cirrhotic	*26*	*16:10*	*48.4 (35-72)*

6.2.2 Measurement of Soluble Transferrin Receptors

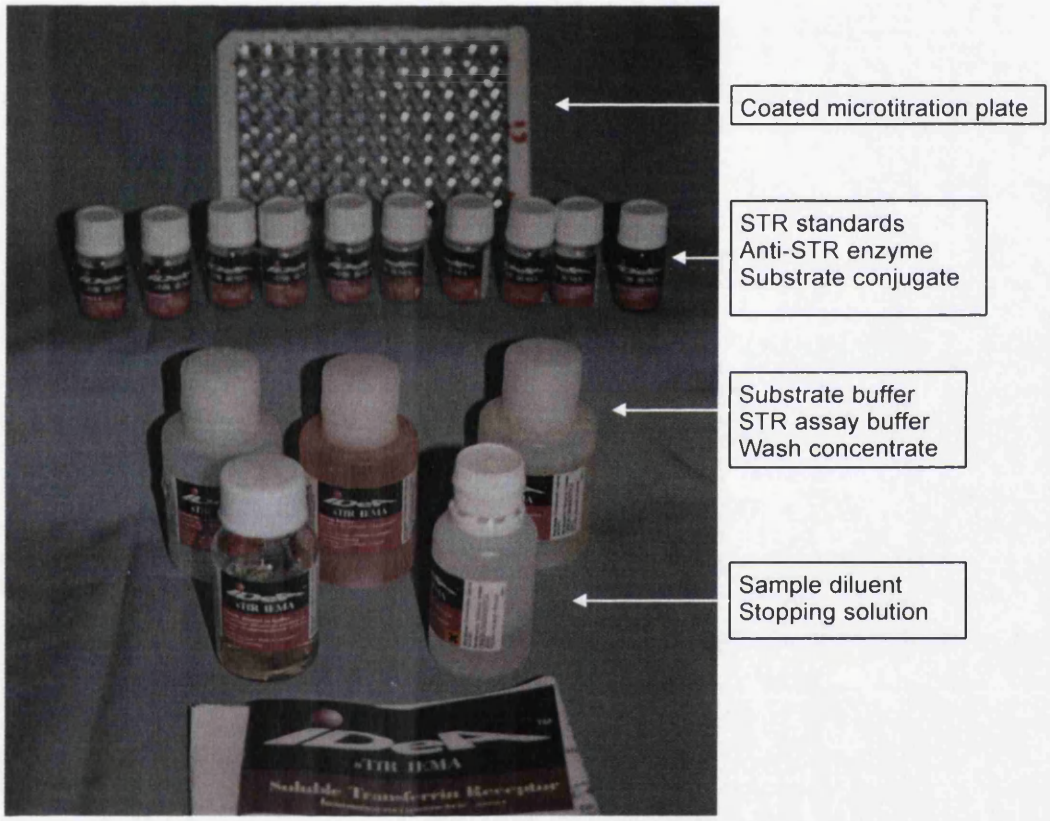
The assay used was a soluble transferrin receptor immunoenzymometric assay.

The assay kit comprised (Figure 6.1):

- A 96 well microtitration plate coated with monoclonal mouse anti-transferrin receptor antibody
- Soluble transferrin receptor standards which were all lyophilised preparations of human origin. The standard values were: 0 mg/L, 0.6 mg/L, 1.9 mg/L, 3.1 mg/L, 5.6 mg/L, 11.0 mg/L. Each of these was reconstituted with 0.5 mls of distilled water.
- 0.5 ml of anti-soluble transferrin receptor enzyme conjugate, a mouse monoclonal antibody stock solution, and this was diluted 1:50 with assay buffer

- 50 ml of soluble transferrin receptor assay buffer
- 0.5 ml of substrate concentrate
- 25 ml of substrate buffer. This was added 1:50 to the substrate concentrate to form the substrate solution.
- 10 ml of stopping solution
- 40 ml of wash concentrate. This was diluted with 960 mls to make 1,000 mls wash solution
- 15 ml sample diluent buffer

Figure 6.1: Soluble transferrin receptor (STR) assay kit (Orion Diagnostica)



The serum samples for analysis were defrosted to room temperature. 20 µL of each sample or standard was pipetted into each microtitration well. 200 µL of soluble transferrin receptor assay buffer was added to each well and the mixture was left to incubate for one hour at room temperature on a plate shaker.

The plate was then washed four times using approximately 250 µL washing solution per well. 200 µL diluted anti-soluble transferrin receptor enzyme conjugate solution was added to each well, and the mixture was left to incubate for one hour at room temperature on a plate shaker.

The plate was then again washed four times using about 250 μL washing solution per well. 200μL substrate solution was added to all wells. The plate was then left to incubate at room temperature for 30 minutes, without shaking. The enzyme reaction was then stopped by adding 50 μL stopping solution to each well, with the addition in exactly the same order as the substrate solution was added. The mixture was then shaken for two minutes. The absorbance was then read in a platereader at 405 nm.

The mean absorbance of the zero standard duplicates was calculated. This was then subtracted from those readings from each sample or standard. A standard curve was then plotted of absorbance at 405 nm versus soluble transferrin receptor concentration of each standard (mg/L). The unknown serum samples soluble transferrin receptor concentrations were then calculated from the standard curve. The normal range for each kit was given as 1.3-3.3 mg/L.

6.2.3 STATISTICAL ANALYSIS

A full iron profile was obtained in each individual. In each group the number of individuals with iron deficiency, features of the anaemia of chronic disease, macrocytosis and altered soluble transferrin receptor status was calculated.

Iron deficiency

In order to interpret any effect of alcohol misuse on the soluble transferrin receptor assay, first the iron indices: serum iron, TIBC, % saturation and ferritin, were analysed in each of the groups to establish the incidence of iron deficiency in the sub-groups. Iron deficiency was indicated by the following:

- Haemoglobin <12 g/dL (men), <11.5 g/dl (women)
- MCV <80 fL
- Iron <10 μmol/L
- TIBC >85 μmol/L
- % saturation <15%
- Ferritin <25 μg/L (men), <10 μg/L (women)

The number of individuals in each group satisfying the above criteria was calculated.

The anaemia of chronic disease

This is seen in the context of chronic inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease and chronic renal failure, and often shows many of the features of iron deficiency above. However serum ferritin is often be elevated as an indication of inflammation, at over 300 μg/L.

The number of individuals with both an elevated ferritin and a haemoglobin less than 12 mg/dL was calculated in each group.

Macrocytosis

Macrocytosis, or an erythrocyte mean corpuscular volume (MCV) greater than 100 fL, is a feature of alcohol misusers and has been used as a marker of abuse (Morgan et al. 1981). The number of individuals in each group with an MCV greater than 100 fL was calculated.

Soluble transferrin receptors

Soluble transferrin receptors: the number of individuals in each group testing both high (a result above the upper limit of normal given by the manufacturer's) and low (a result below the lower limit of normal given by the manufacturer's were calculated.

Inter-group correlations

The relationship between the soluble transferrin receptor and haemoglobin, serum transferrin, iron, TIBC, % saturation, ferritin was then analysed for each group using the Spearman Rank correlations.

Each of these indices was then correlated with CDTect and AXIS %CDT, again using the Spearman Rank test.

These analyses were performed using *Astute: Statistics Add-in for Microsoft Excel.*

6.3 Results

6.3.1 IRON DEFICIENCY

The number of individuals with features of iron deficiency in each group is shown in Table 6.2.

Table 6.2: Individuals with indices consistent with iron deficiency

Study subgroup	Hb <12 g/dL n (%)	MCV <80 fL n (%)	Iron <10 µmol/L n (%)	TIBC >85 µmol/L n (%)	% saturation <15 % n (%)	Ferritin <10µg/L n (%)	All criteria present n (%)
Healthy volunteers (n=49)	0	1 (2.0)	5 (10.2)	2 (4.1)	5 (10.2)	3 (6.1)	0
Hospital inpatients (n=49)	12 (24.5)	4 (8.2)	22 (44.9)	1 (2.0)	14 (28.6)	0	0
Non-alcoholic liver disease (n=74)	15 (20.3)	1 (1.4)	9 (12.2)	2 (2.7)	6 (8.1)	2 (2.7)	0
Non-cirrhotic (n=35)	*7 (20.0)*	*1 (2.9)*	*6 (17.1)*	*0*	*5 (14.3)*	*1 (2.9)*	*0*
Cirrhotic (n=39)	*8 (20.5)*	*0*	*3 (7.7)*	*2 (5.1)*	*1 (2.6)*	*1 (2.6)*	*0*
Abstinent alcohol misusers (n=93)	26 (28.0)	1 (1.1)	12 (12.9)	5 (5.4)	7 (7.5)	1 (1.1)	0
Non-cirrhotic (n=22)	*3 (13.6)*	*0*	*4 (18.2)*	*2 (9.1)*	*3 (13.6)*	*1 (4.5)*	*0*
Cirrhotic (n=71)	*23 (32.4)*	*1 (4.3)*	*8 (11.3)*	*3 (4.2)*	*4 (5.6)*	*0*	*0*
Chronic rheumatoid disorders (n=30)	9 (30.0)	3 (10.0)	6 (20.0)	1 (3.3)	6 (20.0)	1 (3.3)	0
Inflammatory bowel disease (n=30)	17 (56.7)	4 (13.3)	19 (63.3)	0	14 (46.7)	5 (16.7)	0
Chronic renal failure (n=50)	38 (76.0)	2 (4.0)	12 (24.0)	0	2 (4.0)	0	0
Drinking alcohol misusers (n=95)	11 (11.6)	1 (9.1)	4 (4.2)	10 (10.5)	3 (3.2)	2 (2.1)	0
Non-cirrhotic (n=69)	*4 (5.8)*	*0*	*3 (4.3)*	*9 (13.0)*	*2 (3.0)*	*1 (1.4)*	*0*
Cirrhotic (n=26)	*7 (26.9)*	*1 (14.3)*	*1 (3.8)*	*1 (3.8)*	*1 (3.8)*	*1 (3.8)*	*0*

There were no individuals with all the features of iron deficiency in any group. Although a haemoglobin of less than 12 g/dL was present in 20% or more of all groups other than the healthy volunteers and drinking alcohol misusers, there were few other indices of iron deficiency in these groups.

6.3.2 THE ANAEMIA OF CHRONIC DISEASE

Table 6.3 shows the proportion of each group with a ferritin greater than 300 μg/L, a feature suggestive of the anaemia of chronic disease in the presence of a low haemoglobin.

Table 6.3: Individuals with an elevated serum ferritin >300 μg/L

Study subgroup	Ferritin >300 μg/L n (%)	Ferritin >300 μg/L & Hb <12 g/dL n (%)
Healthy volunteers (n=49)	1 (2.0)	0
Hospital inpatients (n=49)	8 (16.3)	2 (4.1)
Non-alcoholic liver disease (n=74)	16 (21.6)	7 (9.5)
Non-cirrhotic (n=35)	*6 (17.1)*	*3 (8.6)*
Cirrhotic (n=39)	*10 (25.6)*	*4 (10.3)*
Abstinent alcohol misusers (n=93)	32 (34.4)	12 (12.9)
Non-cirrhotic (n=22)	*7 (31.8)*	*2 (9.1)*
Cirrhotic (n=71)	*25 (35.2)*	*10 (14.1)*
Chronic rheumatoid disorders (n=30)	0	0
Inflammatory bowel disease (n=30)	5 (16.7)	2 (6.7)
Chronic renal failure (n=50)	15 (30.0)	10 (20.0)
Drinking alcohol misusers (n=95)	39 (41.5)	6 (6.3)
Non-cirrhotic (n=69)	*22 (31.9)*	*2 (2.9)*
Cirrhotic (n=26)	*17 (65.4)*	*4 (7.7)*

The proportion of individuals with an elevated ferritin was more than 30% in the alcohol misusers groups. 12.9% of the abstinent alcohol misusers had anaemia and an elevated ferritin, while although 41.5% of actively drinking alcohol misusers had an elevated ferritin, only 6.3% had both. This suggests that the elevated ferritin in the active drinkers was secondary to active drinking.

20.0% and 6.7% of chronic renal failure and inflammatory bowel disease patients respectively had anaemia and an elevated ferritin suggesting the anaemia of chronic disease, while surprisingly none of the rheumatoid arthritis patients had an elevated ferritin. Approximately 10% of the non-alcoholic liver disease patients had the anaemia of chronic disease.

6.3.3 MACROCYTOSIS

The proportion of individuals in each group with macrocytosis is shown in Table 6.4.

Table 6.4: Numbers (%) of individuals with macrocytosis

Study subgroup	Individuals with MCV >100 fL n (%)
Healthy volunteers (n=49)	1 (2.0)
Hospital inpatients (n=49)	1 (2.0)
Non-alcoholic liver disease (n=74)	6 (8.1)
Non-cirrhotic (n=35)	*1 (2.9)*
Cirrhotic (n=39)	*5 (12.8)*
Abstinent alcohol misusers (n=93)	27 (29.0)
Non-cirrhotic (n=22)	*4 (18.2)*
Cirrhotic (n=71)	*23 (32.4)*
Chronic rheumatoid disorders (n=30)	0
Inflammatory bowel disease (n=30)	0
Chronic renal failure (n=50)	1 (2.0)
Drinking alcohol misusers (n=95)	37 (38.9)
Non-cirrhotic (n=69)	*24(34.8)*
Cirrhotic (n=26)	*13 (50.0)*

The proportion of individuals with macrocytosis is higher in the actively drinking alcohol misusers than the non-drinking populations, as expected. The abstinent alcohol misusers have the highest proportion of the non-drinking populations, and this may be due to the half life of the erythrocyte being three months and the cut-off for abstinence being six weeks. The cirrhotic part of the alcoholic and non-alcoholic populations had higher proportions of macrocytosis than the non-cirrhotic parts. The 38.9% of alcohol misusers with macrocytosis secondary to active drinking may well have iron deficiency, but will not then have microcytic anaemia, as expected.

6.3.4 SOLUBLE TRANSFERRIN RECEPTORS

The proportion of individuals in each group with a soluble transferrin receptor result testing high or low are given in Table 6.5. There were only five individuals in the total study

population with a low soluble transferrin receptor level, but over half of the study population had a high soluble transferrin receptor assay. 20.5% of the healthy volunteers, who had normal iron indices, had high levels of soluble transferrin receptors and 50.0% of the hospital inpatients. The non-alcoholic liver disease group, abstinent alcohol misusers and three chronic disease groups all had similar high rates of 63-77% and the drinking alcohol misusers were higher still at 90.5%.

Table 6.5: Individuals testing high and low for soluble transferrin receptors

Study subgroup	Number (%) STR testing high	Number (%) STR testing low
Healthy volunteers (n=49)	10 (20.5)	1 (2.0)
Hospital inpatients (n=49)	24 (50.0)	2 (4.1)
Non-alcoholic liver disease (n=74)	47 (63.5)	0
Non-cirrhotic (n=35)	*22 (62.9)*	*0*
Cirrhotic (n=39)	*25 (64.1)*	*0*
Abstinent alcohol misusers (n=93)	72 (77.4)	1 (1.1)
Non-cirrhotic (n=22)	*14 (63.6)*	*0*
Cirrhotic (n=71)	*58 (81.7)*	*1 (1.4)*
Chronic rheumatoid disorders (n=30)	19 (63.3)	0
Inflammatory bowel disease (n=30)	21 (70.0)	0
Chronic renal failure (n=50)	30 (60.0)	1 (2.0)
Drinking alcohol misusers (n=95)	86 (90.5)	0
Non-cirrhotic (n=69)	*62 (89.9)*	*0*
Cirrhotic (n=26)	*24 (92.3)*	*0*
Total population (n=470)	309 (65.7)	5 (1.1)

The relationships between soluble transferrin receptors and Hb, iron indices and CDT assays were examined using the Spearman Rank correlation and are shown in Table 6.6.

There was no iron deficiency in any of the groups, so that the relationship between soluble transferrin receptors and any other index should have been independent of the effect of iron deficiency.

For the total population Table 6.6 shows that soluble transferrin receptors are significantly correlated with haemoglobin, MCV, ferritin and the two CDT assays, CDTect and AXIS %CDT.

Table 6.6: '*p*' and correlation coefficient (rs) values for Spearman rank correlations between soluble transferrin receptors and other variables in test groups

		Hb	MCV	Iron	TIBC	% saturation	Ferritin	Trans-ferrin	CDTect	AXIS
Healthy volunteers	*p*	<0.0001	0.028	ns	ns	ns	ns	ns	0.002	0.033
	rs	0.53	-0.27						-0.41	-0.38
Hospital inpatients	*p*	ns	0.0004	ns	0.0280	0.0012	<0.0001	0.0010	0.016	ns
	rs		-0.46		0.27	-0.42	-0.52	0.43	0.31	
Non-alcoholic liver disease	*p*	0.0001	ns	0.0008	0.0028	ns	ns	0.0080	ns	0.0042
	rs	-0.41		-0.37	-0.33			-0.28		0.31
Non-cirrhotic	*p*	*0.010*	*ns*	*0.0028*	*0.0020*	*ns*	*ns*	*ns*	*ns*	*0.009*
	rs	*-0.39*		*-0.47*	*-0.48*					*0.39*
Cirrhotic	*p*	*0.005*	*ns*	*0.030*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	rs	*-0.41*		*-0.31*						
Abstinent alcohol misusers	*p*	0.0018	ns	ns	ns	ns	0.0190	ns	0.0003	0.0014
	rs	-0.30					-0.22		0.35	0.31
Non-cirrhotic	*p*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cirrhotic	*p*	*0.020*	*ns*	*ns*	*ns*	*ns*	*0.005*	*ns*	*0.005*	*0.024*
	rs	*-0.25*					*-0.30*		*0.30*	*0.24*
Chronic rheumatoid disorders	*p*	ns	ns	ns	ns	ns	ns	ns	ns	ns
Inflammatory bowel disease	*p*	0.0003	0.0002	0.0002	ns	0.0002	0.033	ns	ns	ns
	rs	-0.59	-0.61	-0.61		-0.61	-0.34			
Chronic renal failure	*p*	ns	ns	ns	ns	ns	ns	ns	ns	ns
Drinking alcohol misusers	*p*	ns	ns	ns	ns	ns	ns	ns	ns	ns
Non-cirrhotic	*p*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cirrhotic	*p*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Total population	***p***	**0.010**	**0.0009**	**ns**	**ns**	**ns**	**0.014**	**ns**	**<0.0001**	**<0.0001**
	rs	**-0.11**	**0.14**				**0.10**		**0.26**	**0.21**

The 'p' values shown in red indicate a significant value of <0.05, ns indicates a value >0.05. Using the Spearman rank correlation test the degree of association between soluble transferrin receptors and the other variable tested is given by the correlation coefficient rs. A value of +1 indicates complete positive correlation, 0 no correlation, and –1 indicates complete negative correlation.

Within the sub-groups the soluble transferrin receptors negatively correlated with the haemoglobin in the non-alcoholic liver disease groups, the abstinent alcohol cirrhotics and the inflammatory bowel disease groups. The CDTect and AXIS %CDT assays correlated with the soluble transferrin receptors in the healthy volunteers and the abstinent alcoholic cirrhotics.

Within the actively drinking alcohol misusers there was no correlation between soluble transferrin receptors, CDT and any index of iron status.

6.3.5 SERUM CDT ASSAYS

The relationships between both CDTect and AXIS %CDT, and the indices of iron deficiency were tested using the Spearman Rank test. The results are shown for CDTect in Table 6.7 and AXIS %CDT in Table 6.8.

Table 6.7: Spearman rank correlations between serum CDTect and the indices of iron status

		Hb	MCV	Iron	TIBC	% saturation	Ferritin	Transferrin
Healthy volunteers	*p*	<0.0001	0.025	ns	0.043	ns	0.0025	0.0008
	rs	-0.55	0.28		0.25		-0.40	0.44
Hospital inpatients	*p*	ns	ns	ns	0.048	ns	0.014	0.0007
	rs				0.24		-0.32	0.45
Non-alcoholic liver disease	*p*	0.048	0.013	ns	ns	ns	0.036	0.025
	rs	-0.20	0.26				-0.21	0.23
Non-cirrhotic	*p*	*0.035*	*ns*	*ns*	*ns*	*ns*	*0.049*	*0.001*
	rs	*-0.31*					*-0.29*	*0.51*
Cirrhotic	*p*	*ns*	*ns*	*0.018*	*0.039*	*ns*	*0.080*	*ns*
	rs			*0.35*	*0.29*		*-0.23*	
Abstinent alcohol misusers	*p*	0.040	ns	0.0006	ns	ns	0.044	ns
	rs	-0.18		0.33			-0.18	
Non-cirrhotic	*p*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*0.024*
	rs							*0.43*
Cirrhotic	*p*	*ns*	*ns*	*0.0007*	*ns*	*ns*	*ns*	*ns*
	rs			*0.37*				
Chronic rheumatoid disorders	*p*	ns	ns	ns	0.048	ns	0.013	0.008
	rs				0.31		-0.41	0.44
Inflammatory bowel disease	*p*	ns	ns	ns	0.0002	ns	0.018	<0.0001
	rs				0.61		-0.38	0.71
Chronic renal failure	*p*	ns	ns	0.0060	0.032	0.046	0.0026	0.025
	rs			0.35	0.26	0.21	-0.39	0.28
Drinking alcohol misusers	*p*	ns	ns	ns	ns	ns	0.0038	0.016
	rs						-0.28	0.22
Non-cirrhotic	*p*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cirrhotic	*p*	*ns*	*ns*	*ns*	*0.0004*	*0.029*	*0.0009*	*0.0013*
	rs				*0.62*	*-0.38*	*-0.58*	*0.56*
Total population	***p***	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**ns**	**<0.0001**
	R s	**0.09**	**0.34**	**0.33**	**0.20**	**0.20**		**0.28**

Table 6.8: Spearman rank correlations between serum AXIS %CDT and the indices of iron status

		Hb	MCV	Iron	TIBC	% saturation	Ferritin	Transferrin
Healthy volunteers	*p*	<0.0001	ns	ns	ns	ns	ns	ns
	rs	-0.43						
Hospital inpatients	*p*	ns	ns	ns	ns	ns	ns	ns
Non-alcoholic liver disease	*p*	<0.001	0.0002	ns	0.0008	0.0050	ns	<0.0001
	rs	-0.46	0.41		-0.37	0.31		-0.42
Non-cirrhotic	*p*	*0.002*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	rs	*-0.49*						
Cirrhotic	*p*	*0.033*	*0.028*	*ns*	*0.004*	*0.008*	*0.014*	*0.0007*
	rs	*-0.30*	*0.31*		*-0.43*	*0.39*	*0.35*	*-0.50*
Abstinent alcohol misusers	*p*	0.0006	0.0002	0.035	<0.0001	0.0002	ns	0.0001
	rs	-0.33	0.37	0.19	-0.41	0.37		-0.38
Non-cirrhotic	*p*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cirrhotic	*p*	*0.009*	*0.0001*	*ns*	*0.0002*	*0.0003*	*ns*	*<0.0001*
	rs	*-0.28*	*0.43*		*-0.42*	*0.40*		*-0.47*
Chronic rheumatoid disorders	*p*	ns	ns	ns	ns	ns	ns	ns
Inflammatory bowel disease	*p*	ns	ns	ns	0.0082	ns	0.045	0.0171
	rs				-0.43		0.31	-0.39
Chronic renal failure	*p*	ns	0.032	ns	0.0470	0.015	ns	0.014
	rs		0.26		-0.24	0.31		-0.31
Drinking alcohol misusers	*p*	ns	0.017	ns	0.0007	0.024	ns	0.0026
	rs		0.22		-0.33	0.21		-0.29
Non-cirrhotic	*p*	*ns*	*ns*	*ns*	*0.0051*	*ns*	*ns*	*0.037*
	rs				*-0.32*			*-0.22*
Cirrhotic	*p*	*ns*	*0.040*	*ns*	*0.009*	*0.049*	*ns*	*0.0027*
	rs		*0.36*		*-0.47*	*0.34*		*-0.54*
Total population	***p***	**ns**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	rs		**0.48**	**0.24**	**-0.21**	**0.34**	**0.21**	**-0.20**

Overall CDTect had a positive correlation with transferrin, AXIS %CDT a negative ($p<0.0001$). In the total populations both serum CDT assays correlated with iron, TIBC, % saturation and erythrocyte MCV ($p<0.0001$). CDTect also correlated with haemoglobin ($p=0.024$) and AXIS %CDT with ferritin ($p<0.0001$).

Within the actively drinking cirrhotic population, CDTect correlated with total iron binding capacity, % saturation, ferritin and transferrin, but not iron. In the non-cirrhotic population there was no correlation between CDTect and any one part of the iron indices. AXIS %CDT correlated with transferrin but not iron or ferritin.

The total abstinent alcohol misusers population showed CDTect correlated with haemoglobin, iron and ferritin. AXIS %CDT correlated with all indices other than ferritin, however the minimal change part of the group showed no correlation with any of the indices.

In the non-alcoholic liver disease group, the cirrhotic part correlated with all indices other than iron using AXIS %CDT. The non-cirrhotic part of the non-alcoholic liver disease group and the healthy volunteers only correlated with haemoglobin. The hospital patients and minimal change abstinent alcoholic liver disease showed no correlation with any of the indices.

Using CDTect there were correlations with ferritin and transferrin in all groups other than the abstinent alcoholic cirrhotics and drinking minimal change alcohol misusers.

In summary, overall there was a positive correlation between serum CDT and soluble transferrin receptors, and a negative correlation with ferritin. There was, however, no correlation between soluble transferrin receptors and CDT or any determinant of iron status in the active alcohol misuse population. Serum CDT correlated with transferrin, but not consistently with any other determinant of iron status.

6.4 Discussion

Soluble transferrin receptors are elevated in iron deficiency, at a ferritin level less than 12 μg/L (Skikne et al. 1990). There were no individuals meeting the criteria for iron deficiency in the population overall but 65.7% of the total population had a high soluble transferrin receptor level. 20.5% of the healthy volunteers, none of whom were iron deficient, had a high reading suggesting the manufacturer's reference range was inappropriately low in this population.

In the alcohol misusers population the soluble transferrin receptors were elevated in 90.5% of individuals. In this group the soluble transferrin receptors did not correlate with any of the indices of iron deficiency (Table 6.6), so that the reason for the high levels seems unlikely to be secondary to occult iron deficiency. Serum CDT assays correlated with transferrin but not with any other iron index. The effects of altered iron status on CDT were not clear, however there appeared to be no relationship between iron indices, transferrin receptor status and CDT.

In the abstinent population the soluble transferrin receptors were elevated in 77.4% of alcohol misusers and 63.5% of the non-alcoholic liver disease group. The alcoholic cirrhotics were

higher at 81.7%. The soluble transferrin receptors negatively correlated with haemoglobin in all groups, with iron in non-alcoholic liver disease, and ferritin in alcoholic liver disease, as expected. In those sub-groups the elevated soluble transferrin receptors seemed to be affected by iron status.

CDT assays in the abstinent population correlated negatively with haemoglobin and positively with transferrin. CDTect also correlated negatively with ferritin. Despite the variable relationship between the assays and iron status, the assays correlated with soluble transferrin receptors overall and in most sub-groups. In the abstinent population iron status seems to affect CDT status, at least in part.

The serum ferritin is known to be raised during acute alcohol misuse, but decreases rapidly during abstinence (Bell et al. 1994). The proportion of individuals with a raised ferritin was higher in active drinkers than in the abstinent population, 31.9% of the minimal change group and 65.4% of the cirrhotics had a ferritin of greater than 300 μg/L. It has been suggested that soluble transferrin receptors are of maximum value in detecting iron deficiency in populations where the ferritin may not be low due to a 'false' elevation from the acute phase reaction (Mast et al. 1998). This would suggest that occult iron deficiency was present. However, excluding a low serum ferritin as a criteria for iron deficiency, there were still no individuals fulfilling the criteria for iron deficiency.

The soluble transferrin receptors correlated with ferritin in the total population (p=0.014), seen in the hospital inpatients (p<0.0001), abstinent alcoholic cirrhotics (p=0.005) and inflammatory bowel disease (p=0.03). In all these groups the *rs* was negative, as expected, confirming that when the ferritin decreases, the soluble transferrin receptors increase.

The three chronic disease groups had a raised soluble transferrin receptor assay in 60-70% of individuals. 56.7% of inflammatory bowel disease and 76.0% of the chronic renal failure groups were anaemic. Not surprisingly there was a large number of individuals with a raised ferritin in both these groups, attributable to the inflammatory response. In the inflammatory bowel disease group the soluble transferrin receptors correlated negatively with iron indices, but this was not seen in any other group. CDT assays correlated with transferrin and TIBC, and CDTect with ferritin, but there was no other consistent correlation. Even though soluble transferrin receptors were elevated in this group, iron status was not related to the soluble transferrin receptor status, nor were CDT levels.

Overall there were only five low readings, suggesting iron overload (Khumalo et al. 1998), and all of these had a haemoglobin less than 14 g/dL. Two were anaemic, and while two had a ferritin >300 μg/L, none were greater than 400 μg/L.

It has been suggested that increased soluble transferrin receptors may be due not only to iron deficiency, but also to haemolytic anaemia, thalassaemia, and macrocytic anaemia. The most likely explanation here is macrocytosis: 38.9% of drinking alcohol misusers had an MCV greater than 100 fL, and 29% of abstinent alcoholic liver disease group. This may account, at least in part, for the high soluble transferrin receptor assay levels.

In summary, while the soluble transferrin receptor level has been suggested as a sensitive and specific indication of iron status (Ahluwalia, 1998), the levels in this population were raised in all groups despite no overt iron deficiency. There was an overall correlation between CDT assays and soluble transferrin receptors. Within the abstinent population soluble transferrin receptor and CDT levels were partially affected by iron status. However within the alcohol misusers, despite the fact that soluble transferrin receptors were elevated, the reason was not clearly related to iron status, which in turn did not seem to affect CDT status.

7. THE USE OF SERUM CARBOHYDRATE DEFICIENT TRANSFERRIN IN MONITORING

7.1 Introduction

Once alcohol misusers have been identified and treatment instigated, it is essential to have an objective marker of alcohol misuse to monitor progress and to identify any possible relapse. Early detection is important so that intervention can commence before prolonged misuse results in secondary destructive effects, both physical and psychological.

Markers are required that are objective and reflect both long and short term drinking behaviour. In current practice the blood tests used are gamma glutamyl transferase (GGT), aspartate transaminase (AST) and erythrocyte mean corpuscular volume (MCV). They may all be false positive, largely due to liver disease. Serum carbohydrate deficient transferrin has been suggested as a useful marker in monitoring misusers. Serum CDTect has been suggested to be more sensitive to relapses than GGT with changes occurring sooner to indicate drinking status (Anton et al. 1996) and before self-report (Rosman et al. 1995) (Table 3.7).

The use of serum CDT in monitoring has not been as extensively studied as in screening, in particular the use in women and in individuals with significant liver injury.

Aim: to evaluate serum CDT assays in the monitoring of alcohol misusers from detoxification, through subsequent drinking behaviour, and in comparison to other known markers.

Purpose: to establish the role of serum CDT assays in the monitoring of drinking behaviour in individual alcohol misusers.

7.2 Patients and Methods

7.2.1 ALCOHOL MISUSERS

Forty actively drinking alcohol misusers, 24 men and 16 women, who were recruited from in- and out-patient populations of the Royal Free Hospital. These individuals had a history of

alcohol misuse of at least 60g per day for a minimum of one year. They drank a median (range) of 128 (64-360) g of alcohol per day. Individuals were classified, based on liver histology obtained by needle biopsy, into non-cirrhotic and cirrhotic liver disease. There were 18 alcoholic cirrhotics, 22 non-cirrhotics. All 40 were detoxified over ten days, either as in- or as out-patients, and then followed serially for up to six months. The drinking behaviour was documented throughout using self-report, physical signs, breath alcohol and other laboratory markers. Where possible independent verification was sought.

Blood samples were taken three times per week during detoxification, and then at monthly intervals during the next six months. Blood was taken for routine analysis for conventional markers of alcohol misuse: erythrocyte mean corpuscular volume (MCV), serum aspartate transferase (AST) and serum gamma glutamyl transferase (GGT). Sera were stored, in aliquots, at -20^0C, for analysis of serum CDTect, AXIS %CDT and total transferrin. All samples for a given individual were analysed in the same batch, or in the case of one subject, in two batches. The cut-off values used for the CDT assays were those recommended by the manufacturer: for CDTect 20 U/L for men and 26 U/L for women; for AXIS %CDT 6.0%, for transferrin 200-400 mg/dL.

7.2.2 CONTROL GROUPS

Healthy volunteers. Eight healthy volunteers, four men and four women were selected for study; none had a history or evidence of liver disease or of alcohol misuse, nor currently drank more than 20 g alcohol per day. Blood samples were taken for the same analyses as the alcohol misusers, every two months over a six month period, a total of four samples in all.

Non-alcoholic liver disease. Six individuals with biopsy-proven chronic non-alcoholic liver disease. One man and five women were selected for study. Three had cirrhotic liver disease secondary to hepatitis C, cryptogenic cirrhotic and primary biliary cirrhosis. Three were non-cirrhotic, secondary to chronic active hepatitis, primary biliary cirrhosis and drug induced liver disease. None had a history of alcohol misuse and all currently drank less than 20 g per day. Blood samples were taken for the same analyses as the alcohol misusers, at intervals for up to six months.

Table 7.1: Demographic details of the study groups

	Number	♂:♀	Median (range) age Years
Alcohol misusers	40	24:16	47 (28-72)
Non-cirrhotic	*22*	*13:9*	*41 (28-63)*
Cirrhotic	*18*	*11:7*	*49 (35-72)*
Healthy volunteers	8	4:4	35 (30-50)
Non-alcoholic liver disease	6	1:5	56 (18-75)
Non-cirrhotic	*3*	*1:2*	*36 (18-51)*
Cirrhotic	*3*	*0:3*	*69 (62-75)*

7.2.3 STATISTICAL METHODS

The number of individuals in each group with elevated results for each marker was calculated. These were then compared using the Chi-square test. The alcohol misusers were subdivided by gender and histology (non-cirrhotic and cirrhotic). The inter-group differences were compared using Fisher's exact test as the numbers for comparison were less than 30.

The analytical variation was calculated as:

2.8 x standard deviation.

This is intended to take into account both the analytical variation (Jones and Payne, 1997). The standard deviation (SD) is calculated from the baseline value in question multiplied by the coefficient of variation.

7.3 Results

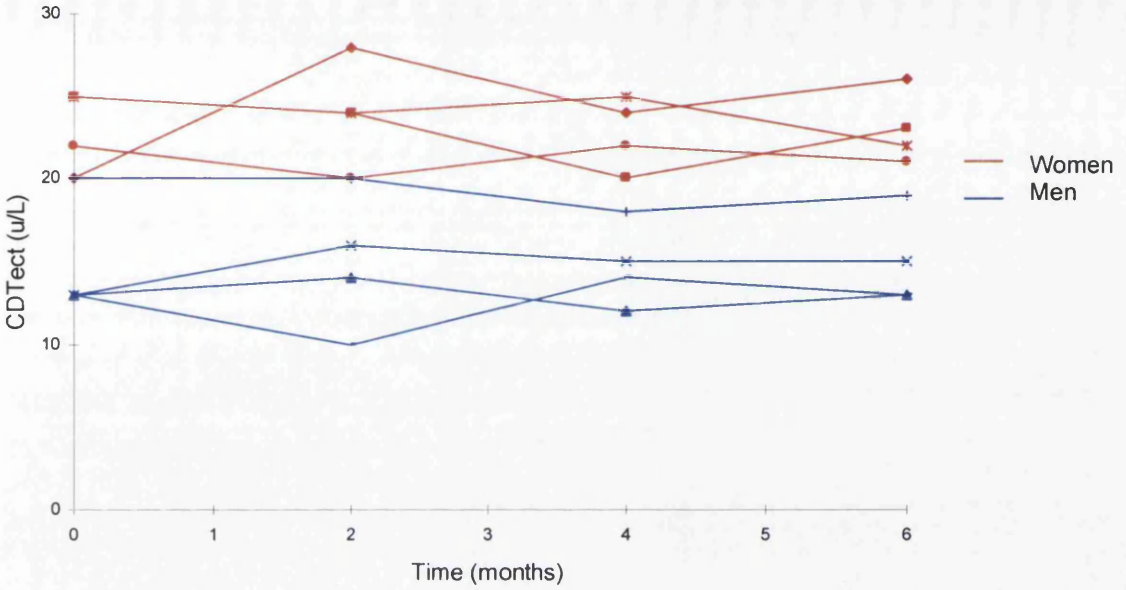
7.3.1 HEALTHY VOLUNTEERS

Four samples were collected for each of the eight healthy volunteers over the six months.

CDTect

The coefficient of variation, or the within assay variation, was 7.5 - 8.5%. Serum CDTect concentrations were, as expected, higher in women than in men (Figure 7.1).

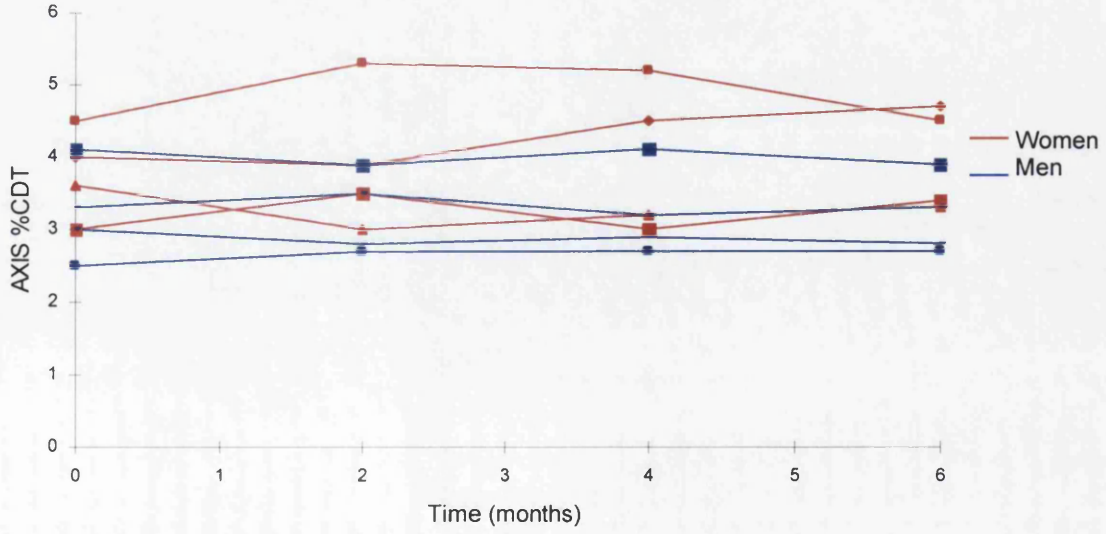
Figure 7.1: Serum CDTect results for the eight healthy volunteers



Seven of the eight healthy volunteers showed no variation in serum CDTect concentration beyond analytical variation over the six month period; one woman showed a single result at two months of 28 U/L which exceeded this variation, no explanation for this was forthcoming.

AXIS %CDT

Figure 7.2: AXIS %CDT results for the eight healthy volunteers

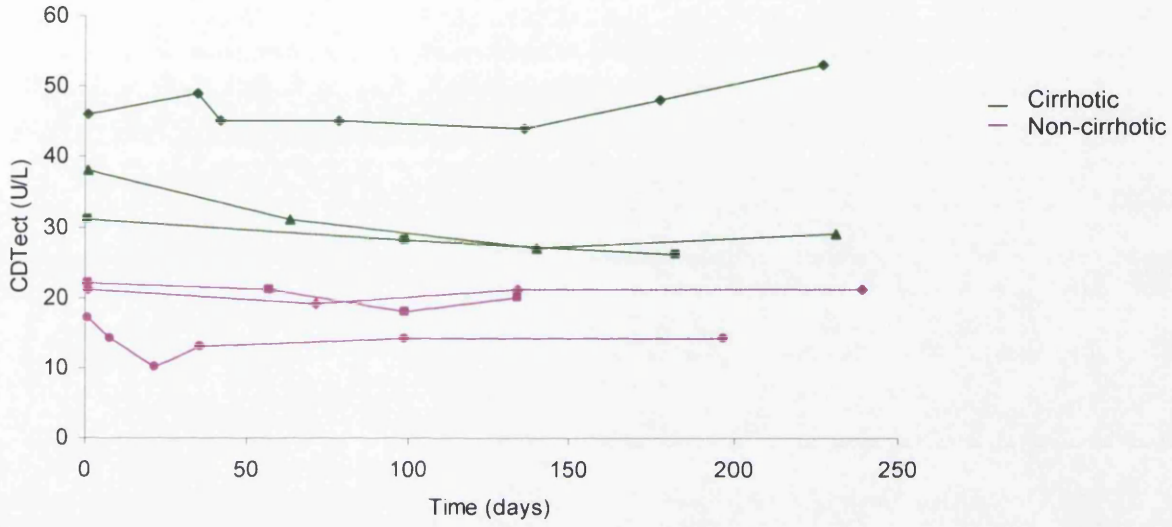


All values were below the manufacturer's cut-off (6.0%) throughout. The coefficient of variation, or the within assay variation, was 3.1-6.0%. No variation beyond analytical variation was observed in any of the eight volunteers over the six month period.

7.3.2 NON-ALCOHOLIC LIVER DISEASE

A total of 29 samples were collected in the six individuals with non-alcoholic liver disease studied over a median (range) of 4 (3-8) months.

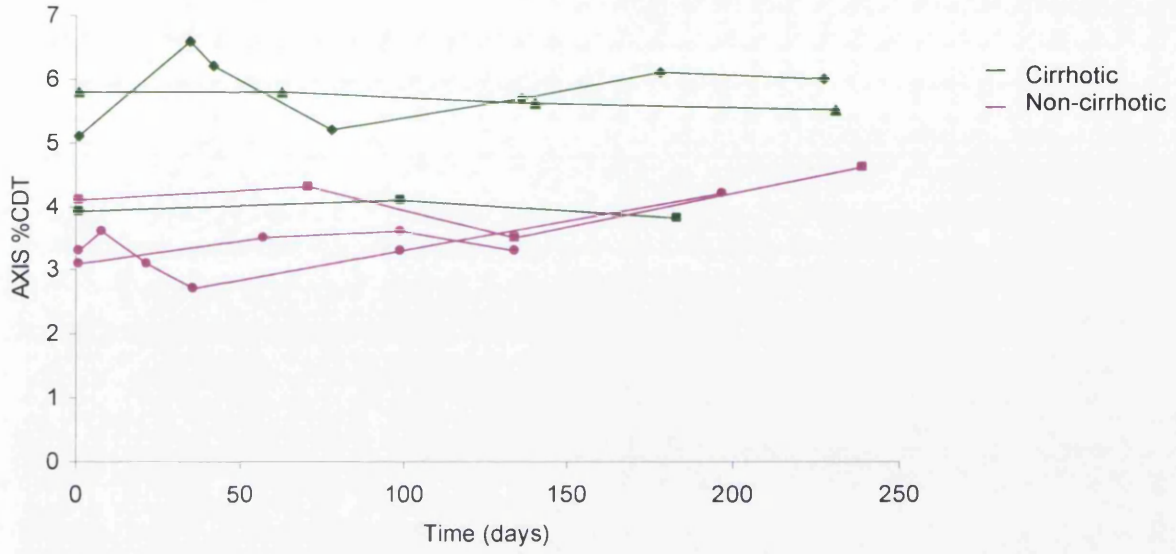
Figure 7.3: Serum CDTect results for non-alcoholic liver disease group



CDTect: the serum CDTect results show that all the individuals had stable results over the six months. None had results that varied beyond the calculated analytical variation. The three cirrhotic individuals, as expected, had serum CDTect results which were above the reference range, all false positive, throughout the period. The three non-cirrhotics all had results which remained within the reference range.

AXIS %CDT: there was greater variation over the six month period for the individuals than when using CDTect. Two individuals had results which exceeded analytical variation: one non-cirrhotic and one cirrhotic woman. Both of these individuals had a varied clinical course over this time. The results for the cirrhotics were generally higher than for the non-cirrhotics. Only one individual exceeded the upper reference limit of 6.0%, the same cirrhotic woman who showed variation in her results.

Figure 7.4: AXIS %CDT levels for the non-alcoholic liver disease group

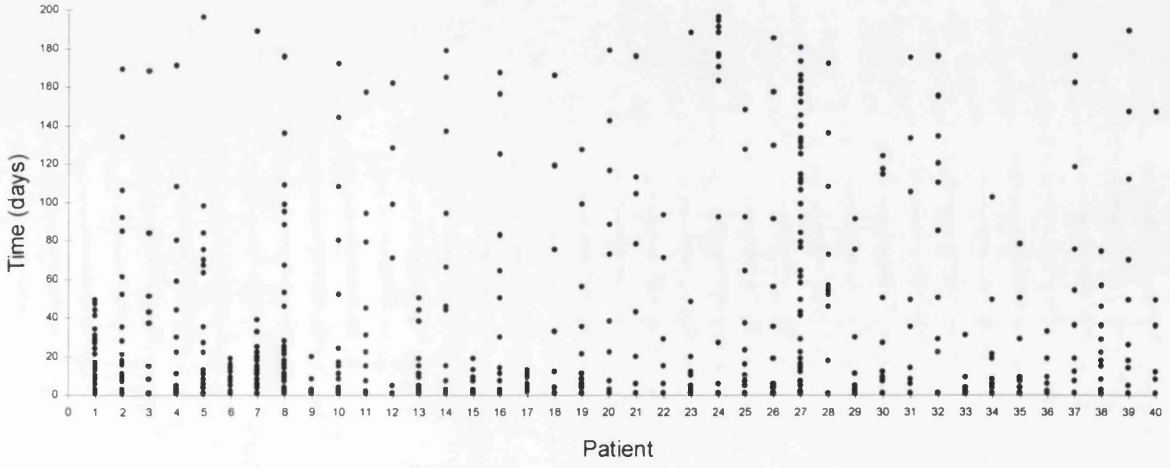


7.3.3 ALCOHOL MISUSERS

7.3.3.1 Sample collection

A total of 433 samples were collected from the 40 alcohol misusers, a median (range) of 10 (5-38) samples per patient. The variation is shown in Figure 7.5.

Figure 7.5: Sample collection profile of alcohol misusers



Each ● represents a data collection point.

The greatest number of samples were collected between days 1-10, during detoxification. Thereafter there was variability between patients, with those who were in-patients, either due to illness (for example patient number 27) or due to a relapse (for example patient 24) having a greater frequency of sample collection than those who remained abstinent following detoxification.

7.3.3.2 Baseline raised values

At day 1 baseline values for erythrocyte MCV, serum AST, GGT, CDTect and AXIS %CDT were elevated in between 39.5 and 90.0% of individuals (Table 7.2).

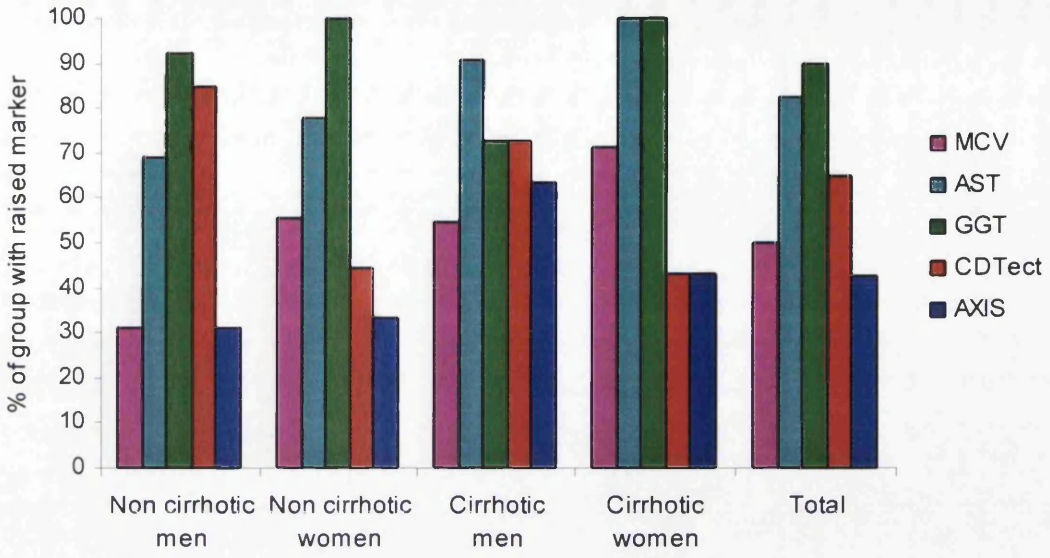
Table 7.2: Number (%) of markers elevated at baseline

Biological marker	Number (%) with elevated baseline value
Erythrocyte MCV	20 (50.0)
Serum AST	33 (82.5)
Serum GGT	36 (90.0)
Serum CDTect	26 (65.0)
Serum AXIS %CDT	17 (39.5)

Of those four individuals negative for GGT, two were positive for serum CDTect, and all were positive for AXIS %CDT. Of the seven negative for AST, three were positive for CDTect and one positive for AXIS %CDT. Of the twenty negative for erythrocyte MCV, fourteen were positive for CDTect and six for AXIS %CDT. No individual was negative for all the markers.

The results for the markers were then divided by liver injury and gender (Figure 7.6). In the non-cirrhotic men GGT and CDTect were both raised in significantly more individuals (p=0.01 and 0.07 respectively) than both MCV and AXIS %CDT. In the non-cirrhotic women GGT was elevated in significantly more individuals than either CDTect (p=0.03) or AXIS %CDT (p=0.004).

Figure 7.6: The % of each group with a raised marker, divided by liver injury and gender



In both the cirrhotic men and women there were no significant differences between the number of individuals with each elevated marker.

For each marker the number of individuals with a raised value in each sub-group was compared. The performance was consistent across the groups with no significant difference seen.

7.3.3.3 Effect of abstinence

Of those with an elevated marker at baseline, Table 7.3 shows the number (%) of individuals who showed a subsequent fall with abstinence. The fall may not have been to the reference range if a relapse occurred before this was possible.

Table 7.3: Number (%) of responders with each marker

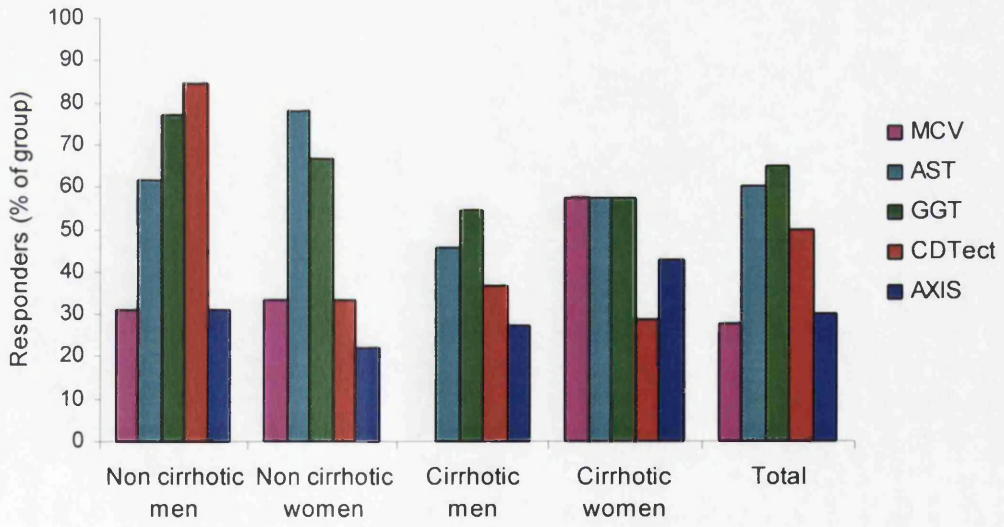
	Number elevated at Day 1	Number falling with abstinence (% of those raised at day 1)
MCV	20	11 (55.0)
AST	33	24 (72.7)
GGT	36	26 (72.2)
CDTect	26	20 (76.9)
AXIS %CDT	17	12 (70.6)

The individuals who had an initial raised assay marker when drinking, which then fell in response to abstinence, were termed 'responders'. Those whose initial result remained elevated in abstinence, or who had an initial result within the reference range were termed 'non-responders'.

The marker with the highest number of responders was GGT, with 26 (65%) of the test population, followed by AST with 24 individuals who were responders (40% of the alcohol misusers population). Both of these were significantly higher than MCV or AXIS %CDT. Using CDTect 20 were responders, or 50% of the alcohol misusers population. This was not significantly different from any of the other markers.

When the number of responders for each marker was compared, as a proportion of those with a raised value at day 1, there was no difference between marker performance (Table 7.3). Figure 7.7 shows the responders, by marker, in each sub-group. The numbers are displayed as percentages of the number of individuals in each group.

Figure 7.7: Responders, as % of each group



In the non-cirrhotic men there were significantly more responders using GGT or CDTect than using MCV or AXIS %CDT. In the cirrhotic men the largest number of responders was using GGT and then AST, both of which were higher than MCV, with which there were no responders. There were no significant differences using either CDTect or AXIS %CDT than any of the other markers. In the non-cirrhotic and cirrhotic women there were no significant differences between the markers.

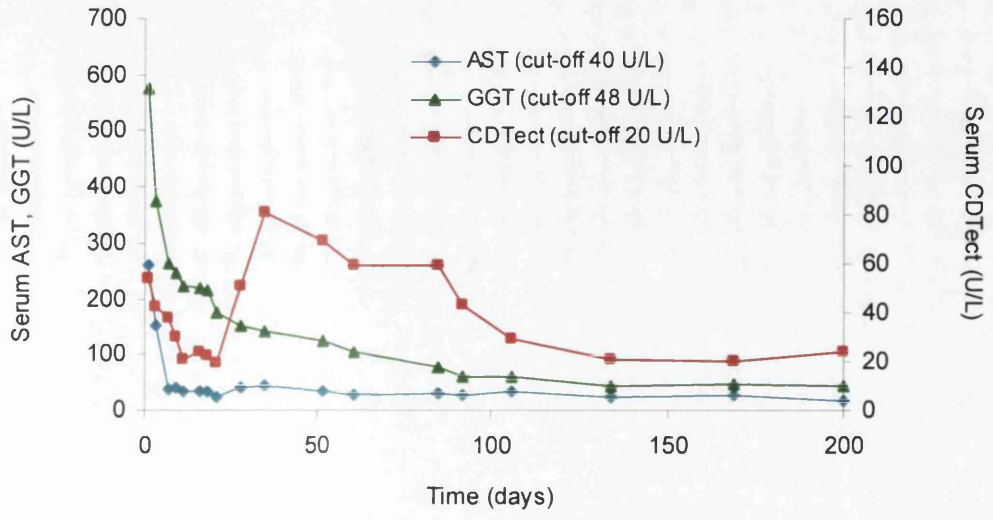
Table 7.4 shows the number of individuals in each group who were responders for all three markers in current UK practice: MCV, AST and GGT. This shows that overall 20% were responders for all three markers, with none positive for all three in the cirrhotic male group. There were only two responders for all three markers and both CDT assays, both of whom were non-cirrhotic. Of the twelve responders for AXIS %CDT, ten were also responders for CDTect.

Table 7.4: Number (%) individuals in each sub-group who were responders for MCV, AST and GGT together, and all five markers

	MCV, AST and GGT responders	All 5 markers responders
Non-cirrhotic men	4 (30.8)	1 (7.7)
Non-cirrhotic women	2 (22.2)	1 (11.1)
Cirrhotic men	0	0
Cirrhotic women	2 (28.6)	0
Total	**8 (20.0)**	**2 (5.0)**

However there may be a variation between responder performance within the individual. Figure 7.8 shows an example of the serum CDTect, GGT and AST in a non-cirrhotic man, who was drinking initially, was abstinent until day 21 when he relapsed until day 66. At the time of his relapse the CDTect rose again to reflect this, while the GGT and AST continued to fall, albeit at a slower rate. He was a responder for CDTect at lower levels than GGT, AST or MCV. In this example the AXIS %CDT had a similar pattern to the serum CDTect.

Figure 7.8: Serum AST, GGT and CDTect profiles in an non-cirrhotic man who was drinking initially and relapsed between days 21 and 66



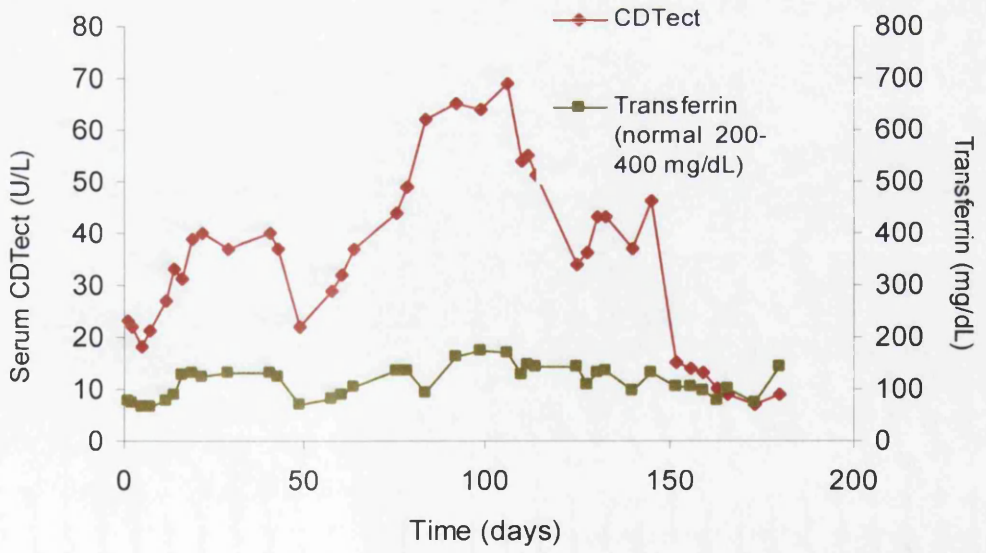
7.3.3.4 False positive serum CDT results

CDTect: six individuals remained with an elevated CDTect in abstinence, or false positive. This represents 15% of the original population. Five of these were cirrhotic. The sixth was a woman with fatty change who admitted to intermittent drinking. Her CDTect was clearly elevated throughout and it is possible she may have been drinking more consistently than intermittently.

AXIS %CDT: 17 individuals had an elevated AXIS %CDT result at day 1. Six (15% of the alcohol misusers population) remained false positive, four were cirrhotics, and two had fatty change, one of which was the same women described above.

Five of the six CDTect false positives and four of the six AXIS %CDT false positives were cirrhotic. Of particular interest was one alcoholic cirrhotic man who tested positive for both serum CDTect and AXIS %CDT (Figure 7.9).

Figure 7.9: Serum CDTect and total transferrin results in a cirrhotic man who underwent orthotopic liver transplant on day 145



He was unwell and an in-patient throughout almost the total six month period. His CDT assays both remained elevated. His condition deteriorated and he underwent orthotopic liver transplantation at Day 145, and following this both his serum CDTect and AXIS %CDT fell into the reference range. This may well have been as a result of blood transfusions given at the time of transplantation surgery, as serum CDT results have been shown to be essentially uninterpretable for six to eight weeks post surgery. His total serum transferrin was low

throughout the six months, despite nutritional supplements, a reflection of his poor protein synthetic function. His serum CDTect profile with the total transferrin levels are shown in Figure 7.9. His serum CDTect is shown, his AXIS %CDT profile followed a similar false positive pattern.

7.3.3.5 False-negative serum CDT at Day 1

CDTect: 14 of the original 40 (35%) alcohol misusers had serum CDTect levels within the reference range at day 1, or tested false negative. Seven of these were cirrhotics, seven had fatty change. Two of these individuals, with relapses, subsequently had raised or true positive serum CDTect. One cirrhotic woman developed an elevated serum CDTect although she was not drinking. All three of these were malnourished on admission, and had low serum proteins including transferrin. They all received nutritional support, either with oral supplements or enteral feeding, and protein levels, including transferrin, rose.

AXIS %CDT: 23 of the forty alcohol misusers (57.5%) had serum AXIS %CDT levels within the reference range initially. 15 were non-cirrhotics, 8 were cirrhotics. All remained negative, regardless of drinking status. Twelve of the fourteen who were false negative for CDTect were also false negative for AXIS %CDT.

Figure 7.10: Serum AST, GGT and AXIS %CDT profiles in a cirrhotic woman who was drinking initially and then abstinent thereafter

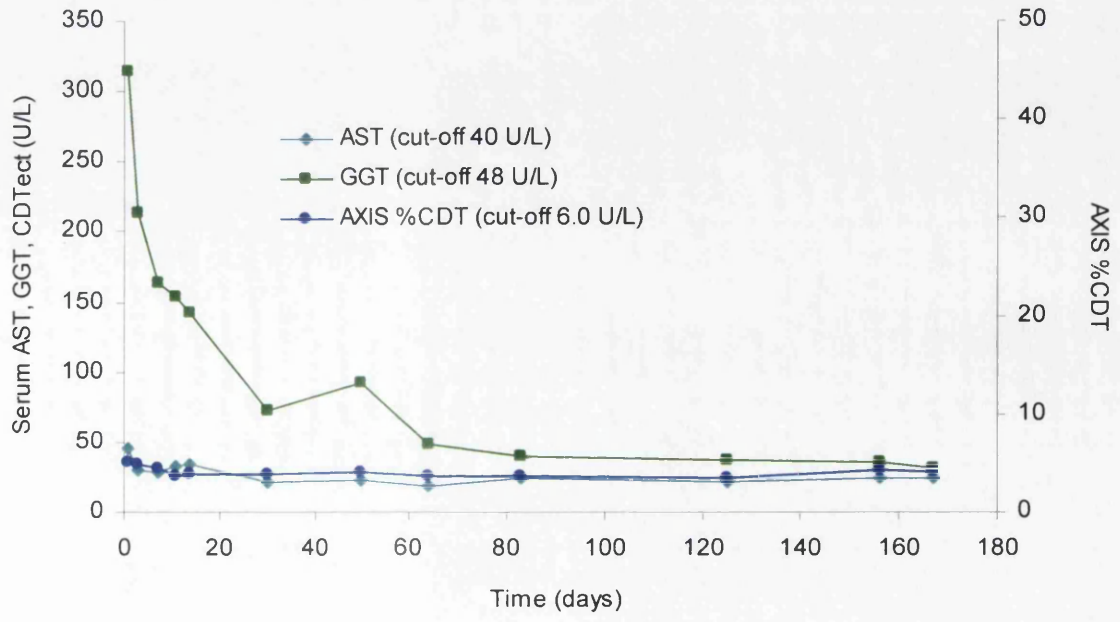


Figure 7.10 shows an example of a cirrhotic woman who was drinking at day 1 and was then abstinent thereafter. Her AXIS %CDT profile is shown, her serum CDTect profile was similar, it also remained within the reference range throughout while GGT accurately reflects the drinking behaviour.

7.3.4 SUMMARY

The control groups, the healthy volunteers and non-alcoholic liver disease groups, showed both CDTect and AXIS %CDT did not vary beyond analytical over the six month study period.

Amongst the alcohol misusers, with one exception, serum GGT was most frequently elevated during active drinking, then AST. Of the CDT assays CDTect was more frequently raised than AXIS %CDT. If subdivided by gender and histology, in the non-cirrhotic men both GGT and CDTect were raised more than MCV and AXIS %CDT, while in the non-cirrhotic women GGT was more frequently raised than CDTect or AXIS %CDT. There were no differences in the cirrhotics. For each specific marker there was no significant difference in the number of individuals with an elevated result between groups.

An individuals with a raised marker which fell during abstinence was termed a responder. GGT had the highest number of responders, but there were no differences in the number of responders if they were considered as a proportion of those elevated at day 1.

Those individuals with a persistently raised marker, despite abstinence, were considered as false-positive. 15% of the test population were false positive for CDTect and AXIS %CDT. Those individuals in whom the marker remained within the reference range, regardless of drinking behaviour, were termed false negative. Of the total alcohol misusers population 35% were false negative for CDTect and 57.5% for AXIS %CDT. However at an individual level, the markers had a variable performance and as Figure 7.8 showed the performance may be false negative or positive for a marker, but there was also one particular marker (CDTect) where the performance was particularly good.

7.4 Discussion

The variation of serum CDTect and AXIS %CDT rarely varied beyond analytical variation in healthy volunteers and in patients with non-alcoholic liver disease, and so any variation outside this was considered significant.

There was variation in marker performance in the study groups. Although GGT was elevated initially in 90.0% of cases, and AST in 82.5%, these markers may remain false positive in liver disease, particularly in cirrhotics, and may therefore be unreliable. It is the responders which are useful in monitoring and overall GGT had the highest number of responders, 65%, followed by AST (60%) and then serum CDTect (50.0%). However, the sub-groups show different markers may be useful for different groups. In the non-cirrhotic men serum CDTect was useful in 85% of cases and GGT in 77%. In the non-cirrhotic women AST and GGT appeared the most useful, although the differences were non-significant. In the cirrhotics all the markers performed less well, largely due to the false positive rate, attributable to the liver disease.

No marker can be relied upon to be reliable in 100% of individuals, and so a combination of markers provide the best monitoring tools. Furthermore it was impossible to predict at an individual level which marker was going to be most useful. All the individuals studied had a least one marker which accurately followed their drinking behaviour. In the non-cirrhotic male group a combination of serum CDTect and GGT would have accurately followed all those studied. This is in keeping to the findings of other published work (Behrens et al. 1988; Helander et al. 1996a), which have largely been in a study population of non-cirrhotic men. These authors have both suggested using a combination of serum CDTect and GGT, Behrens *et al* in particular recommending the use of GGT only if the serum CDTect is within the reference range initially. In non-cirrhotic women AST and GGT proved to be the better combination.

In the cirrhotic population any one of the markers only acted as a responder in only half of the cases. As such at least three markers should be used initially: GGT, AST and one of the CDT assays. In the male cirrhotics serum CDTect had the best performance, while in the female cirrhotics AXIS %CDT had a better performance.

One particular advantage noted by several authors was that serum CDTect levels rose before GGT (Mitchell et al. 1997; Anton et al. 1996) or before self-report (Rosman et al. 1995) in a relapse. This was seen in a number of our patients and is illustrated in Figure 7.8. Helander *et*

al (Helander and Carlsson, 1996b) noted that serum CDTect returned to the reference range sooner than GGT, this was also seen in most of the patients studied and illustrated in Figure 7.8.

Serum CDTect and AXIS %CDT have not been compared in this context previously. In the non-cirrhotic male population there were significantly more responders using CDTect than AXIS %CDT, but there was no significant difference in any of the other sub-groups. The number of individuals false positive were similar using both assays, and both were higher in the cirrhotic groups. There were more non-cirrhotic men testing false-negative using AXIS %CDT than serum CDTect, but this was not seen in any other sub-group.

If CDT was negative at baseline, there were no significant changes in CDT concentration in relation to drinking behaviour using AXIS %CDT. This was also the case with CDTect except in the presence of severe malnutrition, seen in three cases. Here there were low total serum transferrin levels (69-80 mg/dL, normal range 200-400 mg/dL) and so it is possible that, even in the presence of active drinking, the small amount of transferrin produced contained very small amounts of desialylated isoforms. Once a better nutritional status has resulted in increased transferrin production, a higher concentration of desialylated isoforms may be produced in response to alcohol misusers, seen in two individuals who relapsed. Both of these individuals were false negative for AXIS %CDT throughout, so that even if the desialylated isoforms were expressed as a proportion of total transferrin, there were still insufficient desialylated isoforms to accurately reflect drinking behaviour.

In those who were false positive for CDTect, five were cirrhotic and for AXIS %CDT of the six false positive, four were cirrhotic. It is possible that these individuals may have remained false positive because of poor clearance of the desialylated isoforms by the cirrhotic liver. The false positive levels were seen consistently over six months and it is more likely that the cirrhotic liver was only able to produce desialylated isoforms. The cirrhotic man who was false positive for both CDTect and AXIS %CDT was immediately true negative following orthotopic liver transplantation. This has been seen in another series of cirrhotic alcohol misusers undergoing orthotopic liver transplantation (Heinemann et al. 1998). His total transferrin was still low, but it is possible that his new non-cirrhotic liver was able to both clear the abnormal desialylated isoforms and produce normal transferrin, albeit in small amounts.

In summary, in monitoring alcohol misusers several markers should be used. Serum CDT may be useful in conjunction with other markers in current use, the best marker must be selected on an individual basis. The presence of significant liver disease and malnutrition may alter performance.

8. ISOFORM PROFILING OF SERUM USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

There was an unacceptably high false positive rate for CDT in the study groups, using either of the two commercial methods, described in previous chapters. Each of the methods provides an estimate of CDT that is the sum of the CDT isoforms; neither provides details of the relative abundance of individual isoforms. One of the reasons for the high false positive rate is a shift in the isoform profile leading to enrichment of individual isoforms that are not representative of the pattern obtained from true positive sera. Therefore, it was decided to use HPLC to determine the CDT isoform profiles from selected sera in order to test this hypothesis.

High pressure, or high performance, liquid chromatography is ideally suited for the separation of a range of biochemicals including carbohydrates. The stationary phase, either a solid surface, a liquid, anion exchange resin or a porous polymer, is usually held in a metal column that can tolerate the relatively high pressures used during the process. The liquid mobile phase is forced through the column under high pressure.

HPLC has been used as one of the principle research techniques (Jeppsson et al. 1993; Bean et al. 1997; Sillanaukee et al. 1994; Heggli et al. 1996; Martensson et al. 1997; Werle et al. 1997) for identifying individual human transferrin isoforms in serum, although its use has been limited since the method is laborious, time consuming and technically demanding.

Anion exchange chromatography has emerged as the most reliable stationary phase in the HPLC method. The materials and the methodology were adapted from those described previously by Bean *et al* (Bean et al. 1997) and also AXIS Biochemicals Ltd (Personal Communication).

In any assay procedure for any biological macromolecules including HPLC, it is important for accurate quantitation that external standards are included. To this end preparations of individual isoforms are ideal. However these are neither commercially available nor were Pharmacia and Upjohn or AXIS Biochemicals able to provide them for this purpose. They were therefore obtained from another source. The first part of this section (Chapter 8.2 Section I) deals with validation of individual transferrin isoforms, methods for their quantitation and their potential usefulness as calibrators within the HPLC system. The second section (chapter

8.2 Section II) deals with HPLC techniques and the use for detection and quantitation of transferrin isoforms.

8.1 Section I: Purified transferrin isoforms as calibrators: validation and quantitation studies

Samples of purified human transferrin (asialo-, monosialo-, disialo-, trisialo-, tetrasialo- and pentasialo-transferrin) isoforms were kindly provided by Professor Van Eijk of Marasmus University, Rotterdam. They had been isolated and purified by isoelectric focusing alone and were supplied as lyophilised powders with stated protein concentrations.

8.1.1 RECONSTITUTION OF LYOPHILISED ISOFORMS AND PROTEIN DETERMINATION

10mM Bis Tris buffer, pH 7.5,was added to each vial of isoform to bring the approximate protein concentration to 1mg/ml. The protein concentration was then determined for each solution of isoform using a modified Lowry technique, adapted for 96-well plates and using reagents from a Protein Assay Kit. Nunc Maxisorp Immuno-strips were assembled in a frame to make a 96-well plate. A protein standard curve was incorporated into the plate, against which the protein concentrations of the samples could be interpolated. The reference protein was bovine serum albumin (BSA) with a starting concentration of 400μg/ml. Triplicate blank wells were prepared by dispensing, from a Multichannel pipette, 100μl of distilled water into each of three wells A1..A3. BSA at 400μg/ml was dispensed into three wells (Figure 8.1).

Nine serial doubling dilutions were prepared of the BSA. Into each of the wells C1..H6 were dispensed 100μl of distilled water. Using a Multichannel pipette, 100 μl of the BSA in wells B1..B3 were drawn up and mixed with the distilled water in wells C1..C3 to provide the first doubling dilution. Successive dilutions were made in a similar manner by withdrawing diluted BSA and mixing to wells C4..C6. Into all wells, including the blank wells, were dispensed 100μl of Modified Lowry reagent and the plate incubated at room temperature with constant shaking on a plate mixer. 50μl of Folin and Ciocalteau's Reagent was added and the plate replaced on the shaker for 5 minutes during which time full colour development of a purple blue was seen in protein containing wells. The blank wells were colourless. The plate was read on a plate reader set to a wavelength of 720nm to determine the optical densities of the

coloured reaction product, the intensity of which was directly proportional to the concentration of protein in the sample.

Figure 8.1: Protein determination of isoform standards, plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank Wells			BSA 3.125 µg/ml			Trisialo, 1/10					
B	BSA 400µg/ml			BSA 1.56 µg/ml			Tetrasialo, 1/5					
C	BSA 200µg/ml			BSA 0.78 µg/ml			Tetrasialo, 1/10					
D	BSA 100 µg/ml			Asialo, 1/5			Pentasialo, 1/5					
E	BSA 50 µg/ml			Asialo, 1/10			Pentasialo, 1/10					
F	BSA 25 µg/ml			Monosialo, 1/5								
G	BSA 12.5 µg/ml			Monosialo, 1/10								
H	BSA 6.25 µg/ml			Trisialo, 1/5								

The average of the 3 blank wells was subtracted from each reading. The dose response curve for the BSA was constructed. From this curve, the protein concentrations of the samples were interpolated and found to be 40 µg/ml. All isoforms were further diluted to 100µg/ml by the addition of bis-tris buffer and stored deep-frozen in aliquots of 200µl (total protein = 40µg/aliquot) and stored at –70°C before use.

8.1.2 Isoelectric Focussing of Purified Transferrin Isoform Standards

Because the van Eijk transferrin isoform standards had been isolated and purified by isoelectric focusing, this technique was used to confirm that they did indeed form a series of protein isoforms that were distinguishable based on charge (pI). The isoforms were applied to a polyacrylamide isoelectric focussing gel, with ampholytes providing a pH gradient of 4.0 – 6.5. A sample of the AXIS%CDT calibrating standard that was stated to contain a mix of mono-, di- and tri-sialo transferrin isoforms was also run on the gel as both a comparison and putative positive control.

8.1.2.1 Materials and methods

Samples, at protein concentrations of 100μg/ml were thawed. A mixture of isoelectric focusing marker proteins (Table 8.1) was also prepared to provide the calibration proteins on the gel for determining the standard curve against which the pI values for the isoforms could be calculated.

Table 8.1: Isoelectric focusing of pI Markers

Sample	pI Value
Anode pH	4.0
Glucose Oxidase	4.2
Trypsin Inhibitor	4.6
β-Lactoglobulin A	5.1
Carbonic Anhydrase II	5.4
Carbonic Anhydrase II	5.9
Cathode pH	6.5

A 6.5% acrylamide gel was prepared on a standard Gel-bond PAG film using a 25% stock solution of acrylamide solution and ampholines to a pH range of 4.0 – 6.5.

Running Conditions. The gel was run in a cooling bath at 8^0C. A Multi-Temp cooling bath was set to run at a temperature of 8°C. Anode and cathode buffer strips were applied. 5μl of each sample was applied to individual lanes. The isoelectric focusing glass plate was placed on top, aligning the anode and cathode with their respective wicks and the gel was run for 180minutes.

After electrophoresis proteins were fixed by immersing the gel in a fixative solution (125g/L trichloroacetic acid, 40g/L sulphosalicylic acid, in distilled water) for 30 minutes followed by a rinse of the gel in destain solution (7% v/v glacial acetic acid, 12% v/v absolute ethanol, 5g/L cupric sulphate) for 10 minutes. The proteins were then stained with Coomassie Blue by immersing the gel in a bath of stain solution (0.4g/L Coomassie Blue, 10% v/v glacial acetic acid, 27% v/v absolute ethanol, 5g/L cupric sulphate) and leaving the gel to stain overnight. The next day, the whole gel was dark blue and a discriminating de-stain procedure was

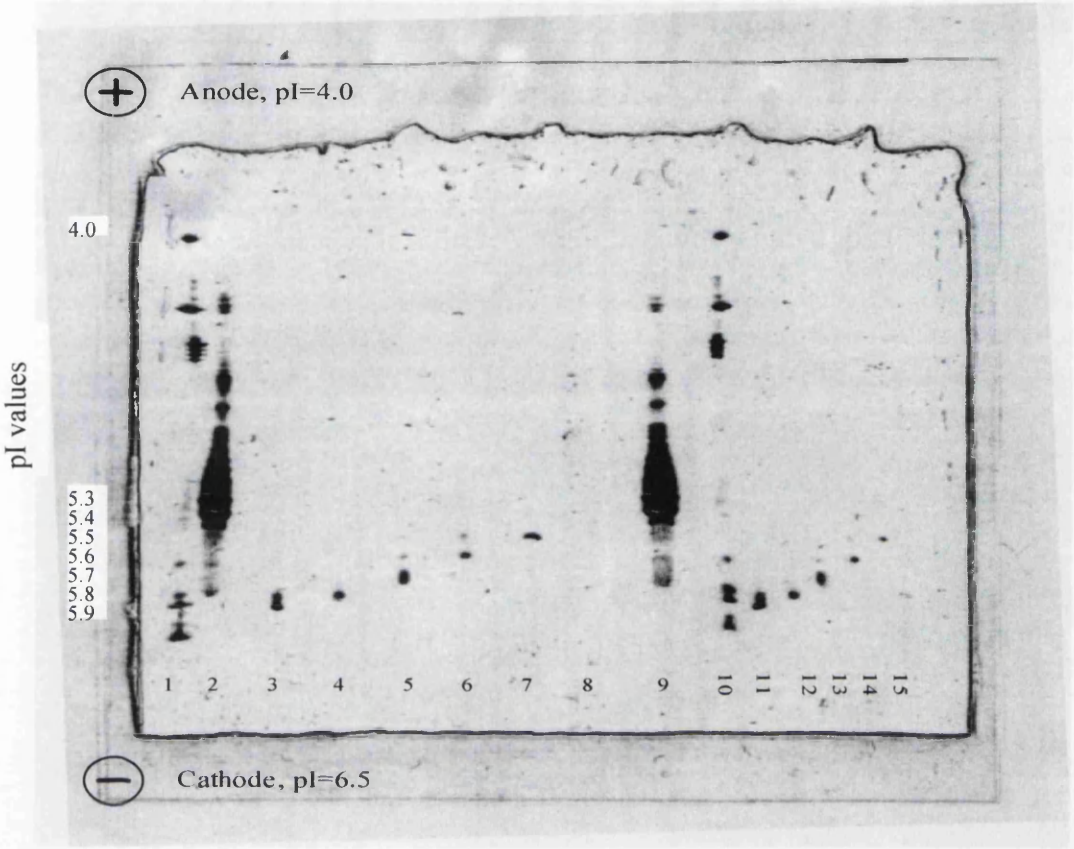
followed. At the end of this period, the individual blue protein bands were visualised against a transparent and colourless gel background.

The positions of the anode and cathode leading edges were identified on the gel as reference points against which migration distances (Rf) would be determined. This was performed by placing the gel on a BioRad G5700 gel scanner that gave high-precision X,Y co-ordinates of the protein bands on the gel. Rf values were measured in millimetres (to 3 decimal places) from the leading edge of the anode. The middle point of each protein band was taken as the mean point for determining the Rf. A standard curve was constructed using the Rf values for each of the pI markers plotted against their respective pI values and linear regression analysis performed to determine the equation that could be used to interpolate the pI values for the sample Rf values.

8.1.2.2 Results

There was sequential separation of the isoform standards (Figure 8.2). However, a sample of the calibrator standards that are a part of the AXIS%CDT kit and should be enriched for mono-, di- and tri-sialotransferrin isoforms had no bands in the pH range where the van Eijk standards appeared. A number of other bands at a more acidic pI (<5.0) values were seen in the AXIS calibrator standard sample. A standard curve was constructed from the pI markers and the pI values of the standards were read from it (Table 8.2).

Figure 8.2: Isoelectric Focusing Gel of Transferrin Isoforms



Key to lane contents:

1 & 10 = pI Markers, 2 & 9 = AXIS%CDT Calibrator, 3 & 11 = Asialotransferrin, 4 & 12 = Monosialotransferrin, 5 & 13 = Disialotransferrin, 6 & 14 = Trisialotransferrin, 7 & 15 = Tetrasialotransferrin, 8 = Pentasialotransferrin.

Table 8.2: Isoelectric Focusing of Transferrin Isoforms Standards

Transferrin Isoform Sample (number of sialic acid residues)	Distance From Anode (mm)	Calculated pI	Reference Value pI (Stibler, 1978)
Asialotransferrin	82.01	5.779 **(5.8)**	5.9
Monosialotransferrin	79.95	5.721 **(5.7)**	5.8
Disialotransferrin	76.90	5.634 **(5.6)**	5.7
Trisialotransferrin	72.82	5.520 **(5.5)**	5.6
Tetrasialotransferrin	69.38	5.422 **(5.4)**	5.4
Pentasialotransferrin	67.07	5.357 **(5.4)**	5.3

8.1.2.3 Conclusion

It was confirmed that the isoform standards had the necessary IEF properties that had been described previously to be associated with transferrin isoforms. Professor van Eijk had described (personal communication) that the isoforms had been prepared from a purified preparation of human transferrin and that the implication was that what he had supplied were, indeed, isoforms of human transferrin.

8.1.3 Immunoassays for detection and quantitation of Carbohydrate-Deficient Transferrin Isoforms

It was proposed to use HPLC to detect the various transferrin isoforms present in serum samples, and this is described in Section II. During these experiments it was required to confirm the presence of transferrin in the eluate of serum samples so that signals, or peaks, obtained during chromatography could be confirmed to be from transferrin. The following experiments related to the confirmation of transferrin at the low concentration found in the HPLC eluate.

8.1.3.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA is an established, sensitive technique for providing qualitative and at least semi-quantitative estimates of molecules in biological fluids. A literature search revealed that it had been used previously (Saitoh et al. 1995; Cochran and Sherman, 1994; Yamashita et al. 1995) as a quantitative method for detecting human transferrin in blood samples. Because there was no commercial kit that was readily available, attempts were made to develop an in-house assay for the purposes of the present study. A competitive inhibition assay was chosen as this was felt to provide the optimum stringency for specificity and sensitivity. The reagents that were used derived mostly from commercial sources used in the Cobra Miras immunoturbidimetry study (see below) and the general approach to the methodology was adapted from other previously published ELISAs.

Availability of suitable reagents meant that the technique could not be developed to a useful state for the present experiments, with particular respect to sensitivity. Therefore, for clarity and completeness, full details of the methodologies used to develop the assay and the results that were obtained are not presented in the present section, but are described in Appendix I.

8.1.3.2 Immunoturbidimetry (Cobas Mira)

Immunoturbidimetry was used to measure transferrin in the AXIS %CDT determination (Chapter 4.2.3) and also to measure total transferrin (Chapter 4.2.4). The technique used the Cobas Mira (Figure 4.5). Using a commercial assay for human transferrin (DAKO) the Cobas Mira was set up to perform this. The kit contained:

- Antibody: rabbit anti-human transferrin at an antibody titre of 2,600 mg/L
- Human serum protein calibrator
- Human serum protein low control
- Human serum protein high control
- Dilution buffer
- Reaction buffer 5% PEG

The Cobas Mira was programmed according to the manufacturer's instructions. 50 µl of sample or control was diluted with 200 µl dilution buffer. This accurately determined the presence of transferrin within the range seen in human sera (2.0-4.0 g/L), but could not accurately determine transferrin at lower concentrations than this, despite altering the

concentration of the transferrin standards. The transferrin potentially present within the eluate would have been diluted first with the reagents during the sample preparation and then by the buffers. The final concentration of transferrin was calculated to be in the range of 10 μg/ml. The calibration and control samples provided in the kit were diluted to this range, but the assay was unable to detect low concentration of transferrin. This method could not be used to confirm transferrin in the eluate.

8.1.3.3 Immunoturbidimetry, (96-well, Semi-micro)

The immunoturbidimetric assay suggested by AXIS Biochemical (Bean et al. 1997) which is the basis of the COBAS Mira machine outlined above, was adapted for use in a 96 well microtitre plate.

The technique relies on the admixture of transferrin samples and an antibody in the wells of a microtitre plate. The concentration of resultant immune complexes can be determined by optical densities of the well contents and interpolating these against the optical densities from a standard curve of known concentrations of transferrin-anti transferrin immune complexes. Because the technique was developed *de novo*, it was important to calibrate and validate each component to optimise its performance as part of validating the HPLC-derived putative transferrin isoforms.

Reagents

Initial experiments utilised Calibrator Transferrin at a concentration of 27μg/ml as reference antigen. Later experiments used human serum transferrin dissolved in dilution buffer at a concentration of 32mg/L. Aliquots of this material were used throughout as the preparation for constructing a standard curve in each plate. Anti-human transferrin (Rabbit polyclonal antibody) to promote the formation of soluble immune complexes.

Method

The volumes of antigen (transferrin standard, HPLC-derived fractions) and antibody were used were arbitrarily pre-determined. 100μl of antigen were dispensed into wells followed by 100μl of antibody (total volume=200μl). Blank wells contained 100μl PBS and 100μl of antibody.

The plate was incubated at room temperature for defined periods and the optical densities of each well determined by reading all wells in a 96-well plate reader set at a wavelength of 340nm. Once the assay had been optimised (see below), each plate was constructed so that it contained transferrin diluted serially that functioned as the standard curve, against which unknown transferrin concentrations could be interpolated.

8.1.3.4 Optimisation Studies.

1. *Transferrin and Rabbit Anti-transferrin Antibody Titration*: Serial two-fold dilutions of rabbit anti-transferrin antibody were prepared in reaction buffer covering the range 1/20 to 1/640. The antibody dilutions formed a group that would be added across the plate. Human transferrin (DAKO calibrator transferrin) was used as the antigen, at a starting concentration of 27μg/ml. Serial three-fold dilutions of the antigen were prepared down the plate (Figure 8.3).

Figure 8.3: Antibody and antigen titration: plate layout

Antibody Titration →

Antigen Titration ↓

	RaTf, 1/20		RaTf, 1/40		RaTf, 1/80		RaTf, 1/160		RaTf, 1/320		RaTf, 1/640	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank		Blank		Blank		Blank		Blank		Blank	
B	Tf, 27μg/ml		Tf, 27μg/ml		Tf, 27μg/ml		Tf, 27μg/ml		Tf, 27μg/ml		Tf, 27μg/ml	
C	Tf, 9μg/ml		Tf, 9μg/ml		Tf, 9μg/ml		Tf, 9μg/ml		Tf, 9μg/ml		Tf, 9μg/ml	
D	Tf 3μg/ml		Tf 3μg/ml		Tf 3μg/ml		Tf 3μg/ml		Tf 3μg/ml		Tf 3μg/ml	
E	Tf 1μg/ml		Tf 1μg/ml		Tf 1μg/ml		Tf 1μg/ml		Tf 1μg/ml		Tf 1μg/ml	
F	Tf 0.3μg/ml		Tf 0.3μg/ml		Tf 0.3μg/ml		Tf 0.3μg/ml		Tf 0.3μg/ml		Tf 0.3μg/ml	
G	Tf 0.1μg/ml		Tf 0.1μg/ml		Tf 0.1μg/ml		Tf 0.1μg/ml		Tf 0.1μg/ml		Tf 0.1μg/ml	
H	Tf 0.03ug/ml		Tf 0.03ug/ml		Tf 0.03ug/ml		Tf 0.03ug/ml		f 0.03ug/ml		Tf 0.03ug/ml	

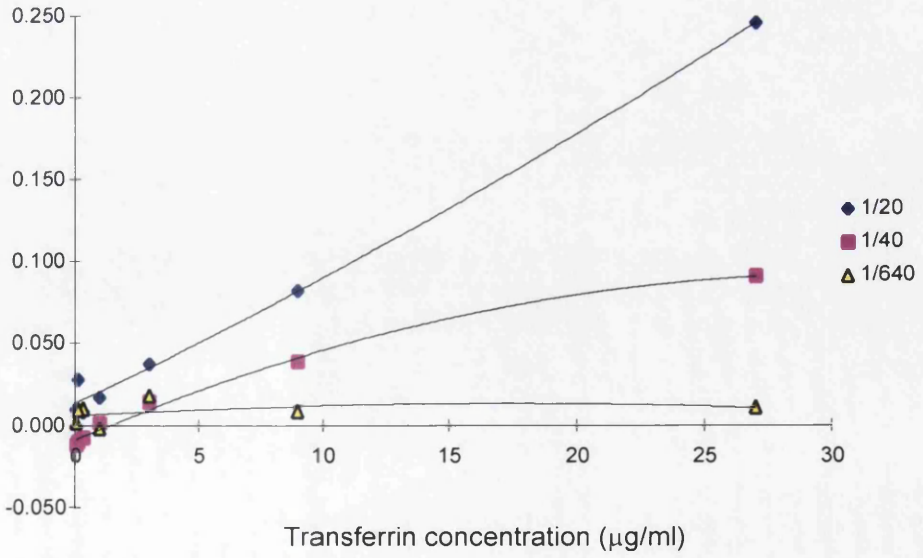
150μl of the top concentration of transferrin were dispensed into all wells of the second row (B1..B12) of the plate. Serial dilutions were made using PBS diluent, starting with row C and using three-fold dilutions in each subsequent row. The transferrin concentrations therefore ranged from 27μg/ml to 0.03μg/ml. Into wells A1..H2 were dispensed 100μl of the rabbit anti transferrin antibody (RaTf) at a dilution of 1/20. The

remaining dilutions of the RaTf were dispensed to wells A3..H4 (RaTf, 1/40) until the most dilute antibody preparation, 1/640 was dispensed to all wells of A11..H12.

The plate was incubated on a plate shaker for 10 minutes and optical densities determined by scanning with a plate reader using a 340nm filter. The average of each of the two blank wells for each antibody dilution was subtracted from each of the wells.

The individual dose-response curves for each antibody dilution were constructed (Figure 8.4), where it can be seen that the antibody dilutions 1/20 and 1/40 resulted in the development of immune-complexes with the range of transferrin concentrations that were used. At antibody dilution of ≥ 1/80, the dose-response curves were too flat to be of any use and it was decided to use an antibody concentration of 1/20 for further optimisation experiments. The dynamic range of transferrin detection from this preliminary experiment was determined to be approximately 1-50μg/ml and should cover the range of naturally occurring serum transferrin levels following HPLC.

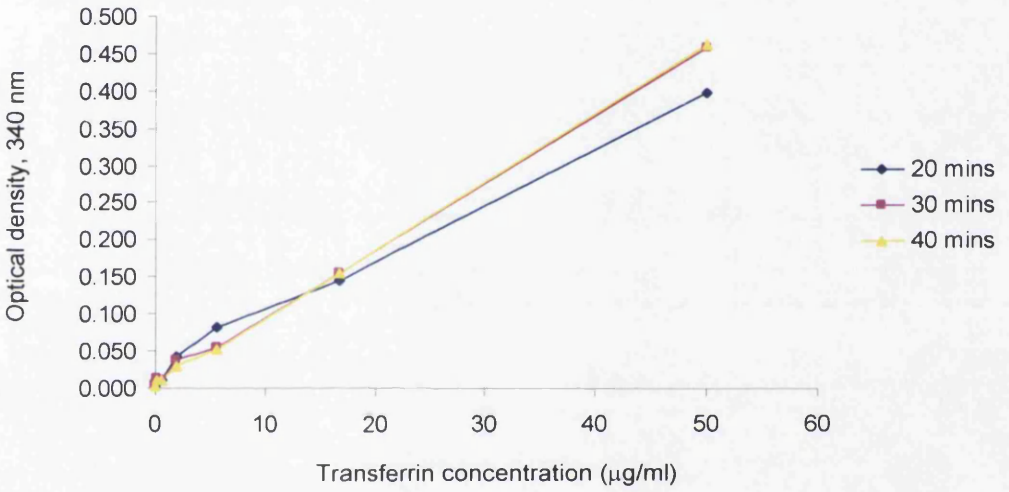
Figure 8.4: Dose response relationship between antibody dilution and antigen concentration in immunoturbidimetric assay for transferrin



2. *Optimisation of Incubation Time For Immune Complex Formation*: Using the optimum dilution of RaTf of 1/20, the optimum time of incubation necessary for immune complex formation and therefore maximum signal was determined. These experiments were undertaken using triplicate wells and Sigma stock transferrin. Serial three-fold dilutions of

human serum, initially pre-diluted to 50μg/ml were prepared in a 96-well plate as before. The range of concentrations covered was 0.069 – 50 μg/ml, with dilutions of transferrin being made as before. RaTf, diluted to 1/20 in Reaction Buffer was added to all wells and the plate incubated on a shaker at room temperature as before. The plate was read at 340nm at times 20, 30 and 40 minutes later and the dose response curves constructed (Figure 8.5) after the average OD values for the 3 blank wells subtracted.

Figure 8.5: Effect of Incubation Time on Immune Complex Formation



The maximum optical densities were reached after 30 to 40 minutes incubation. There was no advantage in prolonging the incubation time beyond 40 minutes. The sensitivity of the assay was again about 1μg/ml.

Further experiments (data not shown) included examination of effects of immune complex formation where the antibody had been diluted in PBS or Reaction Buffer. It was determined that Reaction Buffer was, indeed, necessary for maximum immune complex formation within the constraints of previously optimised parameters.

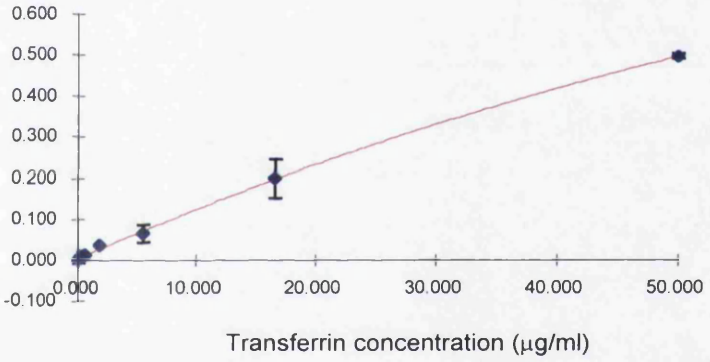
Following these optimisation experiments, the standard immunoturbidimetric assay comprised the following steps:

- Standard curve constructed from human transferrin, 10 serial three-fold dilutions with the highest concentration being 50μg/ml
- 100μl of antigen dispensed to wells in triplicate.

- 100 µl of Rabbit anti-transferrin antibody, diluted to 1/20 in Reaction Buffer added to all wells.
- Plate incubated for 40 minutes.
- Plate read at 340nm

Repeat experiments were performed to establish the reproducibility and sensitivity of the assay. The pooled results of these experiments are illustrated in Figure 8.6. The line of best fit of the standard curve was not linear, but corresponded to a second order polynomial (r^2= 0.992) and this was applied in all subsequent experiments for interpolation of unknown transferrins. The dynamic range of the assay was 1µg/ml to 50µg/ml of transferrin and the coefficient of variation (CV) of the assay was 2.6%.

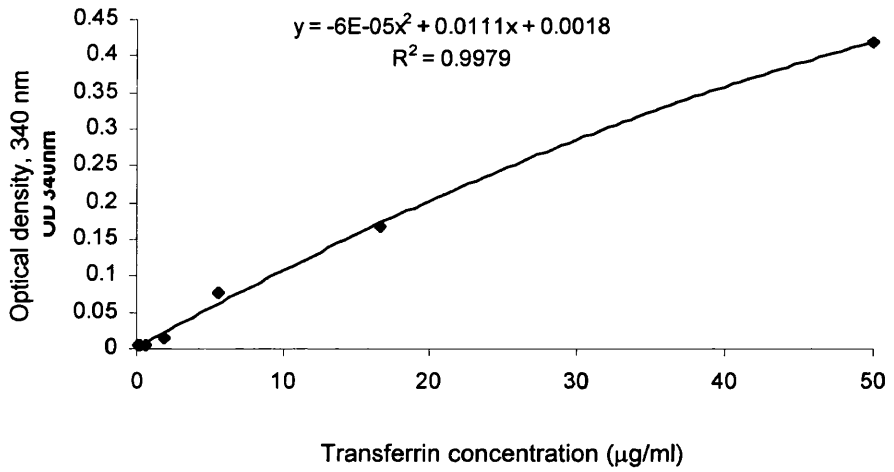
Figure 8.6: Standard Curve of Immunoturbidimetric Analysis for Human Transferrin.



8.1.3.5 Immunoturbidimetric Analysis of van Eijk Standards

One of each of the pure isoform standards was thawed at room temperature and the contents of each vial analysed using the optimised immunoturbidimetric assay described above. Transferrin was confirmed to be present in each sample and the concentrations of each estimated by interpolation form the standard curve (Figure 8.7)

Figure 8.7: Standard Curve for Estimation of Tf Isoform Standards



The polynomial curve fit equation was used to calculate the transferrin concentrations of each isoform and the results obtained are detailed in Table 8.3.

Table 8.3: Calculated Concentrations of Isoform Standards Based on immunoturbidimetric Assay

Transferrin Isoform	Estimated Concentration from immunoturbidimetric assay (µg/ml)	Estimated Concentration from Protein Assay (µg/ml)
Asialotransferrin	17.06	40
Monosialotransferrin	55.69	40
Disialotransferrin	43.85	40
Trisialotransferrin	43.43	40
Tetrasialotransferrin	28.07	40
Pentasialotransferrin	19.34	40

These results, coupled with those obtained from the isoelectric focusing experiments detailed earlier, provide convincing evidence that the van Eijk isoform standards were indeed transferrin isoforms and should be capable of use as calibration standards within HPLC.

8.2 Section II: HPLC techniques and their use for detection and quantitation of transferrin isoforms

8.2.1 MATERIALS AND METHODS

8.2.1.1 Equipment

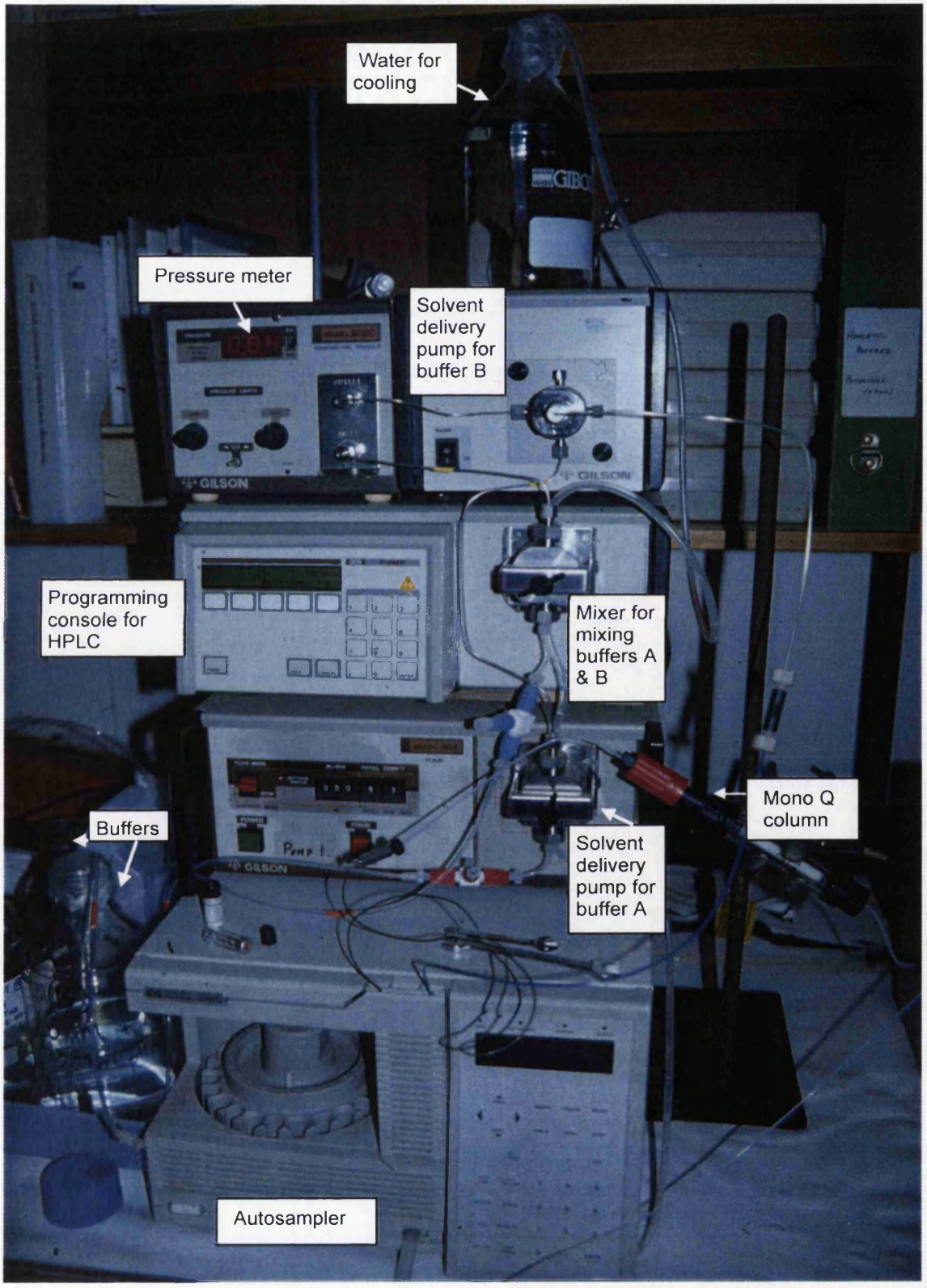
The apparatus consisted of (Figure 8.8):

- A Mono Q HR 5/5 column
- Two solvent delivery pumps
- 811C dynamic mixer
- Pressure meter
- 1050 autosampler
- Syringe loading injection valve with a 100 µl loop
- Ultraviolet Spectrophotometer
- On-line data-acquisition integrator
- Personal Computer
- Hewlett Packard Chemstation software, v3.2a

8.2.1.2 Mono Q Column

A 10ml (10cm x 1cm) column pre-packed with MonoQ anion exchange resin was used. The mono Q beads, with a mean particle size of 10µm, are in the form of a beaded hydrophilic resin (gel) and the protein capacity is 20-50 mg/column. The gel, pre-packed in a glass column, was delivered in a packing solution of 20% ethanol-water (v/v) with sulphate as the counter ion and was washed and equilibrated before use. The packing solution was flushed out with 10mls low ionic strength start buffer comprising 10mmol/L Bis-Tris buffer pH 6.20. Following this a further 10mls of 20mmol Bis Tris was flushed through and then 15mls of Buffer B (20mmol/L Bis Tris pH 5.60 with 300mmol/L NaCl) as the counter-ion. Finally the equilibration was completed with 5mls of Buffer A (20mmol/L Bis Tris pH 6.2). The column was then connected in line with the HPLC circuit (Figure 8.8).

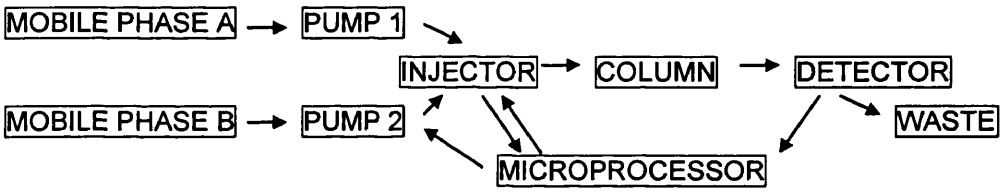
Figure 8.8: HPLC circuit set up for autosampler delivery



8.2.1.3 HPLC system

The column was connected to two peristaltic pumps for establishment of continuous buffer gradients. A 100µl injector was employed in-line for manual injection of samples to the column. Alternatively the autosampler was used, 100µl volumes from sample ampoules were withdrawn and injected automatically. The autosampler had a capacity of 22 ampoules containing prepared samples. Eluates from the column were monitored by spectrophotometry. For iron-saturated transferrin, the maximum absorption of light occurs at 465nm, the iron component of transferrin largely being responsible this wavelength being chosen.

Figure 8.9: Flowchart of HPLC Circuit



The establishment of gradients, run times and other chromatography conditions were under the control of an electronic data analyser and acquisition system, the Hewlett-Packard ChemStation. This was linked to a personal computer and the operator interface was through a Windows v3.1 programme supplied by Hewlett-Packard, ChemStation v 3.3. Real-time tracking of the chromatogram development meant that problems associated with each run could be identified. Output from the spectrophotometer, coupled with the time signals generated by programming the software, enabled HPLC profiles to be stored in computer data files for later reference.

8.2.2 REAGENTS

8.2.2.1 Water

Reverse osmosis water (SQ, Super Quality) was routinely used throughout these experiments instead of distilled water. It was prepared in bulk in a unit that involved a pre-filter unit that was connected to the mains water supply. This stage removed chlorine and particulates. From there, the water went into a reverse osmosis unit and then into a reservoir that contained an

internal mixing pump to prevent stagnation and layering of the water. Water was fed on demand into an ion-exchange unit that de-ionised the stored pure water and provided an extra filtration step to remove particulate material. From this unit the water passed through a carbon filter as a final filtration stage and was collected for use at this stage. The water was constantly monitored for purity and had an acceptable resistance of >20 megaOhm/cm. It is referred to as SQ water.

8.2.2.2 Buffers

In ion exchange chromatography there are variables that can be altered to achieve satisfactory separation between two components: alteration of the ionic strength of the buffers and alteration of the pH of the buffers.

The ionic strength of the buffer solution, or eluant, is responsible, in part, for the elution of charged molecules bound to the column during ion-exchange chromatography. Increase in ionic strength results in increased elution of charged molecules.

Variation in the pH of the buffer results in changes in retention times. This is true over a limited pH range and depends on the acids or bases being chromatographed as well as on the degree of ionisation of the ion exchange groups.

For the present studies a two component (buffer) gradient was established where the principle variables were pH and salt concentration (ionic strength).

Mobile Phase A

This was 20 mmol bis-tris at pH 6.20. 4.18 g Bis Tris was dissolved in approximately 900 mls SQ water and the pH adjusted to 6.20 with 1M HCl. The volume was brought to one litre with SQ water and the pH was re-checked. The buffer was filtered using 0.45μm Millipore filtration to remove impurities. It was then de-gassed, using ultrasound, in a water bath for 15 minutes to prevent possible bubble formation in either the circuit or the column.

Mobile Phase B

This was 20 mmol Bis-Tris at pH 5.60 containing sodium chloride. 4.18g BisTris was dissolved in approximately 900 mls SQ water and NaCl was added at optimum concentration (see below) to provide the elution salt gradient. The pH was adjusted to 5.60 with 1M HCl and

SQ water was added to bring the volume to one litre and the pH re-checked. It was Millipore filtered and de-gassed, as described for Mobile phase A.

8.2.3 SAMPLE PREPARATION

8.2.3.1 Iron Solution to Saturate Transferrin-associated Iron

Each sample was iron saturated so that the transferrin was diferric and any change in pI would reflect an alteration in sialic acid content. The solution used for the iron saturation was made up as a stock x 10 concentrated preparation, frozen in aliquots, and diluted before use.

Each of the components was dissolved in 10 mls of SQ water to make a x10 concentrated solution. This was divided into 5 x 2ml aliquots that were stored frozen at -20^{0}C until use. When iron saturation was to be performed, aliquots of each solution were thawed, pooled and diluted to about 100mls with SQ water and filtered. Tween 20 was added to a final concentration of 0.5 mls per litre (v/v) and the final volume brought to 200 mls with SQ water. Each constituent component was weighed out as follows:

	Molecular Weight	Molarity required	Weight (mg) needed for 100ml of a x10 concentrated solution
Bis Tris	209.24	10 mmol/L	2092.4
Tris	121.14	0.8 mmol/L	96.9
Ferric chloride	270.3	0.15 mmol/L	40.5
Sodium citrate	294.1	0.15 mmol/L	44.1
Maleic acid	116.1	0.4 mmol/L	46.4
Sodium azide	65.01	3.1 mmol/L	201.5

8.2.3.2 Sample preparation stages

These stages are shown in Figure 8.10. Serum samples (1ml) were thawed at room temperature and then iron saturated using the iron solution. 30 µl of iron-solution was added to 150 µl of human serum and was left to incubate for 30 minutes at room temperature. 1.6µl of dextran sulphate (100 g/L in water) was added in addition to 7.5µl calcium chloride (147g/L). This step resulted in the precipitation of lipoproteins. The mixture was vortexed for a few seconds.

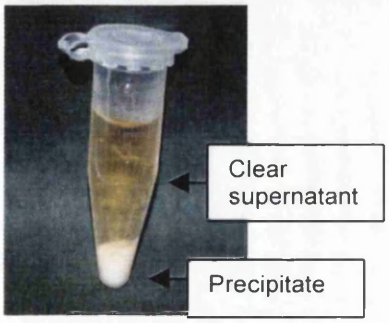
Figure 8.10: The stages of sample preparation

1. Serum thawed at room temperature

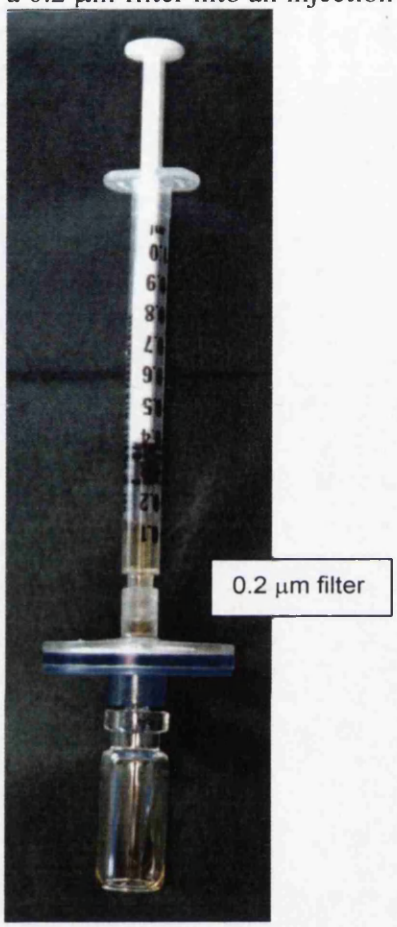
2. 30 µl iron solution added and incubated at room temperature for 30 minutes

3. 1.6 µl dextransulphate and 7.5 µl calcium chloride added and then refrigerated for 1 hour

4. The sample was centrifuged at 5,000g for 15 minutes



5. The clear supernatant was filtered using a 0.2 µm filter into an injection ampoule



6. The final prepared solution in an injection ampoule

It was incubated at 4^0C for 1 hour and centrifuged at 1500g for 15 minutes. The clarified supernatant was decanted, 1.0 ml SQ water added (1:2 dilution of the serum) and the solution filtered through a 0.22μ cellulose filter to remove all remaining particulate matter that might interfere with the column and subsequent chromatography. Depending on the nature of the experiment, either 100μl of sample was then applied to the column using the manual injector, or approximately 300μl sample was dispensed to an ampoule that was then sealed, labelled and added to the autosampler for batch processing.

8.2.4 HPLC METHOD: INITIAL PRE-OPTIMISED STANDARD CYCLE

8.2.4.1 Priming of Column Before Use

Before each run, the column was washed with 10 mls SQ water that had been filtered and de-gassed. The column was then equilibrated with one cycle, including gradient, of the buffers but without serum sample added.

8.2.4.2 Pressure

Solvents were pumped through the system at a mean pressure of 0.65 bar.

8.2.4.3 Eluant Monitoring

Eluant from the column passed through an in-line spectrophotometer set to read absorbance at 465nm, enabling real-time analysis of iron associated protein concentration. The results from the detector were electronically acquired, analysed and displayed using the HP ChemStation.

8.2.4.4 Running Conditions

Preliminary experiments were performed using methods and protocols that were supplied by AXIS Biochemicals (Personal communication) and further detail obtained from the published method of Bean et al. (Bean et al. 1997) that was similar to the AXIS method. Iron saturated samples in Mobile A buffer were applied to the column as 100μl injections from a Hamilton syringe. The pumps were programmed to run as follows:

Initially the solvent was 100% mobile A at 2mls/min for five minutes, during which time the sample flowed through the column and proteins could be adsorbed to the gel. Over the next 30 min Mobile B, containing 0.334M (17.5g/L) NaCl, was introduced so that by 35 min the solvent comprised 75% Mobile A, 25% Mobile B. During this time the gradient and pH change provided the conditions for elution of bound proteins in an isoelectric point related order of elution. The column was then washed with 100% Mobile B for 10 min to elute all remaining proteins, then regenerated with a five minute wash with 100% Mobile A. The total flow rate was 2 mls/min at all stages.

The eluant passed through a spectrophotometer set to read absorbencies at 465 nm, this being the peak absorption of transferrin saturated with ferric iron.

At the end of each day, the system was flushed with SQ water for 20 min followed by methanol:water, 1:1(v/v), for 20 min and the column was left in this solution overnight.

The sample preparation and assay conditions described above had been used successfully by AXIS Biochemicals ASA and Bean *et al.* However, following their methodologies precisely during the present study did not result in any obvious separation or detection of the transferrin isoforms. It was therefore decided to test each step of the sample preparation and HPLC process systematically to both validate and optimise the system.

8.2.5 Optimisation and validation of HPLC studies

The approach that was followed included an examination of the factors involved in HPLC that might result in failure of an apparently working system. Because it was thought necessary to included positive controls within the system, any studies involving serum were compared with a CDT preparation. The source of these derived from CDT standards that were supplied by AXIS Biochemicals in their kit. The isoforms of CDT present in the preparation comprised mono-, di- and tri-sialotransferrin. It was expected that elution profiles from this preparation could be used, in part, to calibrate the column during each run for unknown serum samples. The serum sample that was used in these studies derived from a male, active alcohol misuser, code number = S320, whose serum CDTect was 47 U/l, AXIS %CDT 7.2 % and total transferrin was 375 mg/dl.

8.2.5.1 Spectrophotometer Detector

The simplest explanation for the negative results obtained above was that the spectrophotometric detector was not working. In order to examine its efficiency, there was no need for the column to be in the circuit and so it was removed.

The detector was tested by examining its ability to detect a standard protein, Bovine Serum Albumin (BSA). 100µl of BSA solution (100mg BSA dissolved in 10mls Mobile A) was injected as a single bolus into the circuit and was pumped through at 1ml/min. The eluant passed through the detector that was set to read the absorbance at 280 nm (the optimum wavelength for the majority of proteins). Further serial two-fold dilutions of this BSA solution were made and 100µl of each injected as before. Single peaks, produced by the data acquisition system and displayed and analysed by the ChemStation software were seen approximately 30 sec following injection of the samples (therefore indicating that the 'dead space' of the circuit was 1ml, because the flow rate was 2mls/min), the magnitude of which were proportional to the concentration of the BSA solution (data not shown).

Therefore it was concluded that the detector and programme analyser were both operating properly.

8.2.5.2 Loss of protein following sample preparation

It was possible that the treatment of serum with the iron solution resulted in either loss or de-naturation of protein, so that there was no protein to be detected. Again, the column was not needed for these experiments and so was omitted from the circuit. Instead of using serum, a preparation of CDT 'standards', supplied by AXIS Biochemicals as part of their kit, and at a concentration of 8 mg/L, were used.

CDT standards were iron-saturated as before (Section 8.2.3.1). Aliquots were removed after each of the steps to determine potential loss following sequential treatment with ferric chloride, dextran sulphate, calcium chloride and filtration of the sample. 100µl of each aliquot were injected into the circuit and pumped through the detector, set to read absorbencies at 465nm and the signal data recorded on the ChemStation. Small peaks were seen within about 30 sec following injection of the sample, as before, and the time of appearance and height of the peaks were not influenced by any of the serum preparation steps (data not shown). It was re-

confirmed that the detector and the programme analyser were operating correctly and further that there were no steps in sample preparation that would lead to loss.

8.2.5.3 Sample concentration

A calibration curve was constructed to determine dose/response relationship and sensitivity of the detector. For this purpose the AXIS CDT standard was used and compared with a human serum sample, S320.

8mg/L AXIS %CDT standard positive control was diluted to 2mg/L using Mobile A. This was iron-saturated and treated as though it was a serum sample. Serial 2-fold dilutions were prepared by diluting 100µl of neat sample with 100µl of Mobile A. 100µl of this dilution were then similarly diluted with an 100µl of Mobile A and the process repeated through 13 further dilutions. Similar serial two-fold dilutions were prepared of the iron-saturated serum sample used in the previous experiment.

100µl of each successive dilution was injected, without the column in line, and pumped through using Mobile A as the solvent. The eluant passed through the detector which was set to read absorbencies at 465nm. As before, single peaks for serum were seen approximately 30 sec following injection and the height and area of the peaks were recorded using the ChemStation analysis pack. Dose response curves were constructed to examine the effect of dilution on heights and areas of the peaks for both CDT and serum (Figures 8.11 and 8.12).

Figure 8.11: Spectrophotometer Testing: Dose Response Curve for CDT Standards

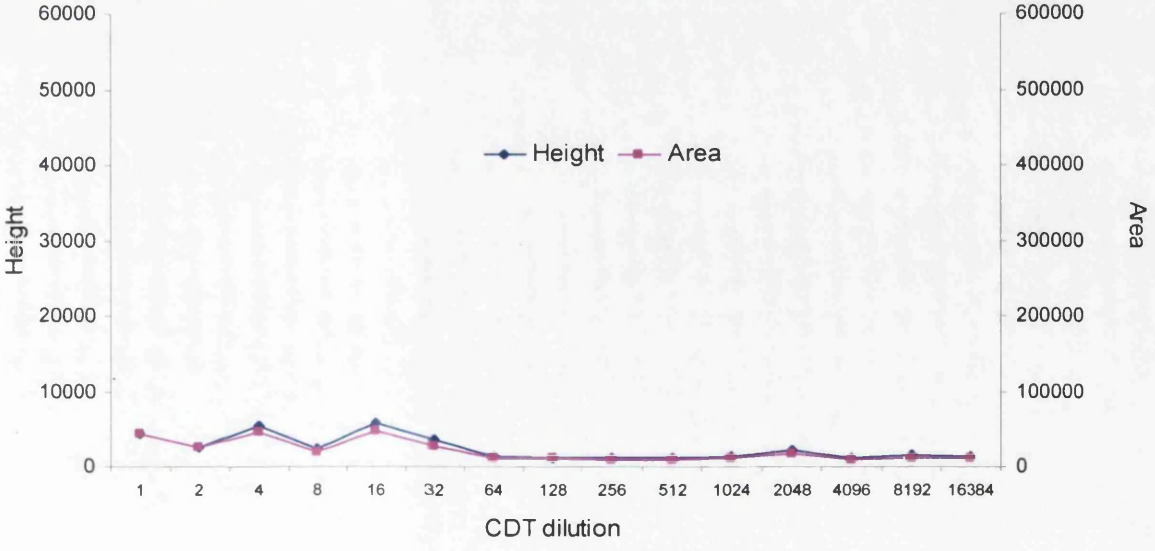
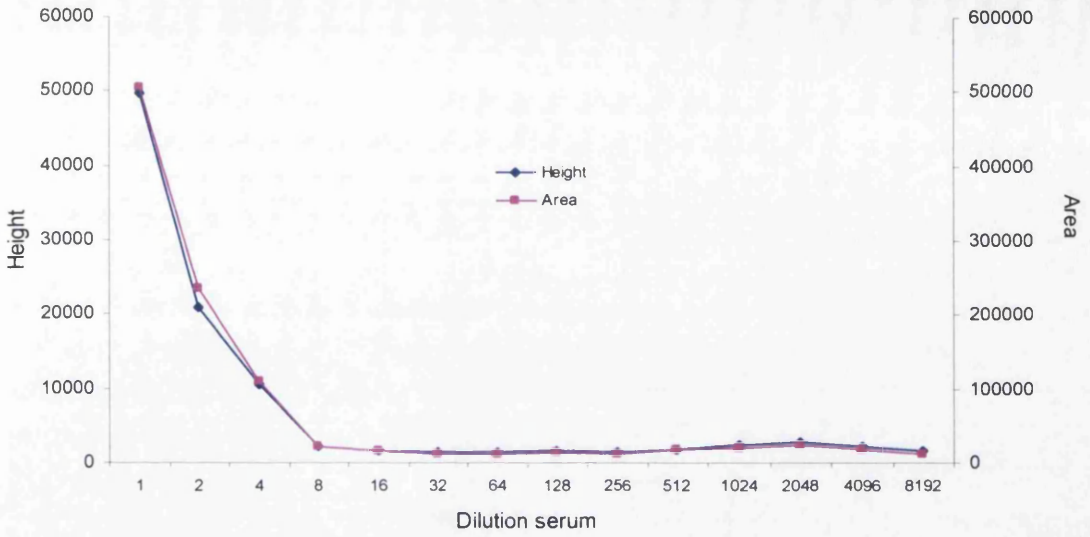


Figure 8.12: Spectrophotometer Testing: Dose Response Curve for a Serum Sample



There was insufficient protein present over all dilutions of the CDT standard calibrator for detection by the spectrophotometer. Only small signals were obtained from the CDT standards, as before. In contrast, relatively high concentrations of serum resulted in strong signals, the strength of which indicated that the CDT standards were too dilute to be of any use. The lowest possible dilution (highest concentration) of iron-saturated serum was 1:2. This resulted in a high signal when pumped through the HPLC circuit and further dilutions of 1:4 and 1:8 were also detectable. The initial iron-saturation sample preparation procedure required a total 1:2 serum dilution but, based on the present study, there seemed no virtue for this and so the final dilution step was omitted from future serum sample preparation steps. Sample preparation was then altered to minimise the dilution of the serum sample by using 1 ml of serum sample and then the volumes of the other constituents of sample preparation were adjusted accordingly. Sample preparation used in the remainder of the experiments was as follows:

To 1 ml of serum sample 200 µl Fe(III)-tris-maleic-citrate solution was added. This was incubated for 30 mins at room temp as previously. 10.7 µl dextran sulphate (100 g/L in water) and 50 µl $CaCl_2$ (147 g/L in water) were added. This was then refrigerated at 4-6^{0}C for 1 hour before centrifuging at 5000g for 15 mins at 4-6^{0}C. 1,100 µL of clear supernatant filtered with a 0.22µm filter. The column was reconnected in circuit.

8.2.5.4 Buffer concentration

The principle variable relating to elution of proteins from an anion-exchange column, apart from the change in pH, is the concentration of salt in the elution buffer (Mobile B). Therefore, the performance of the column was determined by optimising the sodium chloride concentration in Mobile B with respect to maximum resolving power of the column for an optimised serum sample that was injected. Optimal elution would result in well-spaced resolved peaks being detected, within a reasonable (< one hour) but arbitrary time-span.

Mobile B was prepared containing 3M sodium chloride (NaCl, 175.32g/L). Doubling dilutions in NaCl-free Mobile B were prepared so that the range of NaCl concentrations in Mobile B was: 3M, 1.5M, 0.75M, 0.375M, 0.188M, 0.094M, 0.047M

Iron-saturated serum used in previous experiments (S320) was used and the injection volume was 100µl as before. The run time was 50 min for each sample. The more concentrated molarity of NaCl in Mobile B, the 3M Mobile B, showed the fastest peak elution; all the peaks were seen by 12 min. However the peaks were too close together for adequate resolution. With a reduction in the molarity of the NaCl in Mobile B there was an increase in the time taken for each peak to be eluted. The maximum peak resolution occurred with Mobile B containing 0.375 M NaCl.

Further experiments were performed to determine, more precisely, the concentration of NaCl that should be used. Mobile B was prepared containing 0.1M, 0.2M, 0.3M, 0.4M and 0.5M NaCl. A starting concentration of 1M NaCl was prepared in Mobile B (5.84g NaCl dissolved in 100mls Mobile B) and further diluted with NaCl-free Mobile B, according to the schedule below:

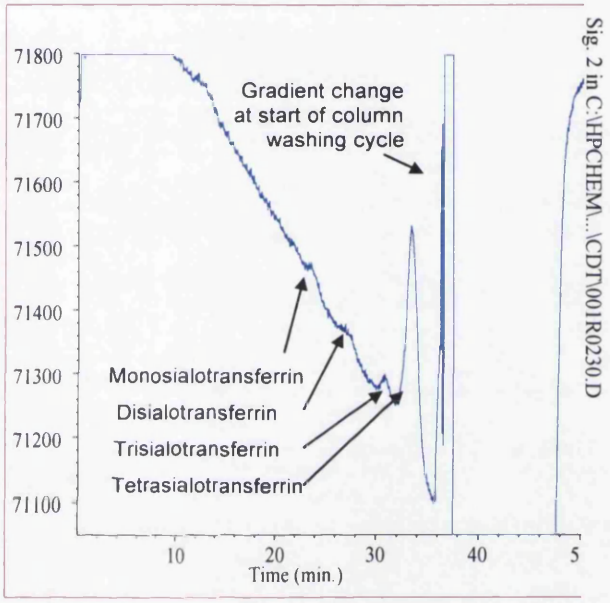
Table 8.4: Volumes of 1M NaCl required to create varying molarity in Mobile B

Volume (mls) of 1M NaCl in Mobile B	Volume (mls) Mobile B added	Molar Concentration of NaCl in Mobile B
50	50	0.5
40	60	0.4
30	70	0.3
20	80	0.2
10	90	0.1

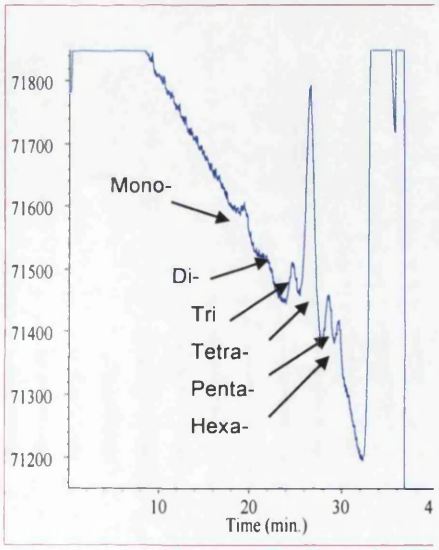
The different Mobile B solutions were used to elute serum samples from the column as before and the chromatograms analysed for optimum resolution of peaks.

Figure 8.13: Chromatograms from Mobile B dilution studies

i) 0.2M Mobile B. Four peaks were seen. These were well-spaced but elution took place over a prolonged time and at 35 minutes any possible penta- and hexa-sialotransferrin had been obscured by the start of the gradient change at the start of the column washing cycle.

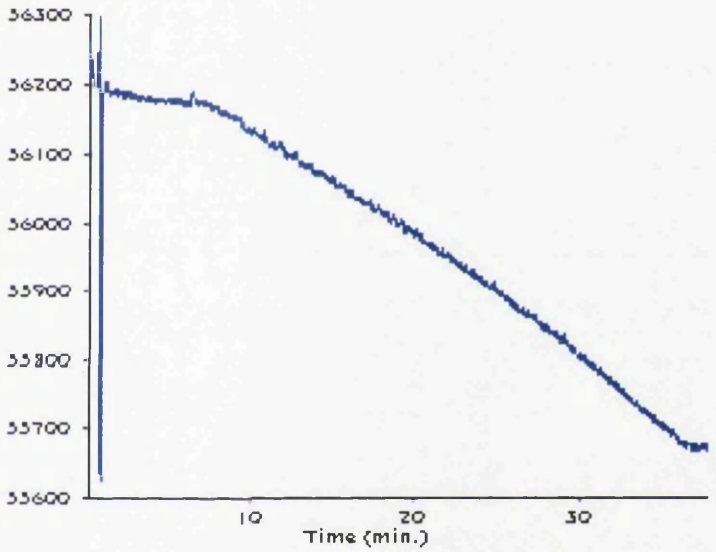


ii) 0.4M mobile B. Six peaks were eluted. These were reasonably well-spaced and there was complete resolution, including penta- and hexasialotransferrin, by 30 minutes



The optimal area and height of peaks associated with consistent retention times was seen with Mobile B containing 0.4M NaCl (Figure 8.13ii). Further, the baseline achieved with these conditions remained stable (Figure 8.14). The line produced is the spectrophotometric reading over a 50 min time period. The spectrophotometer was blanked at the start of the run (Time 0) against 100% mobile A. As the run-time progresses, the composition of the solvent changes and this is routinely reflected in a decreased signal produced by the spectrophotometer. With unchanging solvent conditions, there would be a horizontal base-line.

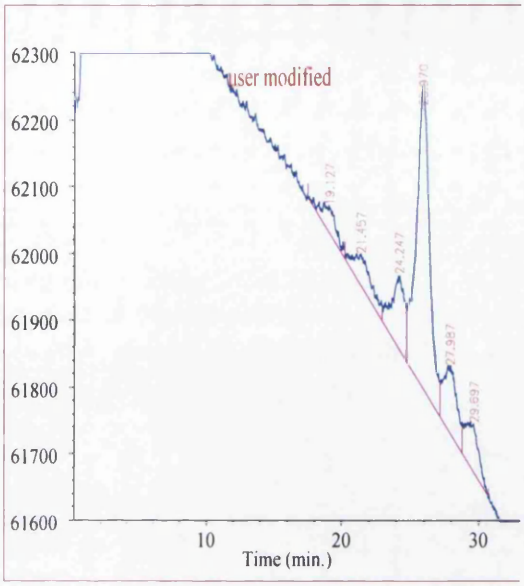
Figure 8.14: HPLC Gradient Baseline



As a further confirmatory step for column optimisation, Mobile B containing 0.4M NaCl was used as the eluting buffer for serum samples diluted to different degrees in Mobile A. The same iron-saturated serum sample that had been used for previous experiments (S320) was serially diluted in Mobile A to give, 1 in 2, 1 in 4 and 1 in 8 dilutions and undiluted serum. 100µl of each, starting with the 1 in 8, were applied to the column which was run under standard conditions. The chromatograms were analysed to identify the maximum signal obtained for each of the eluted peaks without loss of resolving power.

Undiluted serum showed peaks that were well-separated, gave relatively strong signals and were eluted with workable retention times. A typical chromatogram, produced under these running conditions is illustrated in Figure 8.15, where 6 definable peaks were seen with the ChemStation determination of the retention times marked at the apex of each peak.

Figure 8.15: Serum sample S320; HPLC in Mobile B containing 0.4M NaCl



The ChemStation annotation 'user modified' refers to analysis of the chromatogram. A baseline has been drawn in below the peaks and the peak area divided manually to give individual presumptive isoforms. The retention times are shown at the apex of each peak.

8.2.5.5 Gradient optimisation

Elution of proteins during anion exchange chromatography with satisfactory resolution is dependent, in part, on the establishment of a suitable gradient (relative proportion) between the starting buffer (Mobile A) and the elution buffer (Mobile B). If the relative concentration of the elution buffer is too high too early (gradient too steep), proteins will be eluted on top of each other with no clear resolution between the different proteins. Alternatively, where the elution buffer reaches maximum proportions over too long a period of time, good resolution may be obtained but each run would take too long to perform. Optimum conditions require titrating the gradient to achieve maximum resolution of separation of proteins in an acceptable time.

The serum sample studied in the previous experiments (S320), iron-saturated and prepared as described earlier, and using Mobile B at a concentration of 0.4 M, was used. In order to optimise the gradient of Mobile B, the previously used gradient of 25% was varied between 30 and 10% in 5% increments. This was achieved by programming the pumps appropriately and the incorporation of the gradient was started 5 min after injection of the sample. At 30% and 25% the first of the eluted proteins were produced too soon and superimposed on the solvent front. However, at 15% and 10% gradients, the elution of the protein was too late and was

superimposed over the column wash part of the programme. 20% appeared to provide the optimal separation of the first proteins to be eluted and the reference protein within a reasonable time.

Table 8.5: Retention times of individual peaks at different gradients

Gradient	Monosialo-transferrin	Disialo-transferrin	Trisialo-transferrin	Tetrasialo-transferrin	Pentasialo-transferrin	Hexasialo-transferrin
30%	Inadequate separation of peaks for analysis					
25%	19.6	21.6	24.3	25.9	27.9	29.2
20%	21.4	24.5	27.8	29.8	32.4	34.1
15%	24.9	29.1	33.4	Superimposed on the wash cycle		

The peak retention times, heights and areas were compared to finally assess which gradient provided optimal peak characteristics (Table 8.5 and Figure 8.16).

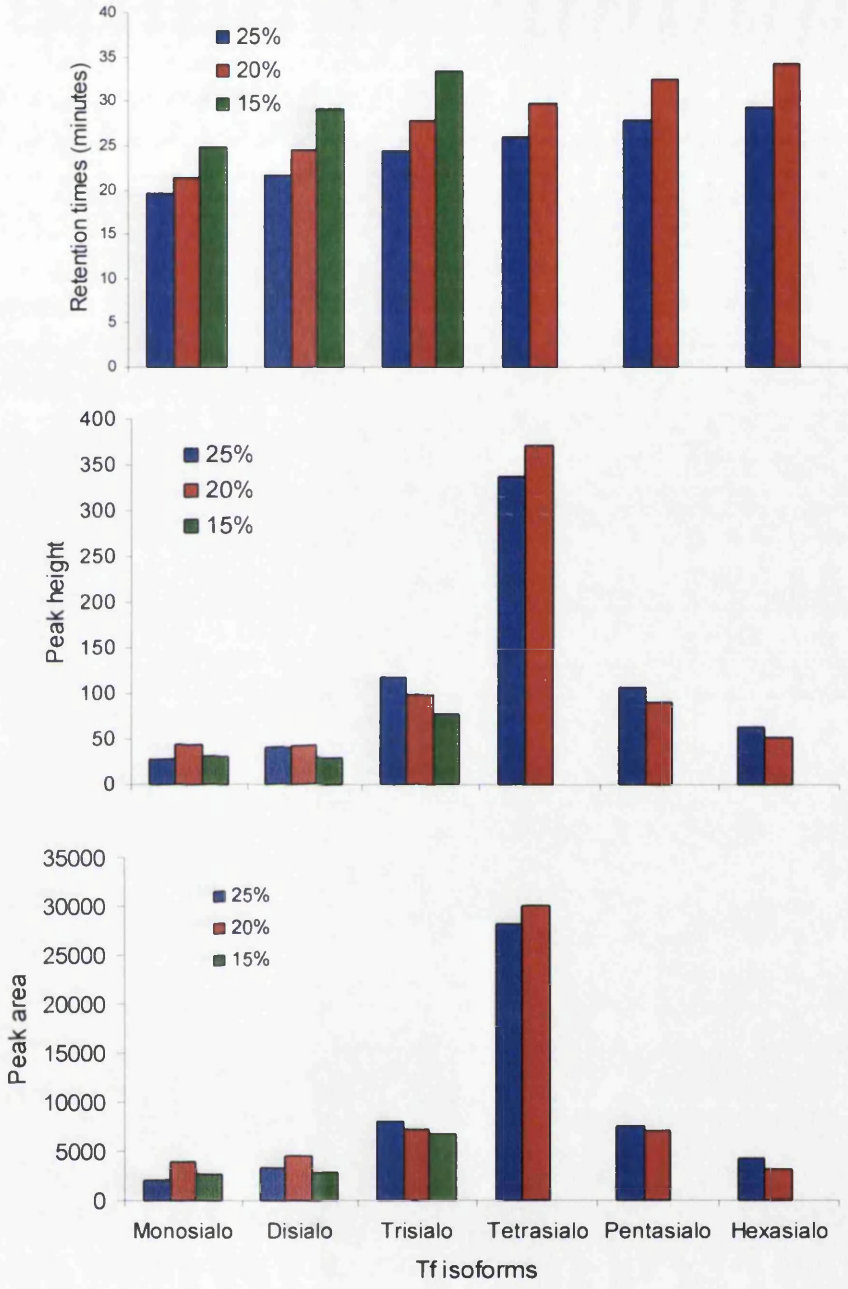
Retention Times. The 30% gradient was too high for adequate resolution of individual isoforms and only a single peak was seen. With 20% and 25% gradients, 6 peaks were eluted over time that corresponded approximately to previously published elution times for particular isoforms. For clarity, these have been labelled with the relevant isoform name, although there is no supporting evidence is offered for this. The 20% gradient resulted in a slightly delayed elution compared to the 25% gradient. The 15% gradient provided elution of three peaks, but subsequent peaks were superimposed on the washing cycle.

Peak Height. There was little to choose between 20% and 25% gradients when examining the heights of each peak. The peak with the maximum height (maximum signal from the spectrophotometer, therefore maximum protein concentration) had a retention time close to tetra-sialotransferrin which has been recognised as the most abundant of the transferrin isoforms.

Area. Again, there was little to choose between the 20% and 25% gradients with respect to the areas of the different peaks, both giving satisfactory resolution of the individual peaks for analysis.

The advantage of the 20% gradient of Mobile B in Mobile A was confirmed.

Figure 8.16: Optimisation of buffer gradient: Putative transferrin isoform profiles



The gradient time was extended by a further ten minutes so that maximum separation could take place of the transferrin isoforms. The final gradient programme is detailed in Table 8.6. There was insufficient protein present over all dilutions of the CDT standard calibrator for detection by the spectrophotometer.

Table 8.6: Final Gradient Characteristics

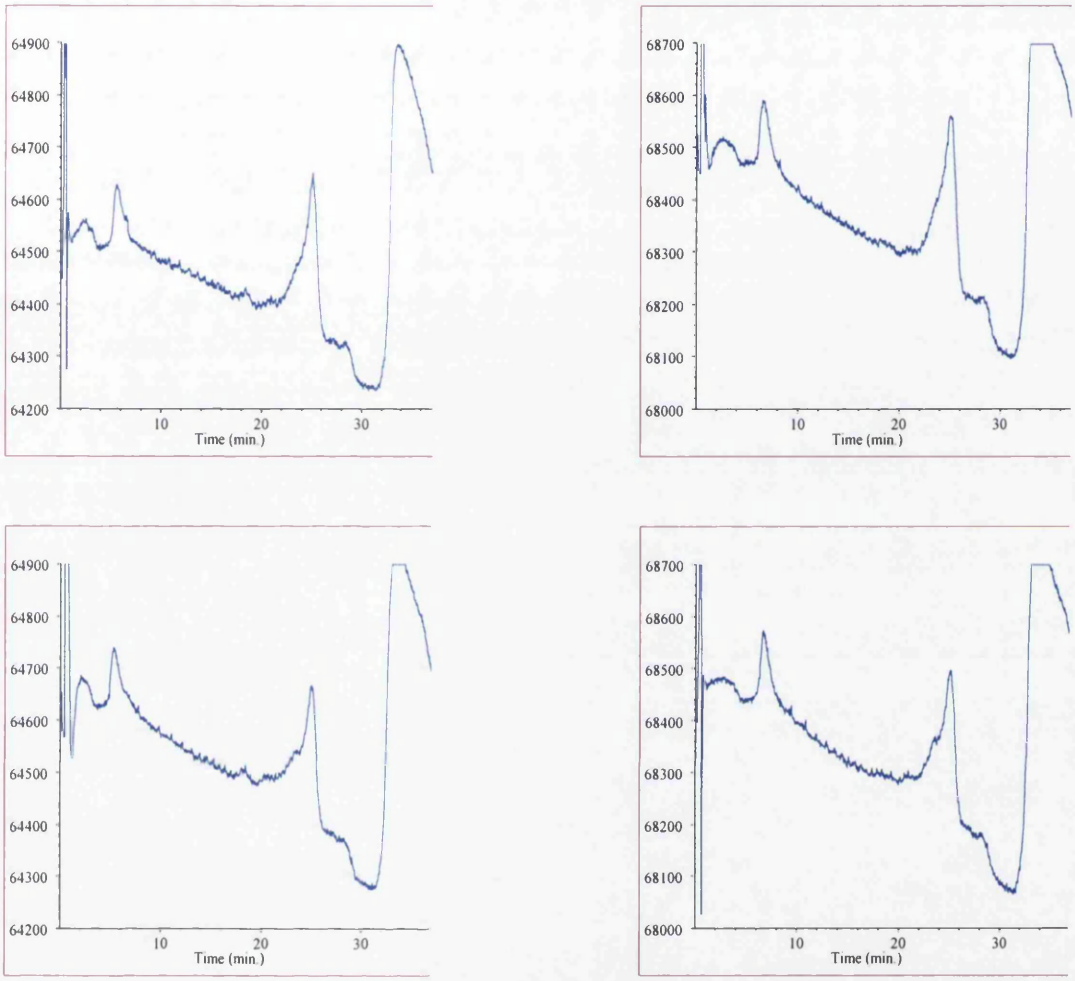
Time (minutes)	Flow (mls/min)	%A	%B
0.0	2	100	0
5.0	2	100	0
35.0	2	80	20
45.0	2	80	20
45.1	2	0	100
55.0	2	0	100
55.1	2	100	0
60.0	2	100	0

8.2.5.6 Reproducibility

The reproducibility of the optimised HPLC system was determined by studying five aliquots of serum from an individual with elevated serum CDT concentrations: CDTect 35 U/L, AXIS %CDT 6.9 % and a normal total transferrin 223 mg/dl. The five samples had all been prepared (iron-saturated) separately on different occasions and HPLC performed using the optimised conditions, described above, including the 20% gradient.

During this experiment the computer collecting the data 'crashed' during the data collection period of the fifth sample. Visually the chromatogram was seen to be similar to the other four; however only the first four can be shown here and it was on these that the calculations were performed.

Figure 8.17: Chromatograms of four serum samples from the same individual



Each of the HPLC chromatograms was analysed using the HPChem station for peak retention times, heights and areas. The mean retention times and the standard errors about the mean, standard error (S.E.), and the coefficient of variation (C.V.) were also calculated for each of the putative isoforms (Table 8.7). These values were compared with those derived from the literature, particularly form Bean *et al* and from AXIS Biochemicals (Table 8.8).

Table 8.7: Reproducibility of HPLC: retention times, height and area

Peak ID*	Retentio Time (mins)	(S.E.)	C.V. %	Height (arbitrar units)	(S.E.)	C.V. %	Area	(S.E.)	C.V. %
1 (MonosialoTf)	**18.600**	0.18	*0.98*	**37.50**	8.18	*21.83*	**4.51**	1.27	*28.11*
2 (DisialoTf)	**21.025**	0.05	*0.24*	**62.50**	9.33	14.92	**7.99**	1.61	*20.16*
3 (TrisialoTf)	**23.625**	0.05	*0.21*	**167.75**	18.19	*10.84*	**22.25**	2.87	*12.89*
4 (Tetrasialo Tf)	**25.100**	0.08	*0.33*	**335.25**	28.81	*8.56*	**50.82**	5.09	*10.02*
5 (Pentasialo Tf)	**27.280**	0.05	*0.18*	**58.00**	4.97	*8.56*	**6.48**	1.26	*19.48*
6 (Hexasialo Tf)	**28.350**	0.06	*0.20*	**68.25**	9.67	*14.17*	**7.96**	1.10	*13.86*

** The Peak Identity is the peak number, eluting successively with time in minutes. Under each of the Peak Identity numbers is the putative transferrin isoform that corresponds with the retention time.*

Figure 8.17 shows that visually the chromatograms were very similar, with the same peak formations. Table 8.8 shows that the retention time standard errors and coefficient of variations for the transferrin isoforms was quite small indicating good repeatability. The height and area standard errors and coefficients of variations of the transferrin isoforms were much larger showing less consistent results.

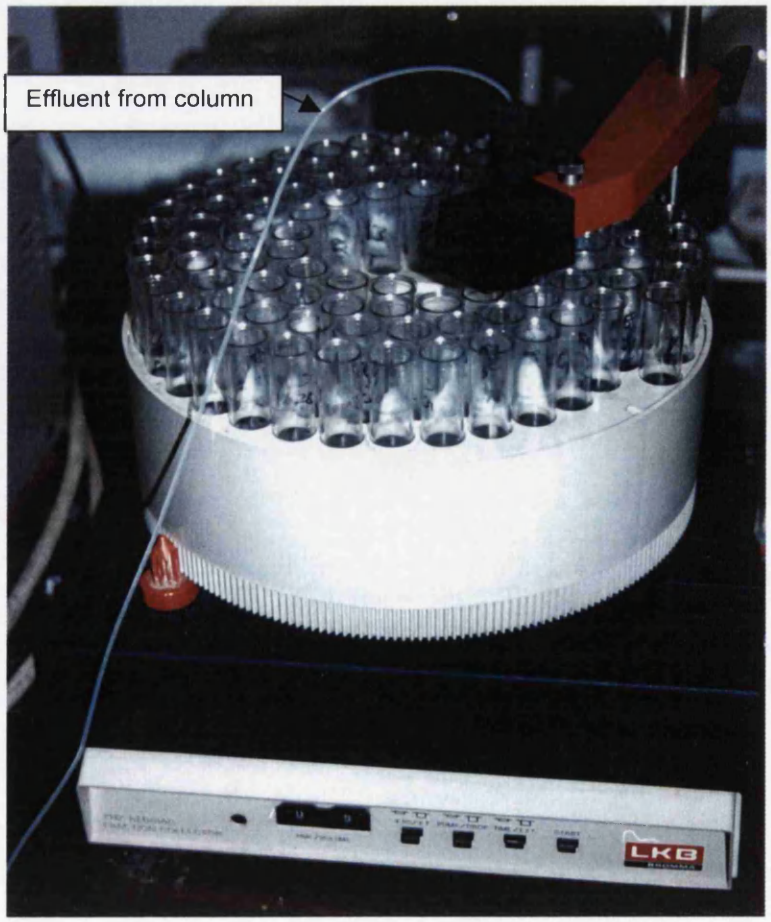
Table 8.8: Comparison of transferrin isoform retention times obtained using HPLC, by different authors

Author	Transferrin isoform retention time (minutes)						
	A-sialotf	Mono-sialotf	Di-sialotf	Tri-sialotf	Tetra-sialotf	Penta-sialotf	Hexa-sialotf
Bean *et al*	Retention times not given						
Jeppsson *et al* (Jeppsson et al. 1993)	9		11	12	14	16	
Werle *et al* (Werle et al. 1997)	8		10.5	11	13	14.5	
AXIS (personal communication)	14.6		16.6	19.2	23.0	26.5	29.2
Present study		18.6	21.0	23.6	25.1	27.3	28.4

The retention times vary according to the HPLC protocol adopted by each author. The consistent finding is that the asialotransferrin is eluted first, and the isoforms follow with increasing number of sialic acids present. None of the above authors has reported the successful identification of monosialotransferrin.

During the HPLC cycle the eluate from the column was collected every 30 seconds into aliquots using a fraction collector (Figure 8.18). These were all analysed for transferrin using immunoturbidimetry. The dead space of the collecting system was calculated to be 2.5 minutes, or the first five collection tubes. From the collection tube number the retention time was estimated that corresponded with each sample (Figure 8.19). The maximum transferrin concentration and retention time was calculated corresponding with the HPLC peak. The mean and standard deviation of the transferrin measured by immunoturbidimetry was then calculated over the five samples (Table 8.9). There are five samples in this part of the experiment as eluate was collected from all samples.

Figure 8.18: Fraction collector



The transferrin concentrations obtained using immunoturbidimetry showed similar results for all five samples. Figure 8.19 shows that, using immunoturbidimetry, transferrin was detected from the column between approximately 18 and 28 minutes. In particular the largest amount of transferrin detected was at 24 minutes in all five samples. This corresponded with the chromatograms obtained using HPLC (Figure 8.17) and confirmed that the peaks obtained during these times using HPLC were of transferrin. The largest immunoturbidimetry concentration was at 24 minutes (mean $\pm$ 1SD 24.1 $\pm$ 0.74 min) in all five samples and this would correspond with tetrasialotransferrin. There were two other detectable peaks: one preceding the large peak at a mean of 20.5 minutes and one after the large peak at a mean of 27.3 minutes. The mean concentration of the largest transferrin peak was 14.6 μg/ml, nearly four times higher than either of the other two peaks (3.8 and 3.2 μg/ml respectively). The immunoturbidimetry does not, however, reflect each isoform HPLC peak at the same definition as HPLC. It provides a qualitative and semi-quantitative determination of transferrin.

Figure 8.19: Transferrin concentrations, measured by immunoturbidimetry, on five aliquots of one serum sample from a given individual

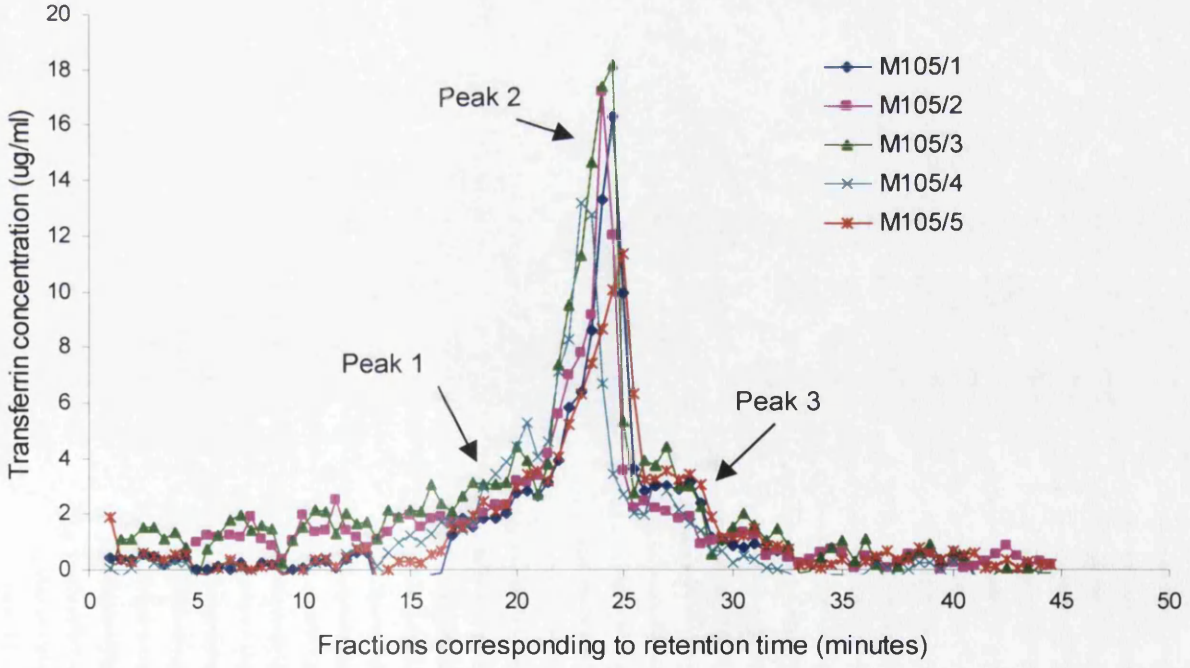


Table 8.9: Transferrin concentration and retention times measured using immunoturbidimetry

Immunoturbidimetry peak	Mean ± 1SD retention time (min)	Mean ± 1SD transferrin concentration (μg/ml)
Peak 1	20.5 ± 0.35	3.8 ± 0.99
Peak 2	24.1 ± 0.74	14.6 ± 2.91
Peak 3	27.3 ± 0.67	3.2 ± 0.90

The identity of each transferrin peak obtained using HPLC could not be confirmed. From other published data it was surmised that the major peak represented tetrasialotransferrin and that the peaks were eluted in order of increasing sialic acid concentration.

8.2.5.7 Summary

Anion exchange HPLC was optimised to produce good resolution of iron-saturated serum samples into a number of peaks that were shown by an independent method (immunoturbidimetry) developed, in house, to be transferrin.

The buffers consisted of Mobile A, 20 mmol bis-tris, pH 6.2, and Mobile B, 20 mmol bis-tris, pH 5.6, containing 0.4M NaCl. The gradient between the two buffers during the elution step was a 20% gradient of Mobile B operating over a 10-minute period.

The precise identity of each transferrin isoform corresponding to the HPLC peaks could not be proven using immunoturbidimetry, but in comparison with other published work it was surmised that it was likely that they were eluted in order of increasing sialic acid content.

8.2.6 HPLC OF ISOFORM STANDARDS

Having optimised the HPLC column for general running conditions of iron-saturated serum, the peak profile obtained was suggestive of transferrin isoforms, by comparison with previously published work. It was felt necessary to attempt independent further confirmation of the precise isoform identity of each transferrin peak. In order to calibrate the column for individual isoform retention times, HPLC of the purified isoform standards was undertaken. Independent verification of the transferrin content was also attempted by testing the relevant HPLC fractions in the immunoturbidimetric assay described earlier.

8.2.6.1 Method

A sample of each of the isoform standards was thawed, iron saturated and processed as serum for HPLC. Each of the isoforms were then chromatographed sequentially using the optimised cycle of HPLC. There was one modification to the process. The resultant eluate from the column was collected using a fraction collector (Figure 8.22), programmed to collect fractions every 30 seconds (as the column flow-rate was 2 ml per minute, the eluate was collected in 1ml aliquots). The presence of transferrin in the fractions was assayed using the immunoturbidimetry assay.

8.2.6.2 Results

No peaks were seen for any of the isoforms. It was postulated that this negative result might be due to either the isoforms being applied in too dilute a form or that the isoforms had adhered to the gel of the column but had not been eluted. The former was considered more likely that the former was correct, based on the spectrophotometer detector check experiments, when CDT calibrator standard had been injected into the HPLC circuit without a column (8.2.5.1).

It was decided, therefore, to concentrate each of the isoforms and run the HPLC again. Twelve aliquots of each isoform were pooled (≅ 480μg protein in 2.4mls) and concentrated approximately 5-fold to about 2mg/ml using molecular sieves (3,000 kDa MW cut-off) centrifuged at 15,000g and the protein concentrations of the concentrated isoforms checked using the modified Lowry technique as before (results not shown).

Each isoform was iron saturated and processed, as previously, injection of 100 μl of the concentrated solution onto the column for chromatography.

A larger number of aliquots of tetrasialotransferrin were available than for any other isoform. As tetrasialotransferrin is present in the highest concentration of all the isoforms and so has the largest peak on a chromatogram, double the aliquots of tetrasialotransferrin were used to give a twelve fold concentration.

Using concentrated samples a small peak could be seen at 24.7 minutes and at 27.9 minutes (Figure 8.20). These correspond with the expected retention times of 25 minutes for

tetrasialotransferrin and 27 minutes for pentasialotransferrin respectively. No signal or peak was detected for any of the other concentrated samples.
The eluate from the HPLC column was collected for each of these samples and immunoturbidimetry was performed to estimate the transferrin concentration.

No transferrin was detected in any of the samples.

8.2.6.3 Conclusions

It was possible to use the standard isoforms in HPLC to confirm the presence of tetrasialotransferrin and pentasialotransferrin but not any of the other isoforms.

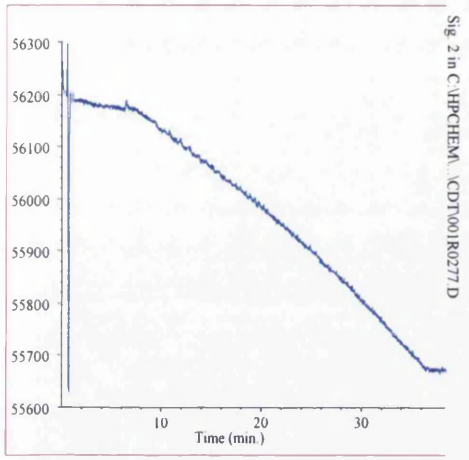
The concentration of standard isoforms in the eluates were too low to be detected using even the modified immunoturbidimetry techniques.

8.2.6.4 Summary

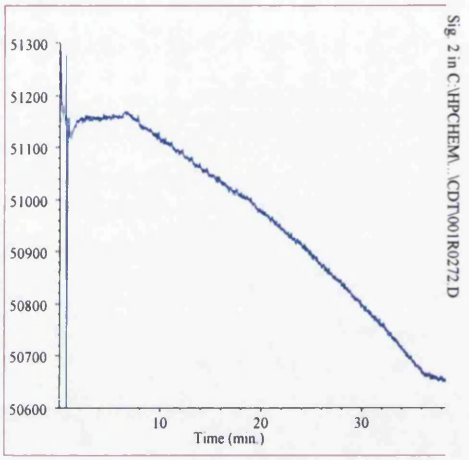
We were able to create conditions whereby an iron-saturated serum sample could be chromatographed using HPLC and obtain peak formations similar to those seen in published work (Werle et al. 1997; Jeppsson et al. 1993; de Jong et al. 1992). The peaks obtained were shown to contain transferrin using immunoturbidimetry. From donated individual transferrin isoforms (Van Eijk standard isoforms) we were able to confirm tetrasialotransferrin and pentasialotransferrin using HPLC but were unable to confirm individual peaks using immunoturbidimetry. It has been shown that asialotransferrin has the highest pI and is eluted first, then each higher isoform in succession. The largest was to be tetrasialotransferrin.

Figure 8.20: HPLC of Concentrated Isoform standards

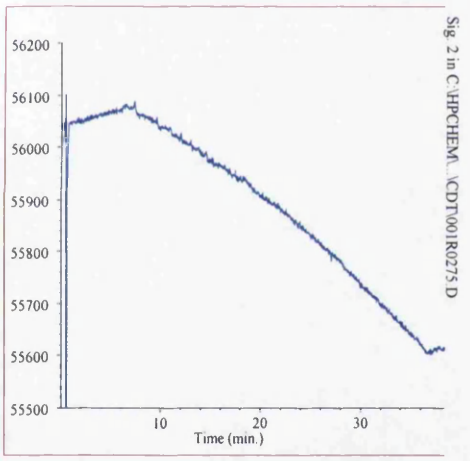
Monosialotransferrin x 5 concentrated



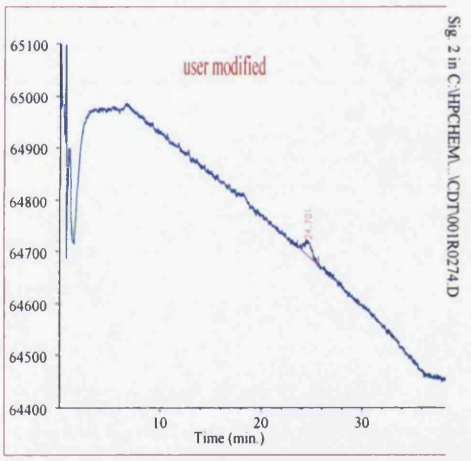
Disialotransferrin x 5 concentrated



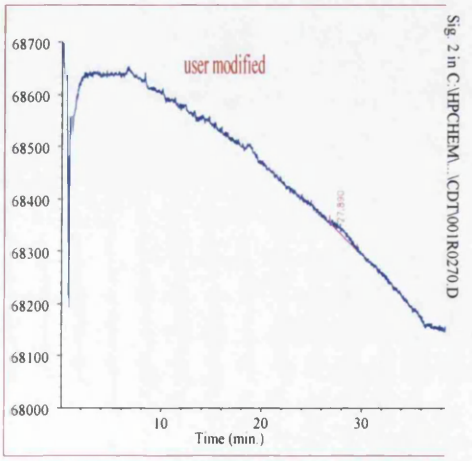
Trisialotransferrin x 5 concentrated



Tetrasialotransferrin x 12 concentrated



Pentasialotransferrin x 6 concentrated



8.2.7 HPLC ANALYSIS OF SERIAL SERUM SAMPLES FROM ALCOHOL MISUSING PATIENTS

8.2.7.1 Test Sample selection

CDT commercial assays have been shown to lack sensitivity and specificity (Chapter 4) but to be potentially useful for monitoring on an individual basis (Chapter 7). With this in mind it was decided to analyse serial serum samples from individuals using HPLC to follow shifts in the isoform patterns during drinking and abstinence where there were known CDT assay values. As there was no independent validation of the various peaks as to which peak was exactly which isoform the patterns of the isoforms were compared serially.

The following alcohol misusers (diagnosis and study identity number) were analysed:

1. **Responder:**
   - Non-cirrhotic male (M002)

2. **False positive non-responders:**
   - Male cirrhotic (M045)

3. **False negative non-responder:**
   - Non-cirrhotic female (M031)
   - Cirrhotic female (M017)

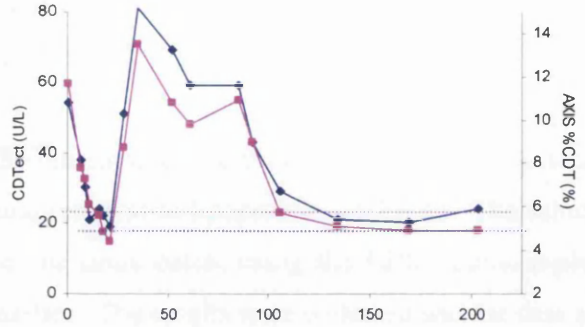
4. **Responder using CDTect but false-negative non-responder using AXIS %CDT:**
   - Cirrhotic female (M028)

5. **False Positive Non-responder:**
   - Male cirrhotic, who following liver transplantation, became a non-cirrhotic true negative (M029)

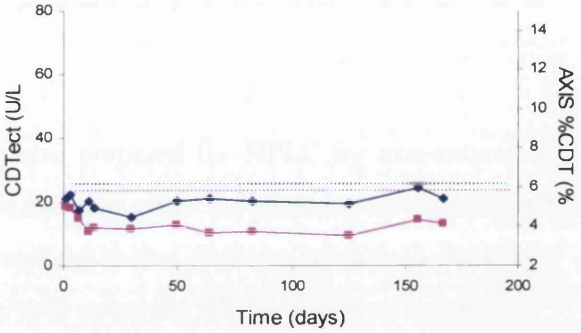
Each of these individuals had been previously analysed for CDTect and AXIS %CDT (Figure 8.21).

Figure 8.21: CDTect and AXIS %CDT values for each individual used for HPLC analysis

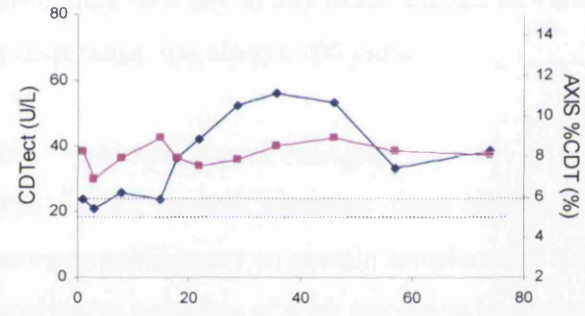
M002: Non-cirrhotic male responder



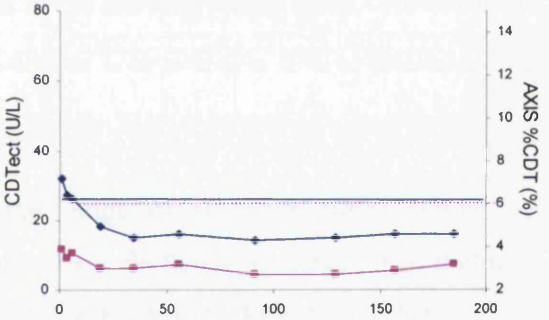
M017: Cirrhotic female, false negative non-responder



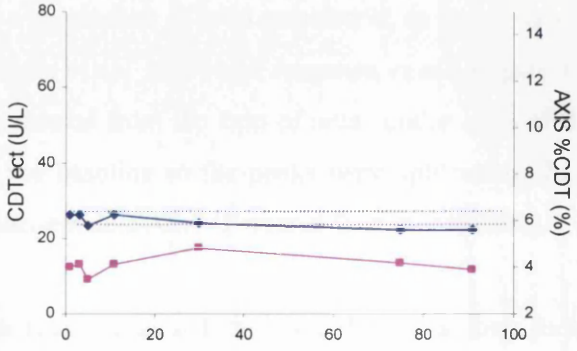
M045: Male cirrhotic, false positive non-responder



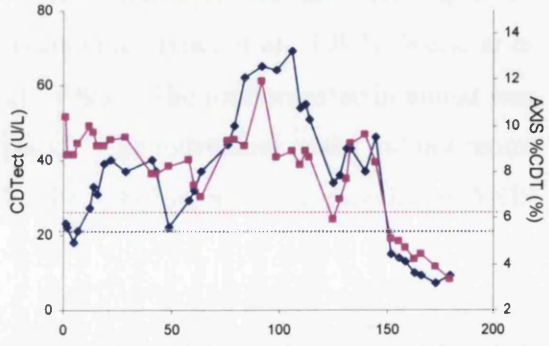
M028: Female cirrhotic, CDTect responder, AXIS non-responder

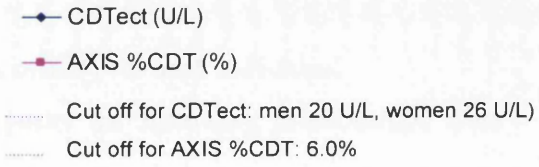


M031: Non-cirrhotic female, false negative non-responder



M029: Male cirrhotic transplanted day 148





8.2.7.2 Analysis

Each individual was followed serially over a six month period with serum samples taken at intervals and analysed for CDT and other markers of alcohol abuse as described in Chapter 7. Additional serum was frozen at each occasion and aliquots of these were used for HPLC analysis.

Serum samples were thawed at room temperature and prepared for HPLC by iron-saturation and removal of lipoproteins (8.2.3.1). The samples for each given individual were all analysed in the same batch, using the HPLC autosampler and optimised running conditions described earlier. The results were collected and the data analysed using the HPChemStation software.

The y-axis was measured in arbitrary units relative to optical density. Variability in circuit efficiency on a day to day basis resulted in variation in the starting y-axis value. However the y-axis range was always 700 units.

i) *Morphological changes.*

The HPLC profiles obtained were assessed in the range of retention times shown by immunoturbidimetry to contain transferrin (17-28 minutes). Particular features noted included qualitative estimates of peak resolution and relative relationships of peaks to each other.

ii) *Isoform areas*

The area of each peak was calculated using the HP Chemstation software, and then expressed as a proportion of total transferrin, as previously by Bean *et al* (Bean et al. 1997), Werle *et al* (Werle et al. 1997) and Jeppsson *et al* (Jeppsson et al. 1993). The total transferrin eluted was calculated from the sum of areas under each of the peaks. The individual peaks did not return to the baseline so the peaks were split using the HP ChemStation, as is the practice at AXIS Biochemicals ASA (Personal Communication).

For each individual, the area of each isoform as a proportion of total transferrin was tabulated. The following graphs were constructed:

i) The areas for each isoform over the six months serially for each individual.

Additionally for the isoform analysis of individual peaks the following relationships were determined:

ii) the sum of presumed a-, mono- and di-sialotransferrin peak areas (equivalent to isoforms measured by CDTect) were correlated (Spearman Rank) with the corresponding serum CDTect levels;

iii) the sum of presumed a-, mono-, di- and half of tri-sialotransferrin peak areas (equivalent to AXIS %CDT) were correlated (Spearman Rank) with corresponding AXIS %CDT levels. Half of trisialotransferrin peak area was determined by calculating the area corresponding to trisialotransferrin and halving it manually and then adding to the sum of the a-, mono- and di-sialotransferrin areas.

iv) Total transferrin, calculated from the sum of the individual peak areas was correlated with the total transferrin (Spearman Rank analysis) derived by independent immunoturbidimetry assay (Chapter 4.1.2.4).

8.2.7.3 Results

General

The results were presented as both morphological assessment of the chromatograms, assessed qualitatively by eye, and by ChemStation computer assisted analysis of each component of the chromatogram. The component peaks (isoforms) of each chromatogram were analysed and correlated with CDTect and AXIS %CDT determinations, described in Chapter 4. Each patient is presented individually. For each of the individuals, between four and six definite peaks were identified with retention times of between 16 and 28 minutes, when transferrin had been independently confirmed to be present. In all individuals the predominant peak, seen at approximately 23 minutes, corresponded with tetrasialotransferrin. Peaks corresponding to asialotransferrin were not detected in any individual.

Selected chromatograms from the HPLC studies have been shown for each individual tested.

M002; non-cirrhotic male non-responder

i). *Morphological assessment of chromatograms (Figure 8.22 a)*

On day 1 when the patient was actively drinking there was a prominent tetrasialotransferrin peak at 23 minutes but no separate peak of trisialotransferrin. During abstinence a separate peak of trisialotransferrin was detectable by day 9. This patient relapsed between days 28 and 66 and during this time the trisialotransferrin peak area diminished but was still detectable. The trisialotransferrin peak became more prominent after day 66 when the patient was again abstinent.

Monosialotransferrin was clearly detectable at day 1, but the peak then became smaller, although still detectable, by day 21. During relapse it became more obvious (days 35 to 85) and then with abstinence again became smaller, until it was less well defined at days 134, 169 and 204. The peak corresponding to disialotransferrin was well defined during drinking at day 1, but then became flatter during subsequent abstinence. By day 21 it was difficult to distinguish from mono-sialotransferrin. The resolution of the peak increased with relapse and was better seen at day 85, only to diminish again with abstinence after this time.

The higher isoforms, penta- and hexa-sialotransferrin, were not seen at day 1. From day 3 however they were detectable although, by the size of their peaks contributed only a minor species and did not appear to change over time.

ii). *Computer analysis of chromatograms (Figure 8.22 b)*

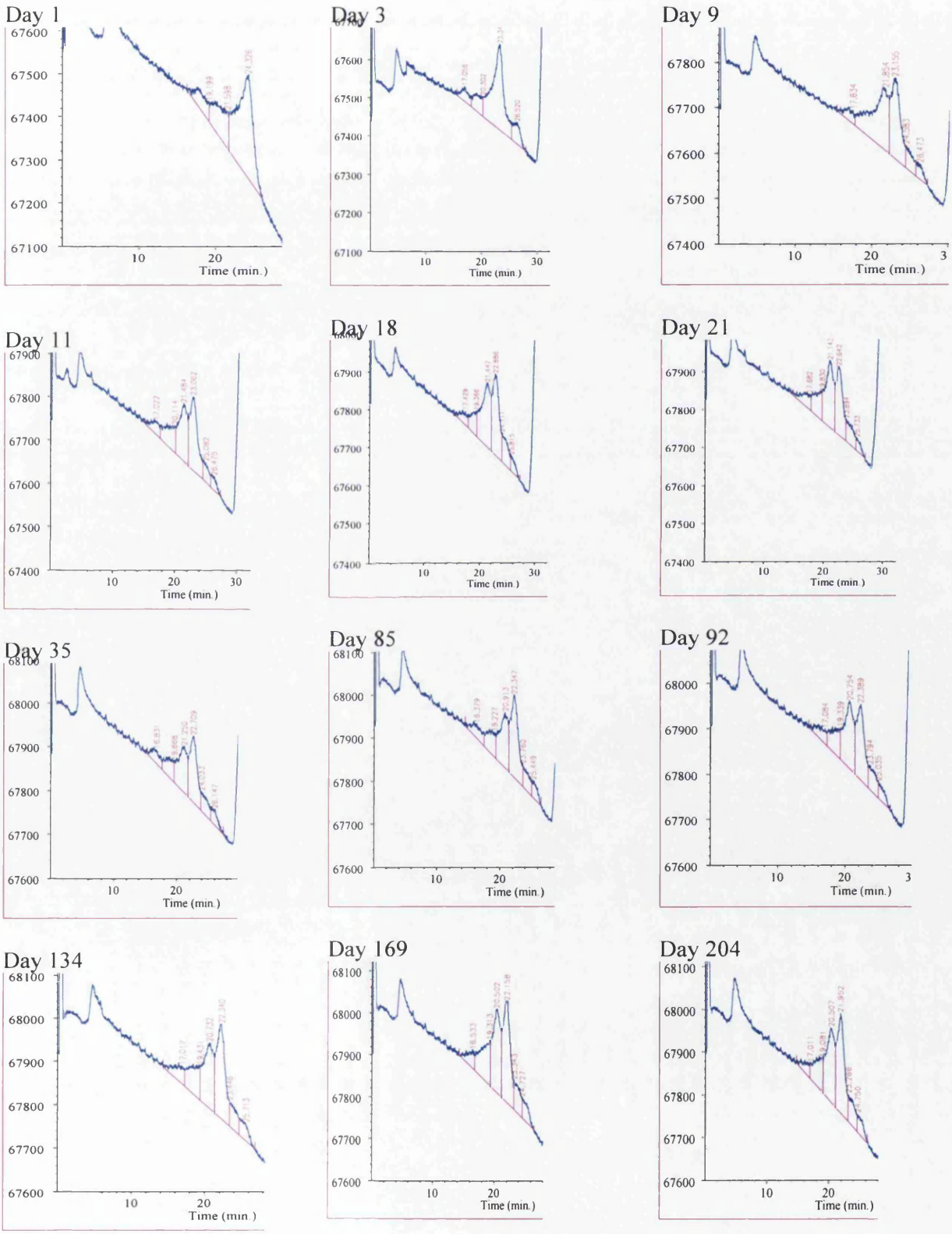
Because individual peak areas were expressed as a percentage of total transferrin and because tetrasialotransferrin was the largest component, changes in tetrasialotransferrin were the most marked. The relatively minor species of monosialotransferrin, disialotransferrin, pentasialotransferrin and hexasialotransferrin were noted as only present or absent because area changes were poorly defined. The proportion of trisialotransferrin to total transferrin seemed to remain stable throughout the study period (Figure 8.27.b.i). However it was not definable on day 1. Once it was detectable the proportion of tetrasialotransferrin appeared to fall dramatically. Tetrasialotransferrin appeared to drop away, this was due to the fact that trisialotransferrin became identifiable as a separate peak and it co-eluted with tetrasialotransferrin. Thus when the areas were divided by eye on the computer a large amount of what had been tetrasialotransferrin became incorporated in trisialotransferrin.

There was no correlation between the sum of mono- and di-sialotransferrin and measured by HPLC and the equivalent isoforms measured using CDTect assay although initially (days 1-28) the correlations mirrored one another. There was no correlation between the sum of mono-, di- and half tri-sialotransferrin when measured using HPLC and by AXIS %CDT.

The total transferrin measured using the independent commercial immunoturbidimetric assay and also by the total area of all the peaks using HPLC (Figure 8.22b.iv) reflected one another but did not correlate significantly.

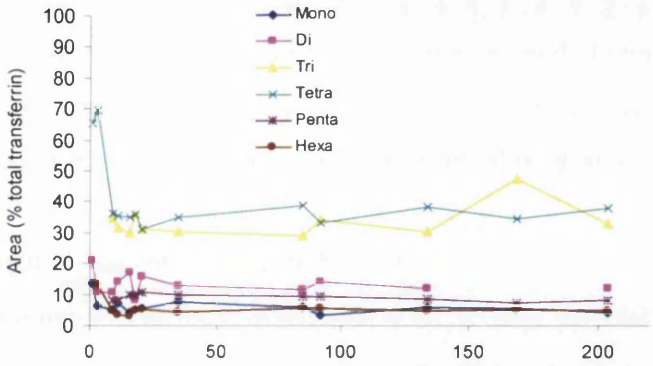
In summary, there were qualitative changes in the HPLC profile between drinking and abstinence, and these partially reversed with relapse. The lower isoforms (mono- and di-sialotransferrin) were more prominent in drinking than in abstinence, with trisialotransferrin more prominent in abstinence. Changes in drinking behaviour were accurately reflected by changes in the CDTect and AXIS %CDT profiles, but these did not correlate with changes in the sum of the isoforms determined by HPLC which these assays are said to detect.

Figure 8.22: Selected chromatograms from M002: non-cirrhotic male responder



b).Isoform peak areas over time and in relation to CDTect, AXIS %CDT and total transferrin for M002

i)



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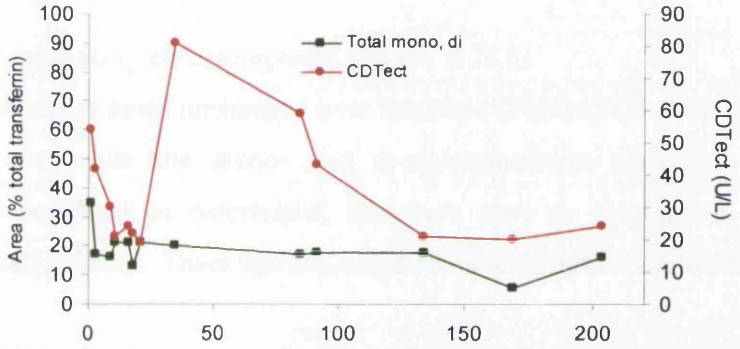
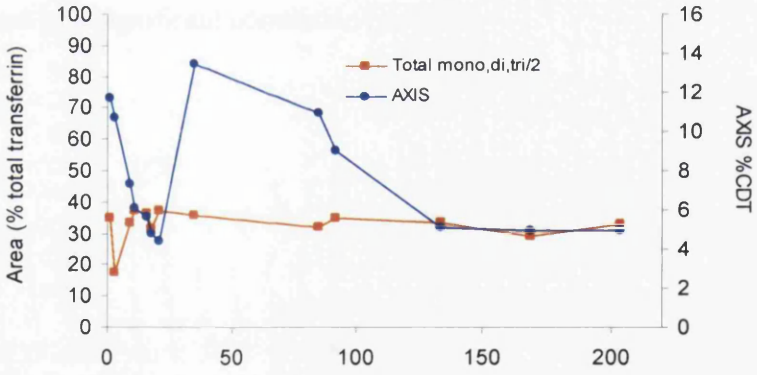
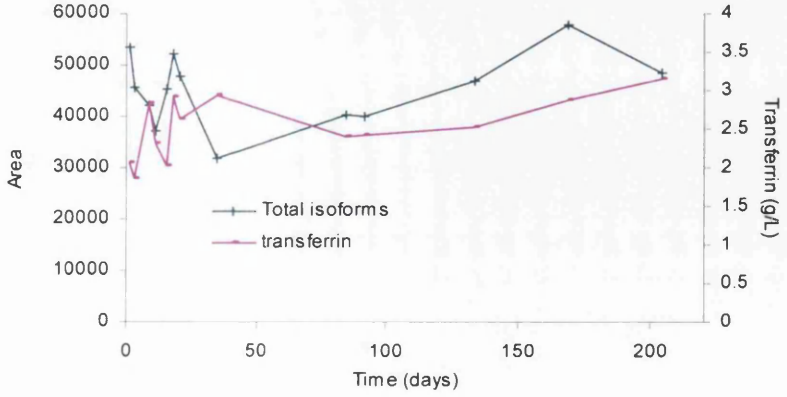


iii)



iv)



M045: male cirrhotic false positive non-responder

This gentleman was drinking at day 1 and thereafter remained abstinent throughout the study period. His serum CDTect and AXIS %CDT levels were raised at day 1, and remained elevated throughout (false positive). The total transferrin was low throughout.

i) *Morphological assessment (Figure 8.23.a)*

Mono- and di-sialo-transferrin were clearly defined. Tri-and tetra-sialotransferrin were eluted together.

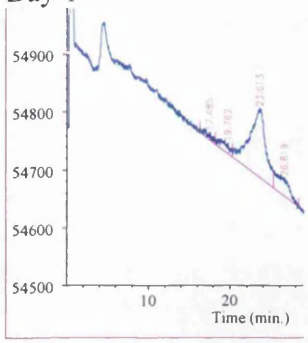
ii) *Computer analysis of chromatograms (Figure 8.23.b)*

The isoforms patterns remained unchanged over this time (Figure 8.23.b.ii). There was a clear increase in CDTect, while the mono- and di-sialotransferrin isoforms did not change. Trisialotransferrin could not be determined, and there were no changes in AXIS %CDT or mono- and di-sialotransferrin. There was not a significant correlation between either of these.

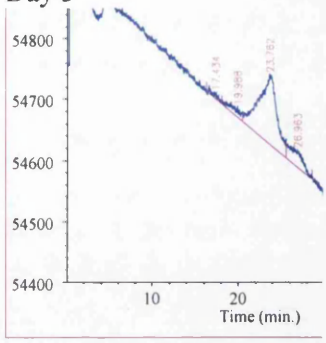
Total isoforms, as measured by total area under the peaks were reflected by total transferrin and this was confirmed by a significant correlation (p=0.011.

Figure 8.23: M045, male cirrhotic false positive non-responder

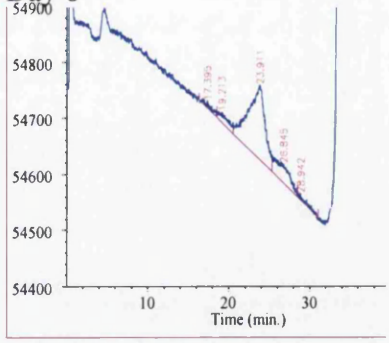
Day 1



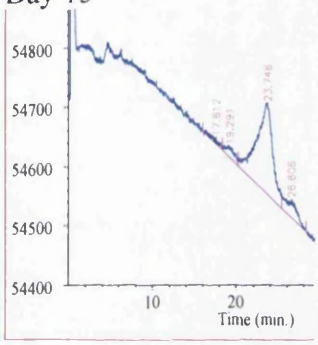
Day 3



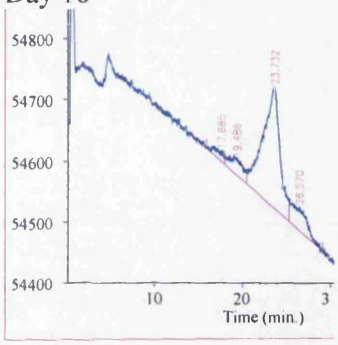
Day 8



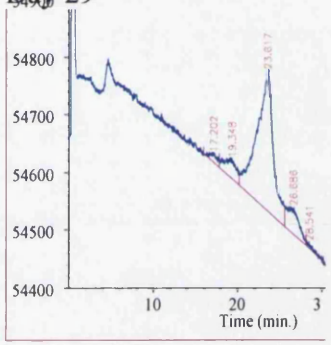
Day 15



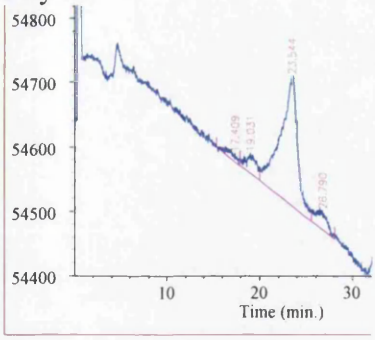
Day 18



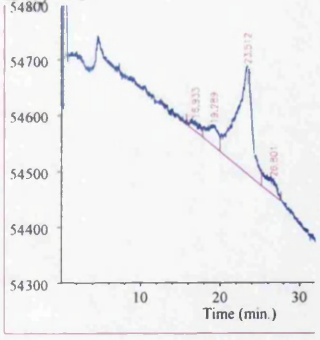
Day 29



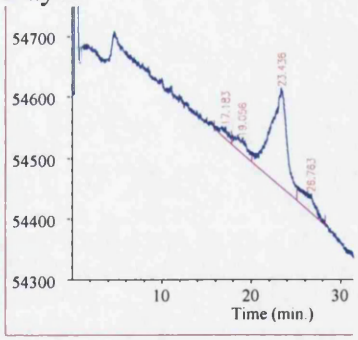
Day 36



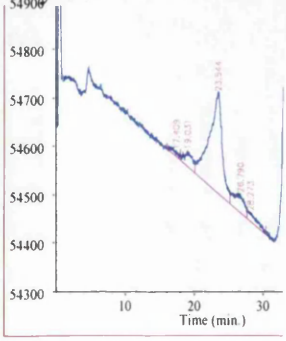
Day 46



Day 57

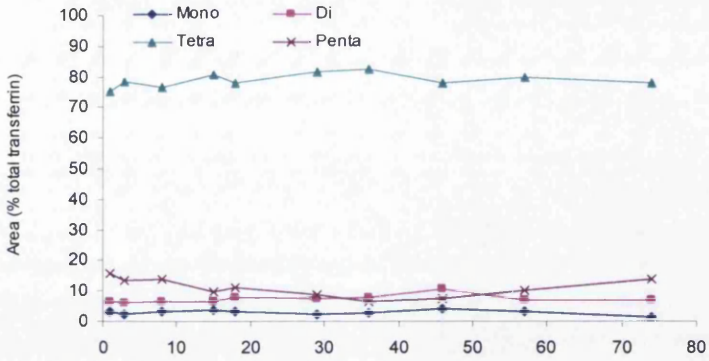


Day 74

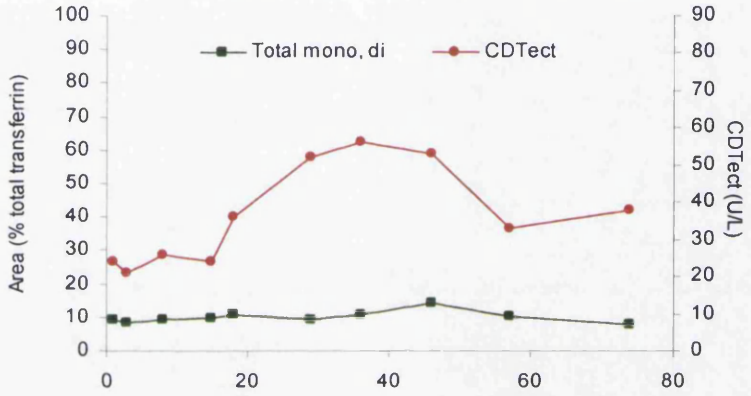


b) Isoform peak areas over time and in relation to CDTect, AXIS %CDT and total transferrin for M045

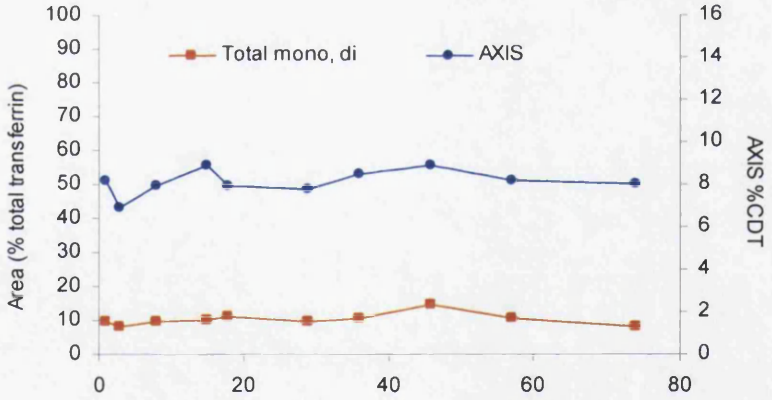
i)



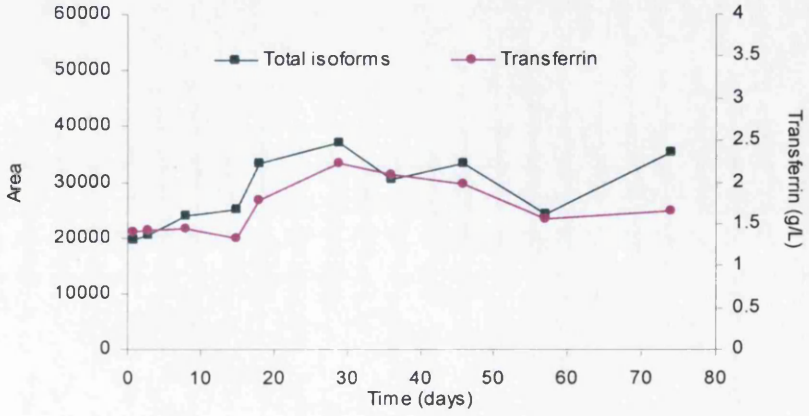
ii)



iii)



iv)



M031: Non-cirrhotic female, false negative non-responder

This woman was drinking at day 1, was abstinent at days 3 and 5, but relapsed intermittently throughout the rest of the study period, other than a period of abstinence of approximately two months from day 31 and including day 75. The transferrin was normal throughout.

i) *Morphological assessment (Figure 8.24.a)*

The mono- and di-sialotransferrin were poorly defined. Trisialotransferrin was seen at day 1, became better defined to day 30, was not so well seen at day 75 and was more prominent at day 91. This better definition coincided with times of drinking. Penta-sialotransferrin could be identified. The overall size of the peak formation decreased from day 1 to day 11, then increased at days 30 and 75, and had diminished again at day 91.

ii) *Computer analysis of chromatograms (Figure 8.24.b)*

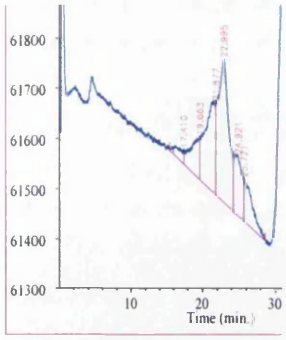
Trisialotransferrin was clearly identified. Serum CDTect and AXIS %CDT remained within the reference range, regardless of drinking behaviour. The changes in serum levels of relevant HPLC isoforms and assay results were similar days 1-11 but overall their values did not correlate.

The changes in total transferrin measured using the total area under the peaks and independently were similar (Figure 8.24.iv) but did not correlate significantly.

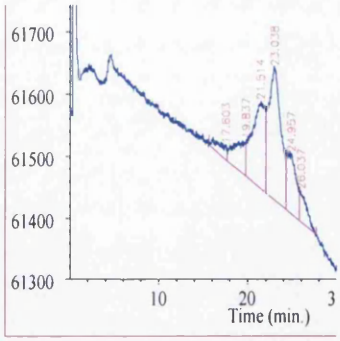
The mono- and di-sialotransferrin were poorly defined throughout in an individual in whom both the CDTect and AXIS %CDT levels remained within the reference range, despite intermittent alcohol misuse. Trisialotransferrin was seen throughout.

Figure 8.24: M031, non-cirrhotic female, false negative non-responder

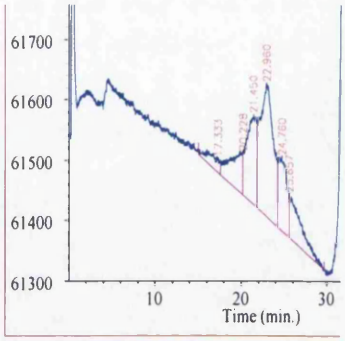
Day 1



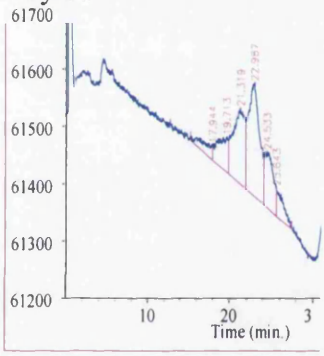
Day 3



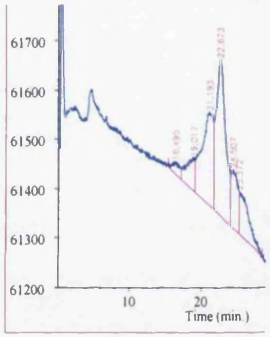
Day 5



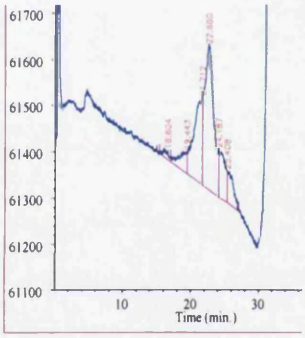
Day 11



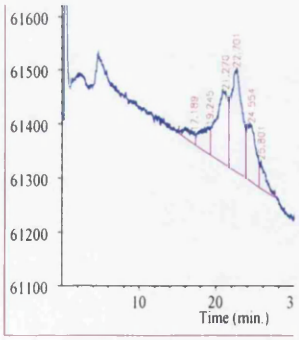
Day 30



Day 75

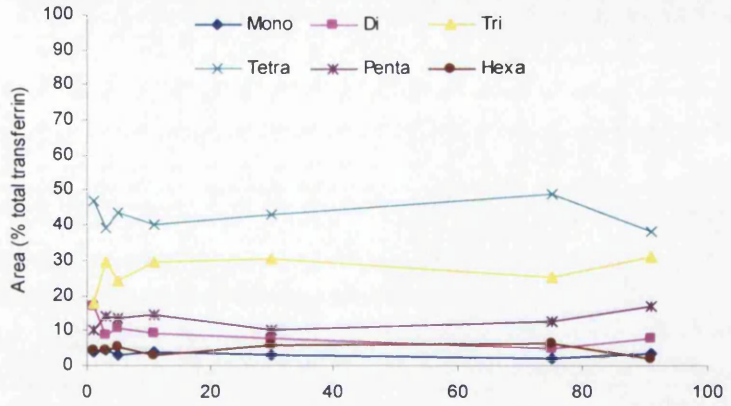


Day 91

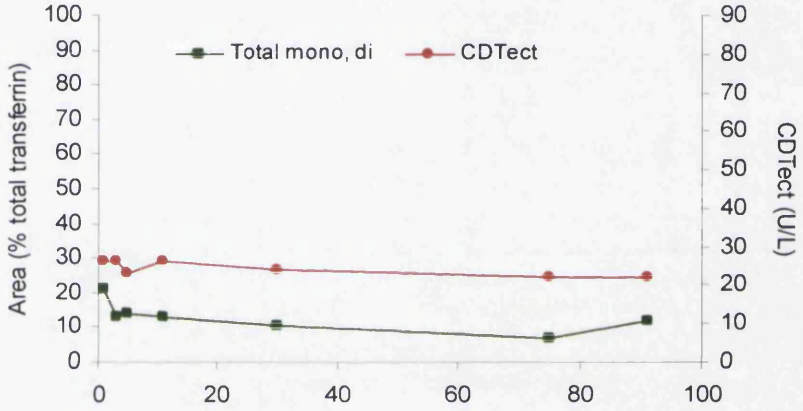


b).Isoform peak areas over time and in relation to CDTect, AXIS %CDT and total transferrin for M031

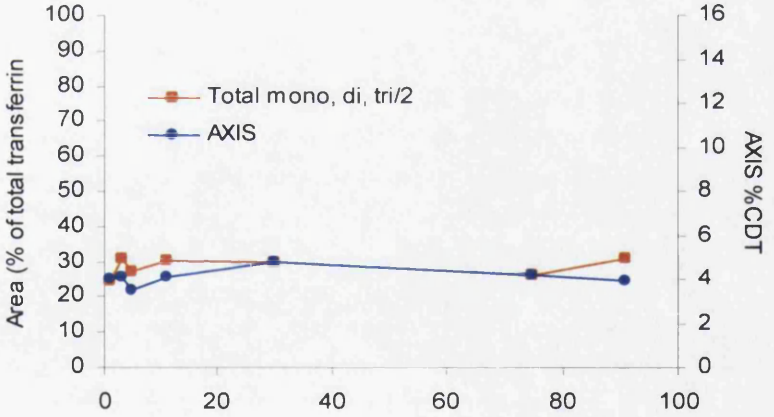
i)



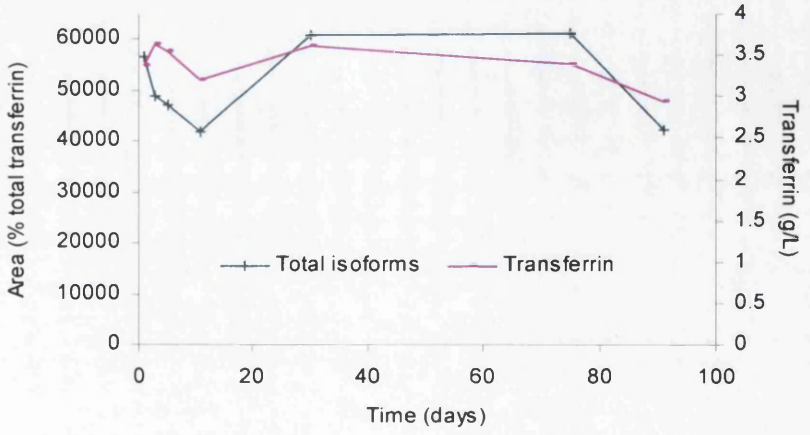
ii)



iii)



iv)



M017: cirrhotic female, false negative

This woman was drinking at day 1 but thereafter remained abstinent throughout the study period. Her serum CDTect and AXIS %CDT levels were within the reference range throughout the study period. Her serum transferrin was low throughout.

i) *Morphological assessment (Figure 8.25.a)*

The overall peak formations increased in size to day 30, decreased again to day 83 and then increased again at days 156 and 167. The mono- and di-sialotransferrin were poorly defined throughout, and trisialotransferrin eluted with tetrasialotransferrin. The pentasialotransferrin was identified and best seen at day 30. There were no changes between drinking and abstinence.

ii) *Computer assisted analysis (Figure 8.25.b)*

The serum CDTect and AXIS %CDT remained within the reference range throughout. The sum of mono- and di-sialotransferrin showed only small changes during the study period. These appeared to correspond with the markers as both CDTect and AXIS %CDT remained stable during the study period but were not statistically significant. The total transferrin measured both by the sum of the areas under the peaks and also independently showed a broad correlation, but were not significantly associated.

Summary for all three non-responders: M045, M031 and M017

Morphological assessment. The non-responder showed little morphological variation over time other than a change in the size of the peak formations. This indicated total transferrin levels and was shown to correlate with total transferrin measured independently. M045 tested false positive and mono- and di-sialotransferrin could be identified. M031 and M017 tested false-negative and the mono- and d-sialotransferrin were not easily identified. None of these individuals showed, using HPLC, a clear morphological difference between drinking and abstinence.

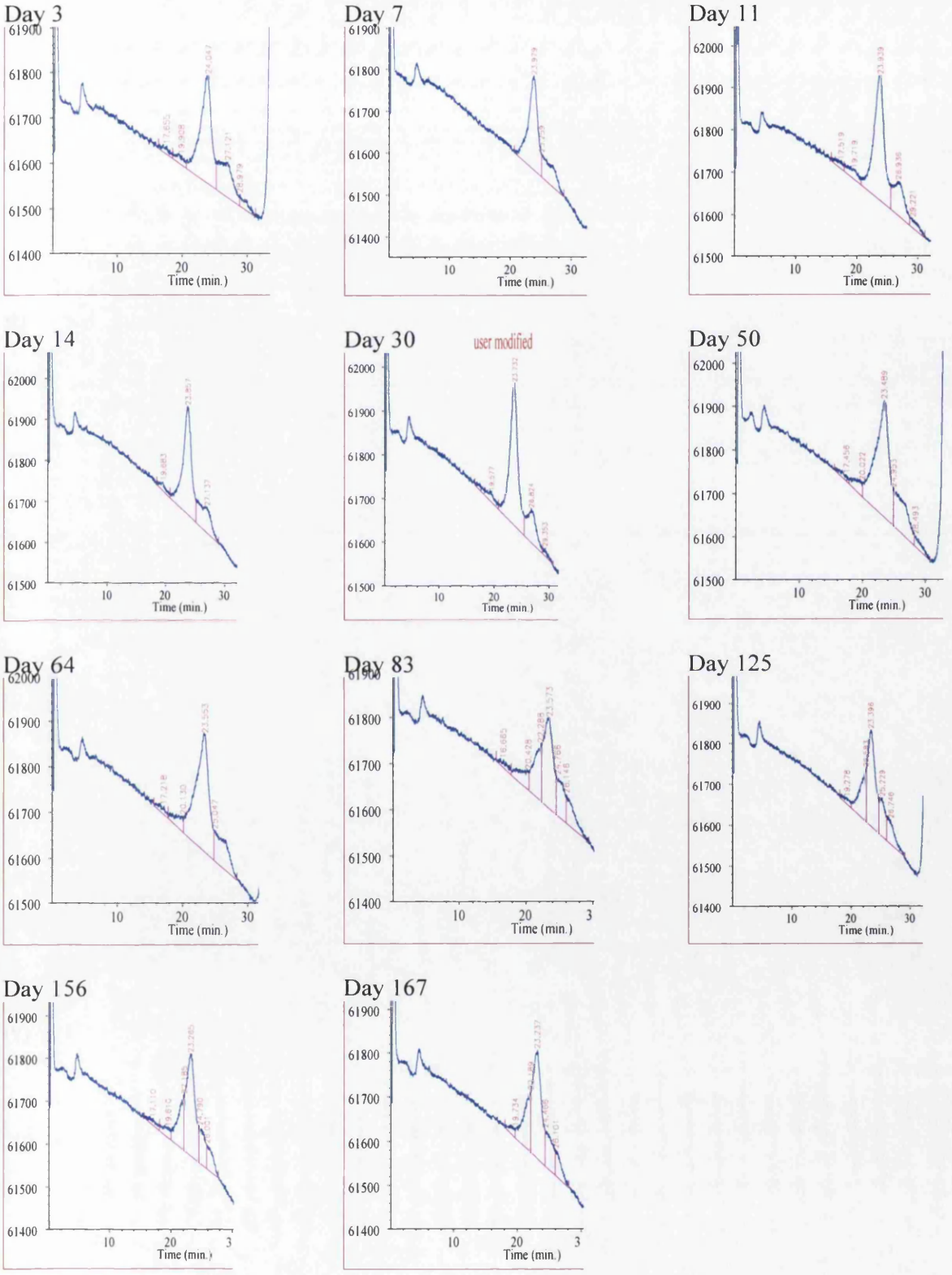
Computer assisted isoform analysis

The CDTect and AXIS %CDT levels remained unchanged, regardless of drinking behaviour, as did the sum of the relevant markers for the false-negative non-responders M031 and M017. However the relationship between the two was not a statistically significant correlation. M045,

the false-positive non-responder, showed changes in the levels of CDTect above the reference range while the levels of isoforms remained stable. There was not a significant correlation here.

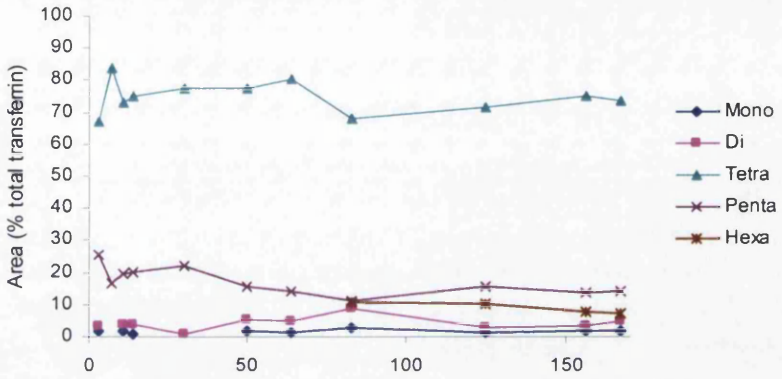
The total transferrin broadly correlated with the total transferrin measured independently, but was only significant for M045. The changes in transferrin levels fluctuated in the other two individuals.

Figure 8.25: M017, cirrhotic female, false negative

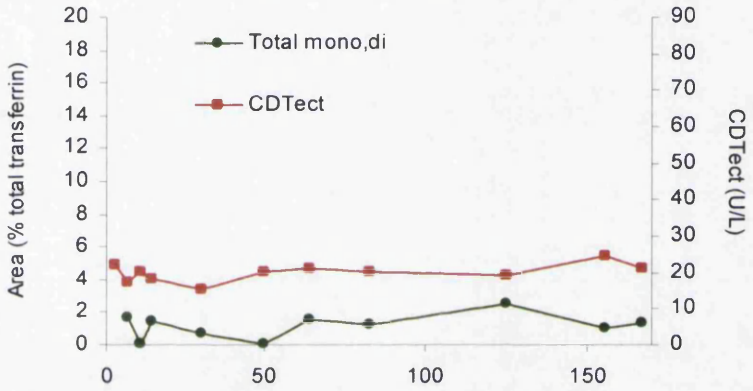


b) Isoform peak areas over time and in relation to CDTect, AXIS %CDT and total transferrin for M017

i)



ii)



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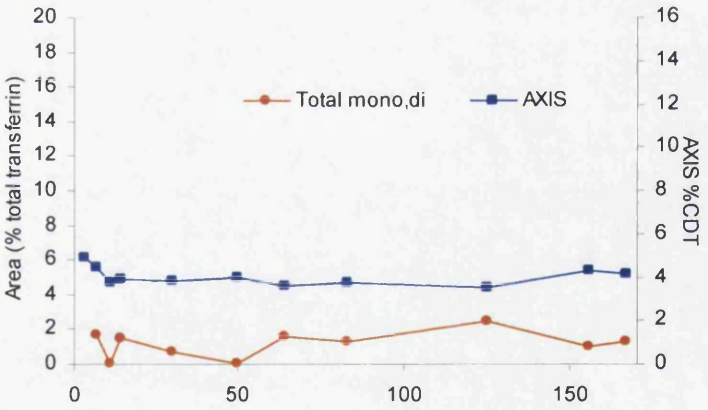
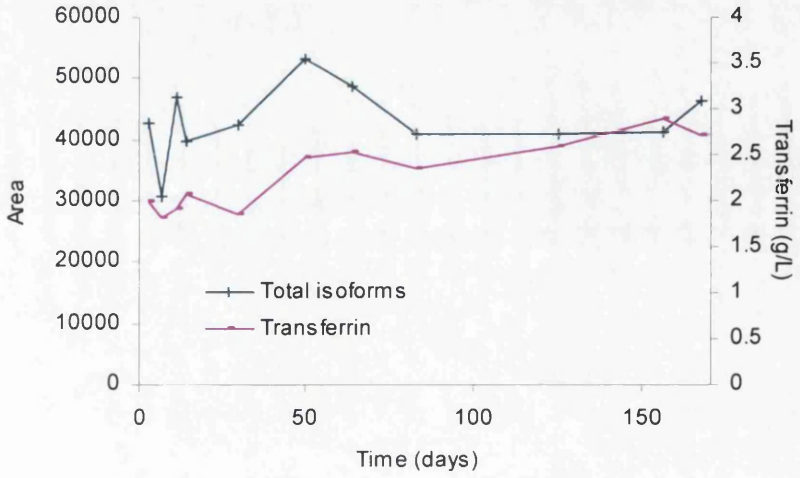


iv)



M028: cirrhotic female, CDTect responder, AXIS %CDT false negative non-responder

This woman was drinking at day 1 and thereafter remained abstinent throughout the study period. Her serum CDTect was initially raised when drinking but then fell to within the reference range with abstinence, a responder, but her AXIS %CDT remained within the reference range throughout the study period, false-negative.

i) *Morphological assessment (Figure 8.26.a)*

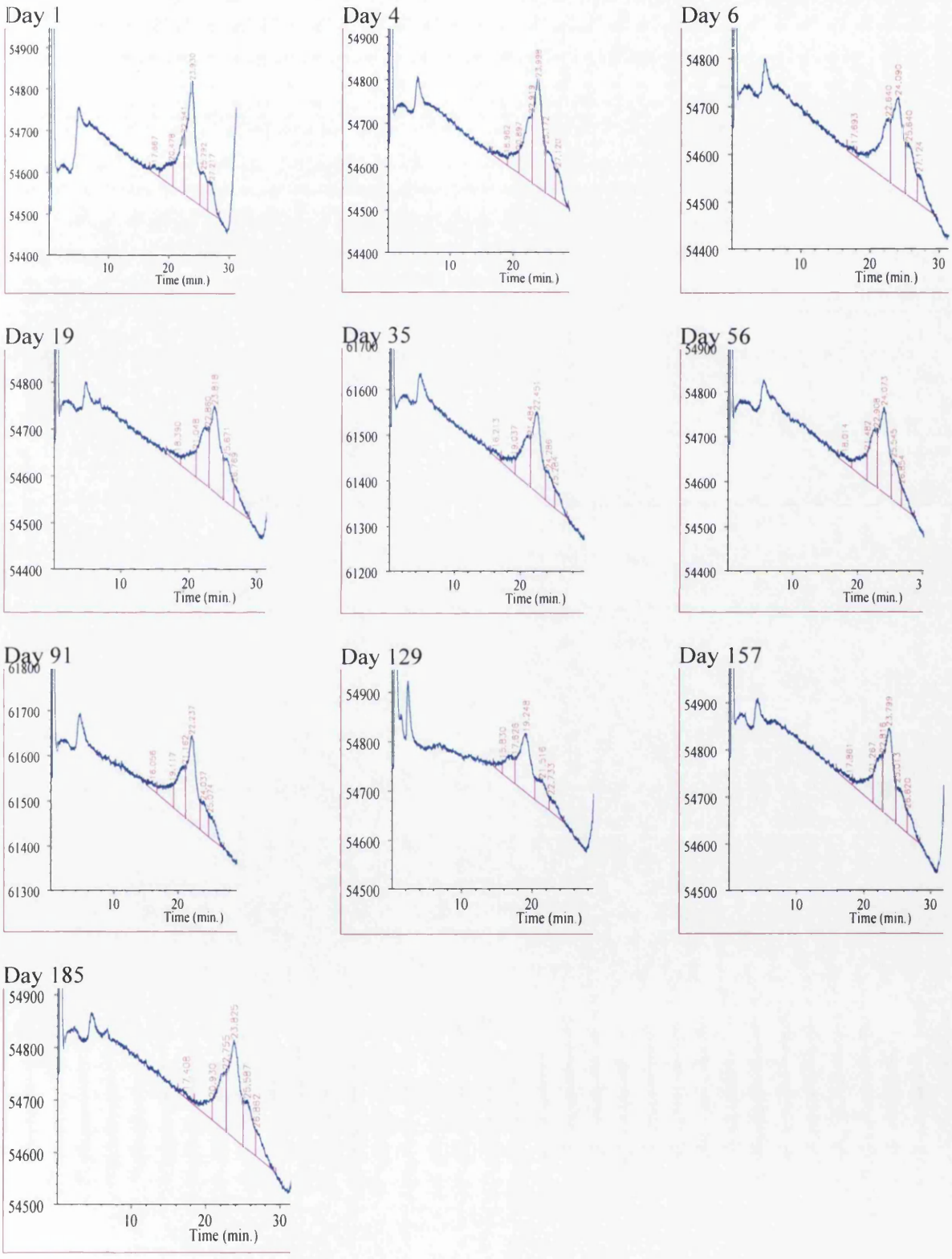
The mono- and di-sialotransferrin were poorly defined. Trisialotransferrin was poorly identified at day 1, but became increasingly more recognisably separate from the tetrasialotransferrin following abstinence and remained stable. Penta- and hexa-sialotransferrin were clearly identified on day 1, then decreased in size to day 35 and then increased again at day 129.

ii) *Computer assisted analysis (Figure 8.26.b)*

The isoforms proportions remained largely stable. The serum CDTect reflected the drinking behaviour but there was no correlation with the sum of the mono- and di-sialotransferrin isoforms. The AXIS %CDT and the sum of mono-, di and half of tri-sialotransferrin both remained unchanged, but the values did not correlate significantly. The total serum transferrin concentration obtained by direct measurement and by extrapolation from peak areas correlated significantly (p=0.016).

Her total transferrin was initially 3.68 g/L which then fell, but remained within the reference range during the study period. Her serum CDTect was elevated during drinking and then fell with abstinence. This may be explained, at least in part, by a high levels of the mono- and di-sialotransferrin which then fall as the total transferrin falls. The proportions of the mono- and di-sialotransferrin compared to the total transferrin remain the same. AXIS %CDT measures isoforms as a percentage of the total transferrin and so these changes were not reflected in an alteration in the measured AXIS %CDT levels. The computer assisted analysis of isoforms were measured as a percentage of total transferrin and so changes in the mono- and di-sialotransferrin were not seen. The total transferrin was seen to change when measured by both methods.

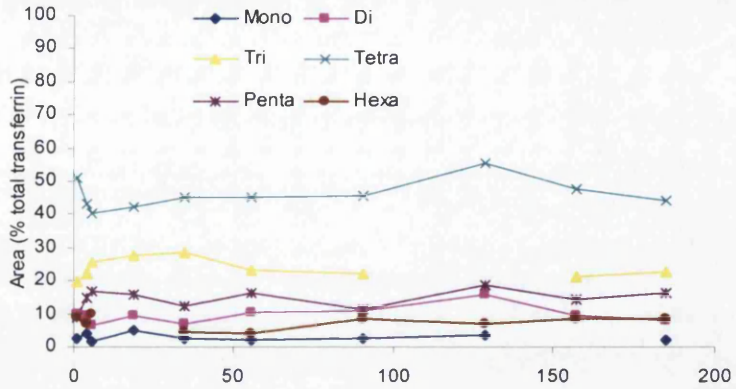
Figure 8.26: M028, cirrhotic female, CDTect responder, AXIS %CDT false negative non-responder



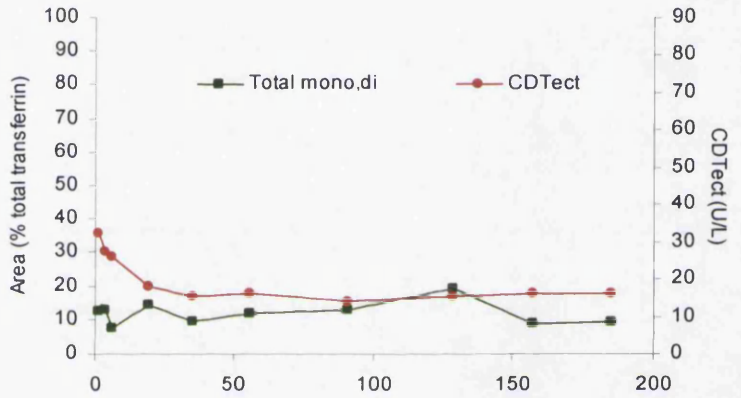
b) Isoform peak areas over time and in relation to CDTect, AXIS %CDT and total transferrin for M028

M028

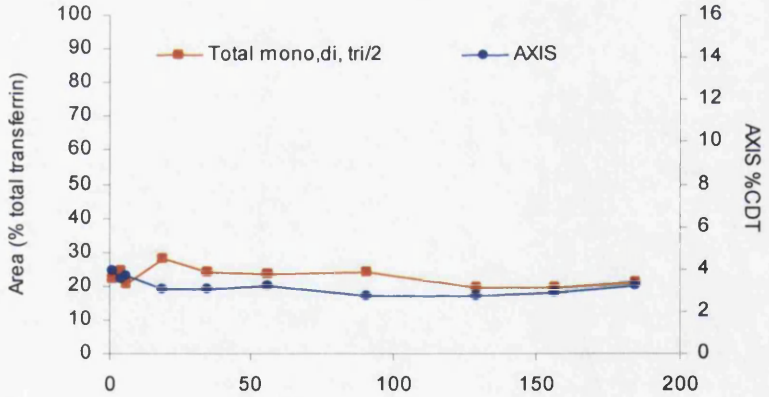
i)



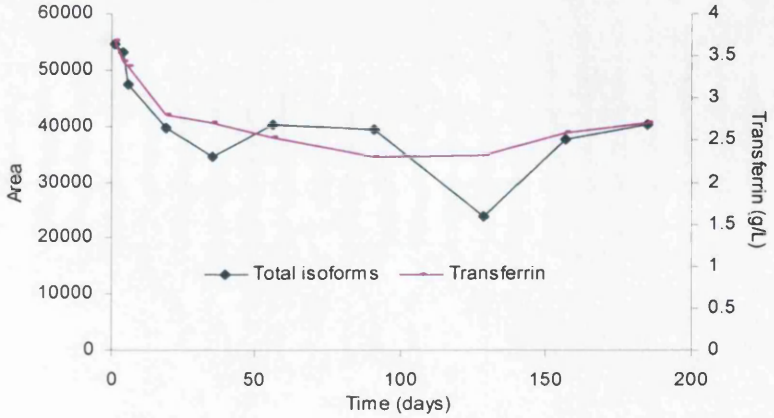
ii)



iii)



iv)



M029, male cirrhotic, false positive until undergoing orthotopic liver transplantation at day 148

This individual was drinking at day 1 but then remained abstinent throughout the rest of the study period. He was hospitalised virtually throughout the study time with deteriorating liver disease and malnutrition and underwent orthotopic liver transplantation on day 148. Additional observations were made during the post-transplant period.

i) *Morphological assessment (Figure 8.27.a)*

The peak profiles were small in comparison to other individuals studied. Penta- and hexa-sialotransferrin were identified. All the peaks were better seen post transplant when the level of total transferrin had increased. Changes pre-transplant were small, in part due to the small nature of the peaks. Trisialotransferrin can be poorly identified at days 128 and 145, but is better seen at day 344 post transplant. The chromatograms of days 1 to 145 were similar while at day 344 it was very different.

ii) *Computer assisted analysis (Figure 8.27.b)*

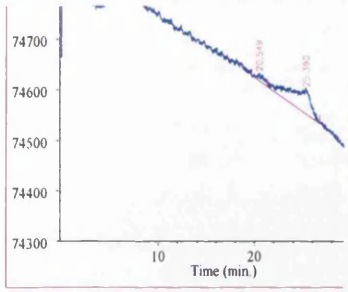
There were composite peaks of tri- and tetra-sialotransferrin. The levels of CDTect and AXIS %CDT were both above the reference range until he underwent transplantation, when they immediately fell within the reference range. There was no significant correlation between the sum of the isoforms from the HPLC chromatograms and the respective CDT measurements using the CDTect and AXIS %CDT assays.

This individual was drinking at day 1, but then remained abstinent through the rest of the study period. The most noticeable change in the chromatograms were the total area indicating changes in total transferrin. There were large fluctuations in the serum CDTect level which was above the reference range until he was transplanted. Some of these changes may be accounted for by changes in the total serum transferrin, although AXIS %CDT which expresses CDT as a proportion of total transferrin, still showed changes in level. The changes in CDTect and AXIS %CDT were much greater than the changes morphologically and so it is not surprising that the relevant HPLC isoforms did not correlate with the markers.

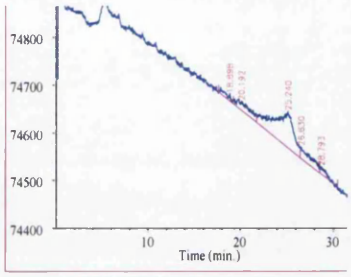
The total transferrin measured using HPLC and independently correlated significantly.

Figure 8.27: M029, male cirrhotic , false positive until transplanted on day 148

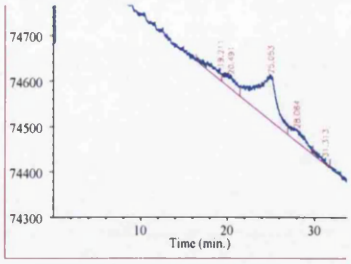
Day 1



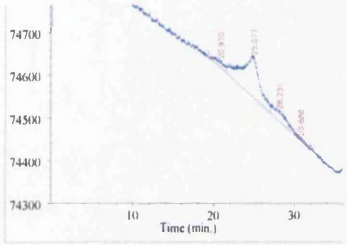
Day 12



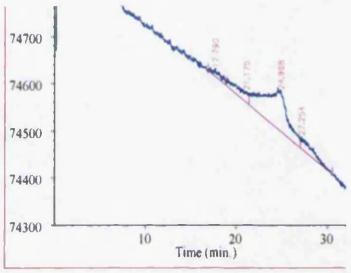
Day 22



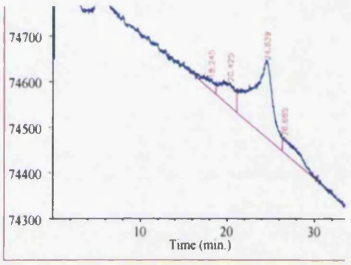
Day 41



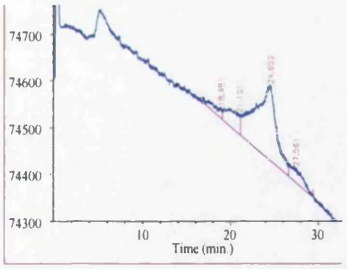
Day 61



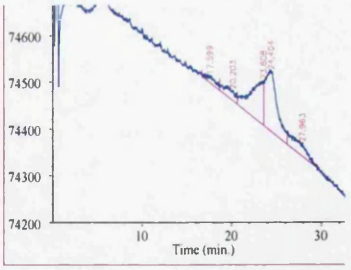
Day 76



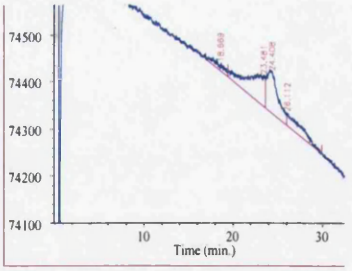
Day 99



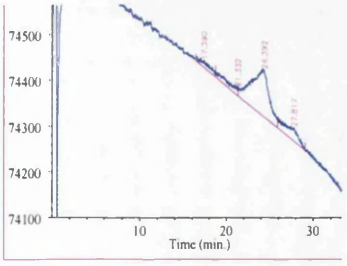
Day 128



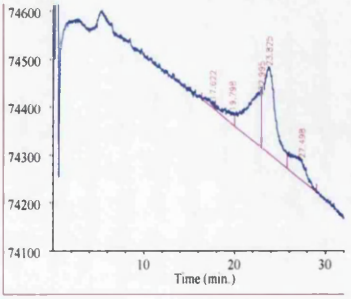
Day 145



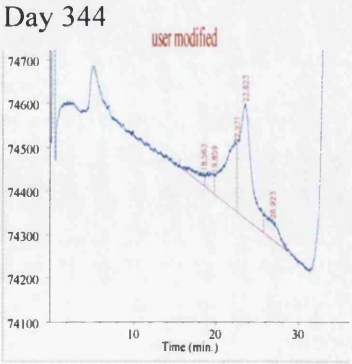
Day 152 (Post-OLT)



Day 180

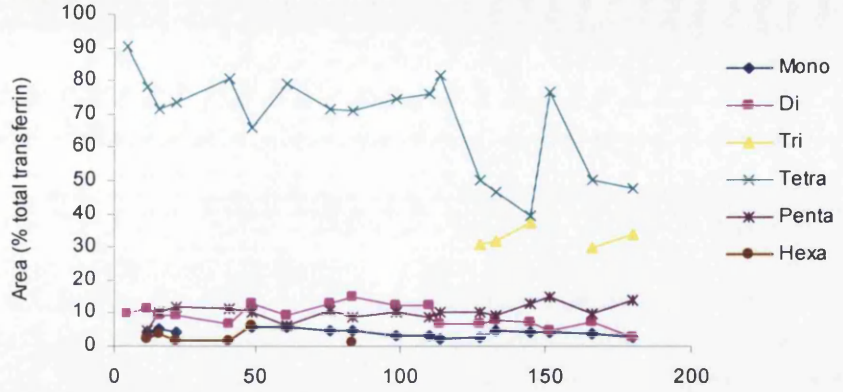


Day 344

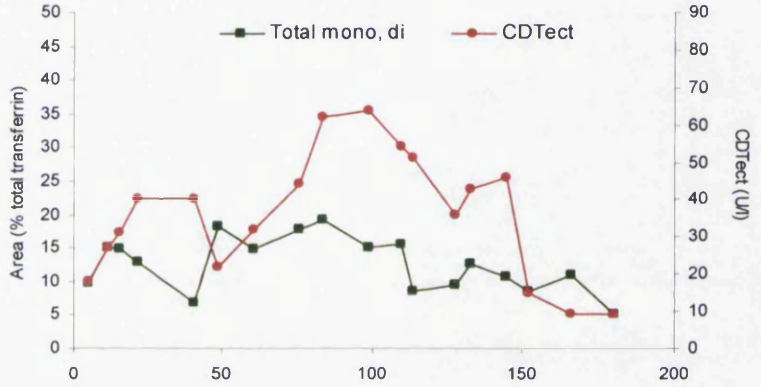


b) Isoform peak areas over time and in relation to CDTect, AXIS %CDT and total transferrin for M029

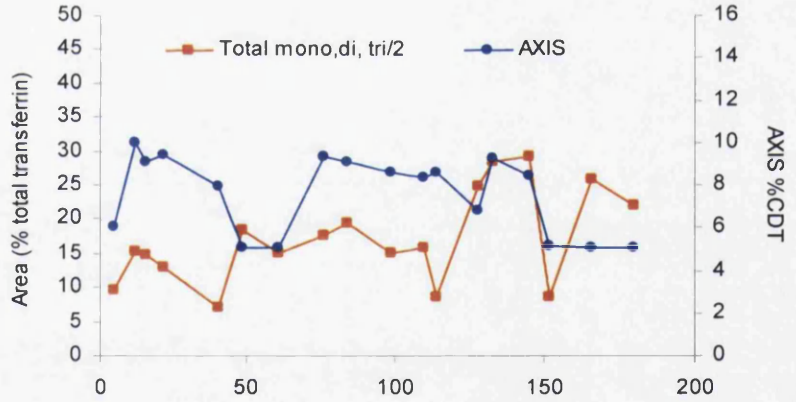
i)



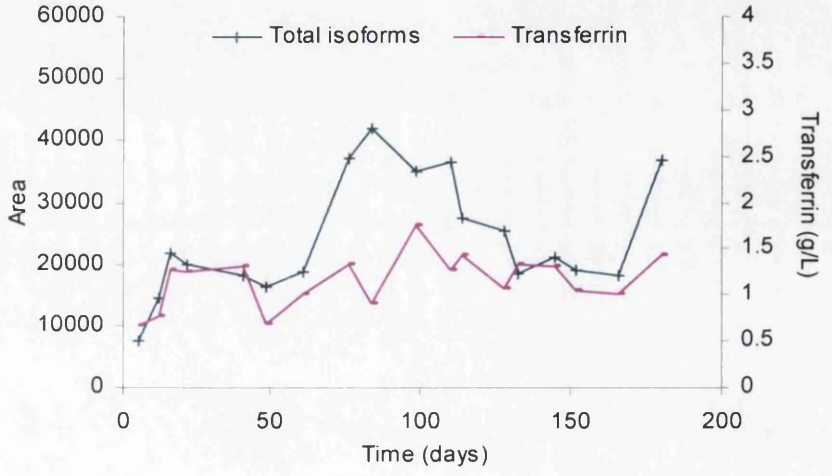
ii)



iii)



iv)



In summary, the peak formations were small and this reflected a low total transferrin but increased post-transplant. The chromatograms pre-transplant were all similar, while post-transplant showed changes by day 344. CDTect and AXIS %CDT were elevated pre-transplant but fell immediately post-transplant reflecting the influx of transfused blood. The markers and the relevant isoforms did not correlate significantly, while total transferrin measured from the chromatograms and independently showed a significant correlation.

8.2.7.4 Summary of HPLC chromatogram analysis of serial samples

The responders' chromatograms showed changes between drinking and abstinence. Mono- and di-sialotransferrin were seen during drinking and decreased with abstinence with corresponding changes seen in CDTect and AXIS %CDT levels. This was also seen in M028, a responder for CDTect. AXIS %CDT also detects trisialotransferrin and half is incorporated in the serum level. It could be anticipated that at raised levels of AXIS %CDT there might be more prominent trisialotransferrin and that this would decrease with AXIS %CDT levels. In the case of M028 the levels of AXIS %CDT remained unchanged while the trisialotransferrin actually increased during abstinence.

The false negative non responders (M031 and M017) showed no clear changes between drinking and abstinence. Mono- and di-sialotransferrin were poorly seen. This would be expected from the low levels of serum CDT detected using the CDTect and AXIS %CDT. Trisialotransferrin was seen in the non-cirrhotic individual (M031) but decreased in abstinence, it was not seen in the cirrhotic individual (M017). The higher isoforms, penta- and hexa-sialotransferrin, were well defined and it is possible that there was a general shift towards the higher transferrin isoforms.

The false positive non-responders (M045 and M029) showed no changes between drinking and abstinence. Mono- and di-sialotransferrin were present but trisialotransferrin was not consistently. Penta- and hexa-sialotransferrin were unchanged throughout. M029 had small overall peaks due to the low overall levels of transferrin, and mono- and di-sialotransferrin were seen as small. Trisialotransferrin was seen when very unwell and may be unrelated to drinking behaviour. Once transplanted trisialotransferrin can be seen in M029.

There were no clear changes in the non-responders. Within the responders changes can be seen within the individual to identify drinking and abstinence. Mono- and di-sialotransferrin were

better seen in those with a raised level of serum CDT, regardless of drinking behaviour. This was expected. The presence or absence of trisialotransferrin is one of the most prominent features of the chromatograms. Changes can be seen with drinking behaviour in the two responders, but no clear pattern can be identified for all those tested.

Overall with higher levels of transferrin the peak areas are increased and are decreased with lower levels.

The computer assisted analysis of isoforms showed a poor correlation with the levels of CDTect and AXIS %CDT. The isoform levels were measured as areas and then expressed as a percentage of total transferrin, rather than as absolute values. Changes seen graphically represent changes in the proportions of the overall transferrin. This could explain some of the poor correlation with CDTect levels. AXIS %CDT levels reflects the levels as a proportion of the total transferrin so it was disappointing that a better correlation was not seen. The computer assisted analysis involved measurements made manually and so human error may have been a factor.

There was a good correlation between total transferrin measured by the two methods.

8.3 Discussion

Tetrasialotransferrin was the predominant isoform, giving the largest peak, and it was assumed that those isoforms with earlier retention times corresponded with mono-, di- and tri-sialotransferrin. Werle *et al* and Jeppsson *et al* were able to detect asialotransferrin but not monosialotransferrin. Bean *et al* could not confirm asialotransferrin or monosialotransferrin to be present. Jeppsson *et al* confirmed the individual isoforms using isoelectric focusing but none of the other authors cite a method of confirmation.

Some individuals measured serially showed changes that did not correlate with serum CDT status, measured either with CDTect or AXIS %CDT. This may have arisen as the proportion of peak area attributable to mono-, di- and trisialotransferrin was small and any changes in these were small. However small changes were seen but graphically these were independent of the changes in serum CDTect and AXIS %CDT.

Serum CDT assays have been shown to give false positive and false negative results (Chapter 4). It may be that, at least in part, some of the poor correlation between the HPLC isoforms and CDTect and AXIS %CDT may be that the assays are not detecting some or all of the isoforms. This has been suggested by Arndt *et al* (Arndt et al. 1998) who used isoelectric focusing to validate each of the stages in the CDTect assay. The CDTect assay used was the RIA method, a fore-runner and different from the EIA method we used. They found that the CDTect minicolumn retained some disialotransferrin so that some results would be falsely low.

The HPLC method developed here gave chromatograms which, in the limited number of individuals studied, showed isoform changes between drinking and abstinence in responders to a greater extent than non-responders. These suggest that alcohol affects the transferrin isoform distribution in some individuals but not in others and a more extensive analysis would be required to identify the specific groups.

9. A STATISTICAL EQUATION FOR THE DETECTION OF ALCOHOL MISUSERS

9.1 Introduction

Chapter 4 showed that the sensitivity and specificity of carbohydrate deficient transferrin (CDT), using either CDTect or AXIS %CDT, was not sufficiently high to be advocated as a screening tool alone, even in highly selected populations. Chapter 7 confirmed that, in the context of monitoring, CDT may be useful on an individual basis, but cannot be recommended in all alcohol misusers.

Comparative studies have shown CDT to have a sensitivity and specificity similar to gamma glutamyl transferase (GGT) (Poupon et al. 1989; Schellenberg et al. 1989; Anton and Moak, 1994; Wickramasinghe et al. 1994; Yersin et al. 1995; Helander and Tabakoff, 1997; Lesch et al. 1996; Bell et al. 1994) and that aspartate transaminase (AST) and erythrocyte mean corpuscular volume (MCV) are also useful screening tools (Gjerde et al. 1988; Stibler et al. 1988; Kwoh-Gain et al. 1990). In practice markers are used in combination and this has been shown to increase sensitivity (Anton and Moak, 1994; Lof et al. 1994; Helander et al. 1996; Huseby et al. 1997). Markers have been used in combination with a weighted score to increase the diagnostic value (Sillanaukee, 1992; Hartz et al. 1997) but these have not incorporated carbohydrate-deficient transferrin.

Aim: We investigated the best way, statistically, to utilise the combination of these markers in the creation of an equation

Purpose: To create an equation to predict the chances of an individual being an alcohol misuser.

9.2 Patients and methods

A study population, described previously in chapter 4.3.1 as the retrospective collection, was used for analysis. The markers used were MCV, AST, GGT and CDT (both CDTect and AXIS %CDT). An equation to estimate the probability that an individual was an alcohol misuser was

created using a multivariate analysis and logistic regression model of all these markers combined. The alcohol misusers formed one group and were used as the 'gold standard'. The non-drinking population was treated as the alternative. Each of the assays in each individual was scored 'raised' or 'normal' if above or within the reference range, respectively. Different equations were created for men and women and for CDTect and AXIS %CDT and were titled the 'discriminant function'.

9.3 'Discriminant Function' equations

Four different equations were compiled:

- Using CDTect, GGT & MCV in males
- Using CDTect, GGT & MCV in females
- Using AXIS %CDT, GGT & MCV in males
- Using AXIS %CDT, GGT & MCV in females

Each equation was devised as:

$$\text{probability (drinker)} = \frac{e^y}{1 + e^y}$$

9.3.1 Using CDTect

Males:

$$\text{Log}_e \frac{p}{1-p} = y = -3.92 + 1.54 \text{ (if raised MCV)} + 0.89 \text{ (if raised CDTect)} + 3.45 \text{ (if raised GGT)}$$

Females:

$$\text{Log}_e \frac{p}{1-p} = y = -2.17 + 1.54 \text{ (if raised MCV)} + 0.89 \text{ (if raised CDTect)} + 2.05 \text{ (if raised GGT)}$$

9.3.2 USING AXIS %CDT

Males:

$$\text{Log}_e \frac{p}{1-p} = y =$$

-4.32
+ 1.26 (if raised MCV)
+ 1.66 (if raised AXIS %CDT)
+ 3.44 (if raised GGT)

Females:

$$\text{Log}_e \frac{p}{1-p} = y =$$

-2.36
+ 1.26 (if raised MCV)
+ 1.66 (if raised AXIS %CDT)
+ 2.01 (if raised GGT)

These equations were first applied to the population from which they had been derived, the retrospective collection. They were then applied to a test population, the contemporary collection, for comparison. In each population the sensitivity and specificity of these equations was calculated.

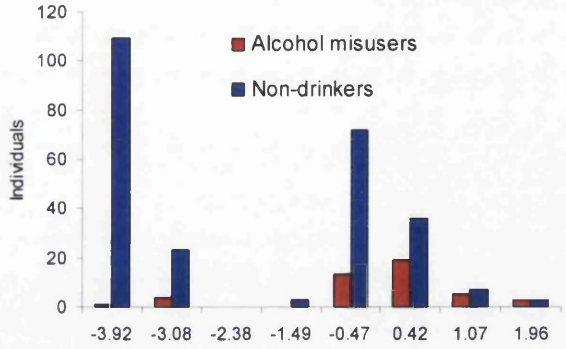
9.4 Results

The results for the alcohol misusers were plotted with the results for the non-drinking population for each equation (Figure 9.1). By the nature of the equations, once they were applied to the population from which they were derived, the results occurred in a step-wise fashion, rather than as a continuum. Figure 9.1 shows that the 'discriminant function' equation did not clearly differentiate the alcohol misusers population, with higher 'discriminant function' values from the total non-drinking populations. However, the non-drinking population contained diverse groups of 496 individuals: healthy volunteers, non-alcoholic liver disease and abstinent alcohol misusers. This then formed a spectrum bias against the 94 healthy volunteers.

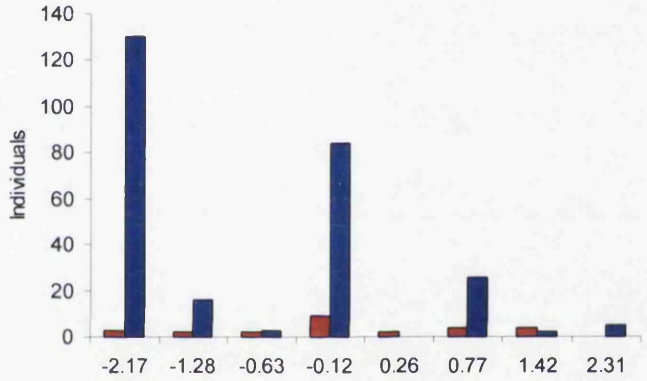
Therefore the results were re-analysed using the active alcohol misusers as the test group, as previously, but using only the healthy volunteers as the control group. These are shown in Figure 9.2. When the healthy volunteers 'discriminant function' equations were plotted against the alcohol misusers alone, in the male equations there were two discrete populations, but the female equations showed more diffuse values for the alcohol misusers.

Figure 9.1: 'Discriminant function' equation values in the alcohol misusers and total non-drinking population

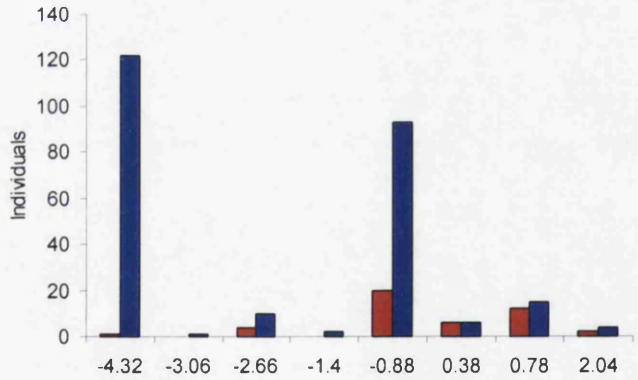
CDTect men



CDTect women



AXIS %CDT men



AXIS %CDT women

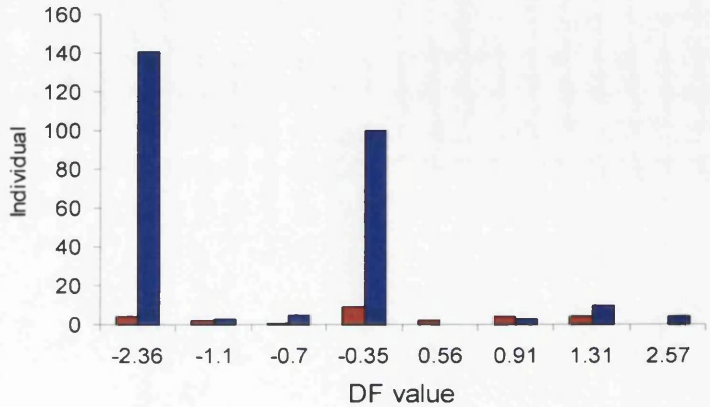
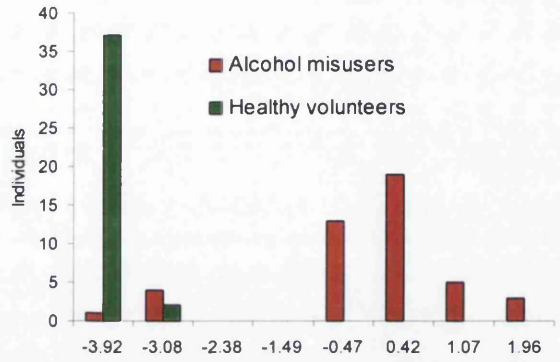
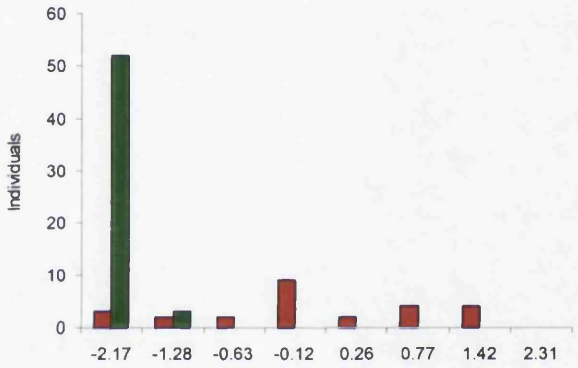


Figure 9.2: 'discriminant function' equation values for the alcohol misusers and healthy volunteers

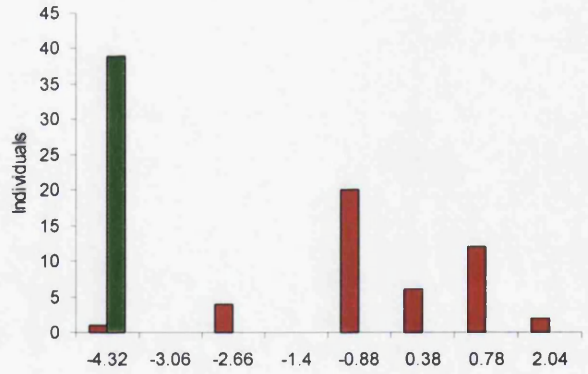
CDTect men



CDTect women

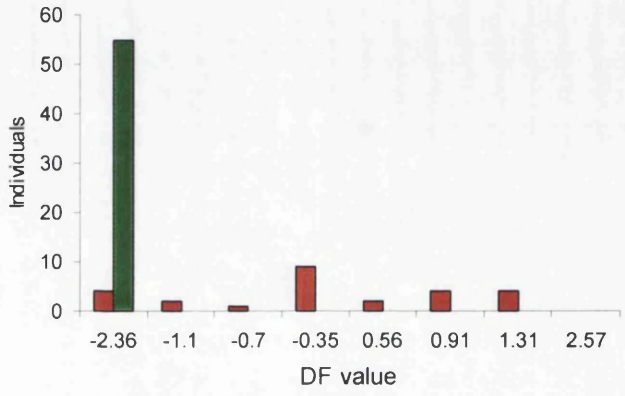


AXIS %CDT men



AXIS %CDT

women



9.4.1 CHOICE OF CUT-OFF

Ideally a cut-off value used to define an alcohol misuser would clearly separate the drinking from the non-drinking populations. As the drinking and non-drinking populations did not form two discrete populations, the choice of cut-off was not obvious, particularly in the female population. The equations gave values in eight defined steps for each (Figures 9.1). To try to establish the optimal cut-off, each of the possible outcome results for each equation was used as a cut-off. A result above the cut-off in the alcohol misuse population was defined as true positive, below the cut-off as false negative. A result above the cut-off in the abstinent population was defined as false positive and bellow as true negative. Using these results the sensitivity and specificity for each was calculated. Ideally a sensitivity and specificity of 95% was required for each equation so that they could be confidently used to identify alcohol misusers. In these calculations the control population was the total abstinent group. The sensitivity and specificity was plotted against the cut-offs, for each equation (Figure 9.3).

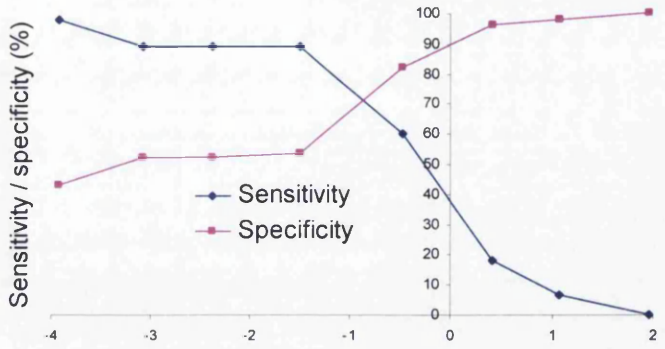
Figure 9.3 again demonstrates that there was not one single cut-off for each equation that could be used to define alcohol misusers. From the plots the best compromise cut-off for each equation would be at about 65%. This would be unacceptable in terms of either sensitivity or specificity. Two cut-offs would be required for each group, one to give a sensitivity of 95%, to give a 95% chance that the individual is an alcohol abuser, and another to give specificity of 95%, to give a 95% chance that the individual is not an alcohol abuser.

These results were interpreted with reference to the relevant equations. For the women's equations the cut-off would be so low that any result obtained, including all three markers being within the reference range, would result in the individual being classified as a drinker, or 100% sensitivity. For the male equations the sensitivity cut-off would be such that if any one of the blood results were positive then the cut-of would be exceeded and the individual defined as an alcohol misuser.

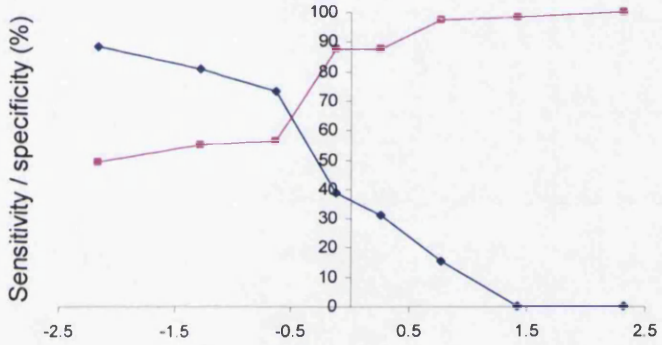
The cut-offs to give 95% specificity for the CDTect equations, either men or women, would allow any one test to be positive, or the combination of MCV and GGT, and the individual would still be classified as true negative. For AXIS %CDT any single test, or the combination of MCV and GGT or MCV and AXIS may be raised, and the individual tested as true negative.

Figure 9.3 Sensitivity and specificity of 'discriminant function' equations at each cut-off

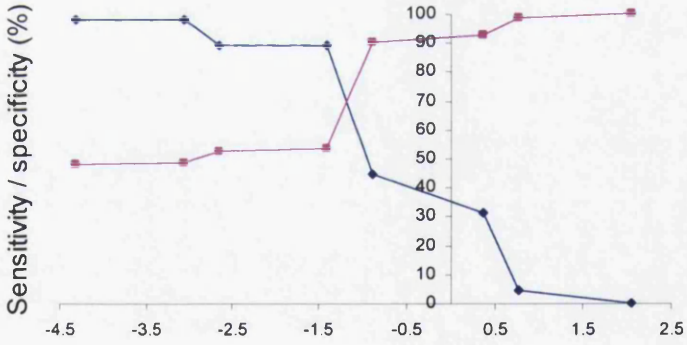
CDTect in men



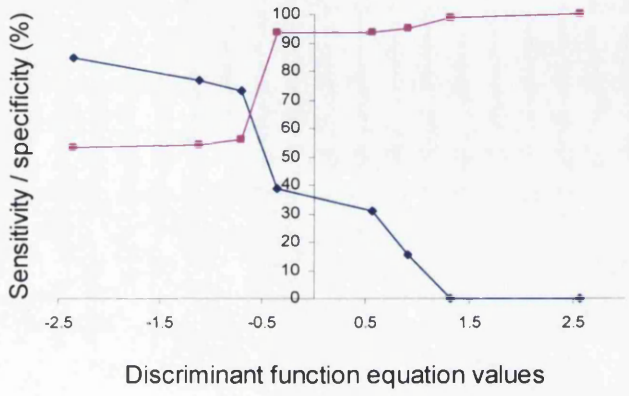
CDTect in women



AXIS %CDT in men



AXIS %CDT in women



9.4.2 APPLICATION OF 'DISCRIMINANT FUNCTION' EQUATIONS TO THE TEST POPULATION

The equations were tested by applying them to a new population. The population used was the 'contemporary collection', as described in chapter 4.4.2. The sensitivity and specificity was then calculated at each possible cut-off for each equation, as with the retrospective collection.

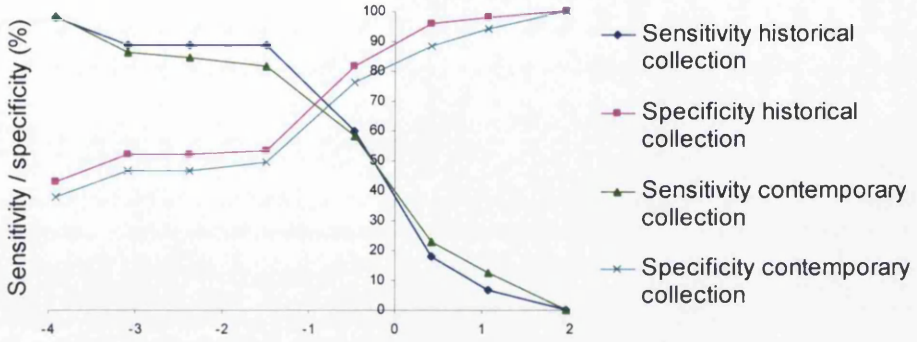
To compare these results with those obtained with the retrospective collection, the sensitivities and specificities derived from the application to this test population were again plotted against the cut-offs. These results were compared with those of the retrospective collection (Figure 9.4).

In this population, as with the retrospective collection, there was no single cut-off for each of the equations from which 95% sensitivity and specificity could be obtained. The sensitivity and specificity results for CDTect (men) were very similar for the two populations. For the CDTect (women) the intercept between sensitivity and specificity was at 77%, higher than in the retrospective collection. Both of the AXIS equations showed a similar intercept between sensitivity and specificity for both populations but at different cut-offs.

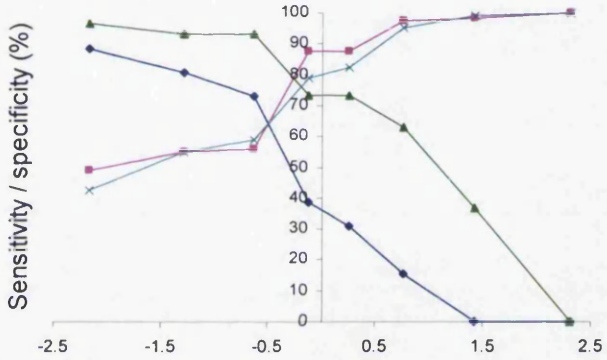
In order to maximise the potential of the equations further optimisation of the cut-offs was required.

Figure 9.4: Sensitivity and specificity of 'discriminant function' equations for both the retrospective and contemporary collections

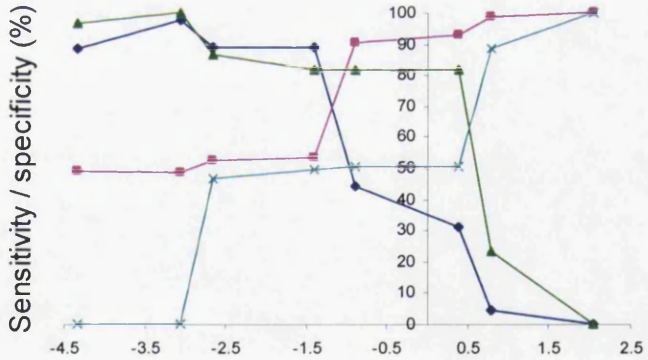
CDTect in men



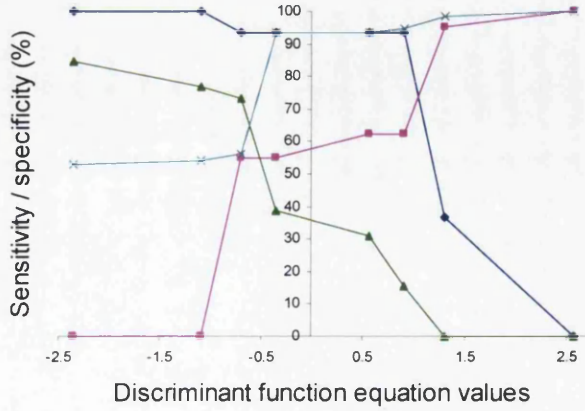
CDTect in women



AXIS %CDT in men



AXIS %CDT in women



9.4.3 OPTIMISATION OF CUT-OFFS USING THE HEALTHY VOLUNTEER POPULATION

9.4.3.1 Method

Alteration of the cut-off used to define a raised or positive 'discriminant function' value had been explored. Previously values either above or below the manufacturer's or hospital laboratory reference-ranges were used for the markers in the equations. The effect of using a value above or below a reference range determined by the results obtained from the healthy volunteers group was investigated.

The retrospective collection was first re-assessed. The mean + two standard deviations of the healthy volunteers was used to determine the cut-off for each marker within the equation. Then, using these calculated cut-offs the four equations were applied and the sensitivity and specificity calculated as before for each possible 'discriminant function' value. This was then repeated for 1 and 3 standard deviations

9.4.3.2 Results

Table 9.1 shows the calculated mean + one, two and three standard deviations of the healthy volunteers level for each marker. These were used to define marker cut-offs in each of the four equations.

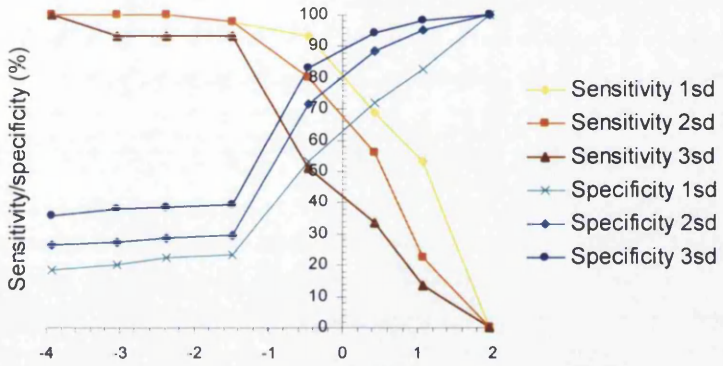
Table 9.1: Marker cut-offs calculated from the healthy volunteers group

	Mean + 1SD	Mean + 2SD	Mean + 3SD
MCV	92.1	95.3	98.5
GGT	21.6	27.8	34.0
CDTect (males)	16.7	21.0	25.4
CDTect (females)	21.2	26.3	31.5
AXIS %CDT (males)	4.8	5.1	6.7
AXIS %CDT (females)	4.7	5.4	6.1

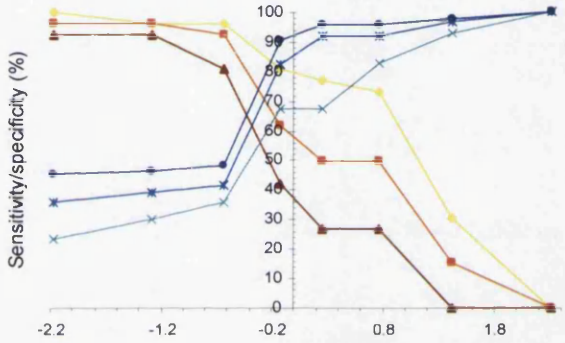
The equations were then applied to the study population. From these the sensitivity and specificity were then calculated. The results are shown in Figure 9.5.

Figure 9.5: Sensitivity and specificity at all 'discriminant function' values, using marker cut-offs calculated from 1, 2 and 3 standard deviations of the healthy volunteers

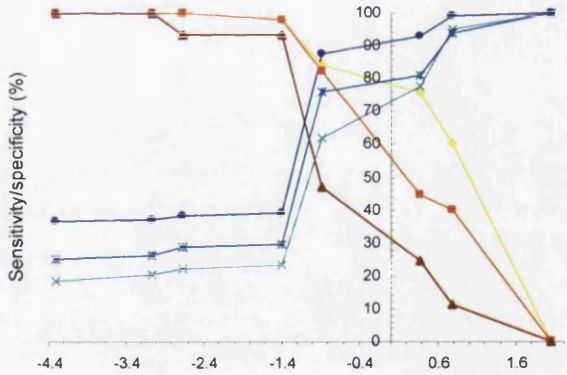
CDTect men



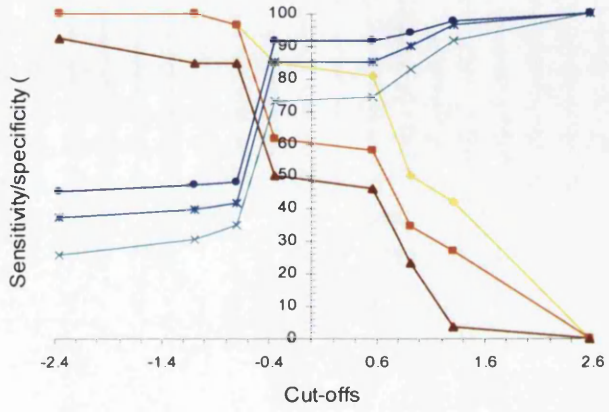
CDTect women



AXIS %CDT men



AXIS %CDT women



In chapter 4 it was demonstrated that at a lower cut-off the sensitivity is raised and the specificity is lowered. The alteration of the marker cut-offs used within the equations showed that a lower cut-off for the markers results in a larger number of individuals having a positive or raised assay result. This in turn results in a higher 'discriminant function' score, or a larger number of drinking individuals classified as true positive, a raised sensitivity, and a larger number of the non-drinking population testing false positive, or a lower specificity. This was demonstrated with all four equations (Figure 9.5).

At any one cut-off it was impossible to achieve 95% sensitivity and specificity. The intercept between the sensitivity and specificity plots was approximately 75% for each equation, which remains unacceptable and so again it was impossible to find a single cut-off for each equation. Using this method of defining the reference range did not give any advantage over using the laboratory and manufacturer's reference ranges.

However, using the healthy volunteers to define the assay reference range offers the advantage of being applicable to users outside the Royal Free Hospital where laboratories may have alternative defined reference ranges. There does not seem to be any other advantage offered.

9.5 Revised equations

The equations have been shown to be sub-optimal with particular regard to the difficulty finding a cut-off to give an acceptable results in terms of sensitivity and specificity, even in the population from which the equations were derived before they are then applied to a test population. The limiting factors were that the equations used either a raised or normal result of each marker, and gave results in 'steps' of values, rather as a continuum. Furthermore an individual with a particularly high marker result was not discriminated from an individual with a result which was mildly raised. This may have accounted for some of the poor specificity results as there was little discrimination in this diffuse groups of individuals which included healthy volunteers and abstinent alcohol misusers.

The equations were revised using the absolute value for each blood marker used, as opposed to a result above or within the reference range. In the previous set of equations the size of the non-drinking population was much greater than the size of the drinking population. It also contained several different sub-groups: healthy volunteers, hospital inpatients, non-alcoholic liver disease and abstinent alcoholic liver disease. Statistically this can result in 'spectrum

bias' when it is used in comparison with the smaller and more uniform alcohol misusers. The equations were re-defined using as a control population both the healthy volunteers and also all of the non-drinking population as two different equations. As the actual result of the marker assay was used different equations were not devised for men and women.

9.5.1.1 Using all non-alcohol abusers as the Control Population

$$\text{Log}_e \frac{p}{1-p} = y =$$

-14.96
+ 0.13 x MCV
- 0.00018 x AST
+ 0.00058 x GGT
+ 0.0.4 x CDTect

9.5.1.2 Using the Healthy Volunteers as a Control Population

$$\text{Log}_e \frac{p}{1-p} = y =$$

-7.79
+ 0.068 x MCV
+ 0.0023 x AST
+ 0.0014 x GGT
+ 0.018 x CDTect

9.5.1.3 Using all non-alcohol abusers as the Control Population

$$\text{Log}_e \frac{p}{1-p} = y =$$

-14.12
+ 0.11 x MCV
- 0.00011 x AST
+ 0.00074 x GGT
+ 0.36 x AXIS %CDT

9.5.1.4 Using the Healthy Volunteers as a Control Population

$$\text{Log}_e \frac{p}{1-p} = y = -7.83 + 0.064 \times \text{MCV} + 0.0027 \times \text{AST} + 0.0014 \times \text{GGT} + 0.15 \times \text{AXIS \%CDT}$$

9.5.2 APPLICATION OF THE 'REVISED EQUATIONS' TO THE RETROSPECTIVE COLLECTION

Each of these equations were then applied to the retrospective population and the sensitivity and specificity calculated. Individuals with particularly high marker results gained higher 'discriminant function' values. Once the equations had been applied to population, the mean + two standard deviations of the healthy volunteers was used to define the reference-range of 'normal' values (Table 9.2) so that the sensitivity and specificity could be calculated.

Table 9.2: Cut-offs calculated from the mean + 2 standard deviations of the healthy volunteers used to calculated sensitivity and specificity

	CDTect		AXIS %CDT	
	Using all the non-drinking groups as controls	Using the healthy volunteers as controls	Using all the non-drinking groups as controls	Using the healthy volunteers as controls
Mean + 2 standard deviations of the healthy volunteer population	-1.87	-0.93	-1.95	-0.97

The two equations each for CDTect and AXIS %CDT were then compared using the Chi-square test to determine if there was any difference in efficacy between using the healthy volunteers and all the non-drinking group as controls.

Table 9.3: Sensitivities and specificities of the revised 'discriminant function' equations applied to the retrospective population

CDTect

Study subgroup	Using all the non-drinking groups as controls		Using the healthy volunteers as controls	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Healthy volunteers (n=94)		100		100
Hospital inpatients (n=88)		94.3		93.2
Non-alcoholic liver disease (n=197)		81.7		55.3
Non-cirrhotic (n=101)		*87.1*		*57.4*
Cirrhotic (n=96)		*76.0*		*53.1*
Abstinent alcohol misusers (n=140)		62.9		54.3
Non-cirrhotic (n=48)		*66.7*		*58.3*
Cirrhotic (n=92)		*60.9*		*52.2*
Drinking alcohol misusers (n=71)	73.2		83.1	
Non-cirrhotic (n=40)	*80.0*		*87.5*	
Cirrhotic (n=31)	*64.5*		*77.4*	
Total	**73.2**	**83.1**	**83.1**	**69.6**

AXIS %CDT

Study subgroup	Using all the non-drinking groups as controls		Using the healthy volunteers as controls	
	Sensitivity (%)	**Specificity (%)**	**Sensitivity (%)**	**Specificity (%)**
Healthy volunteers (n=94)		98.9		98.9
Hospital inpatients (n=88)		96.6		95.5
Non-alcoholic liver disease (n=197)		85.3		58.9
Non-cirrhotic (n=101)		*93.1*		*60.4*
Cirrhotic (n=96)		*77.1*		*57.3*
Abstinent alcohol misusers (n=140)		64.3		56.4
Non-cirrhotic (n=48)		*66.7*		*64.6*
Cirrhotic (n=92)		*63.0*		*52.2*
Drinking alcohol misusers (n=71)	71.8		78.9	
Non-cirrhotic (n=40)	*82.5*		*87.5*	
Cirrhotic (n=31)	*58.1*		*67.7*	
Total	**71.8**	**84.0**	**78.9**	**71.7**

The new equation provides specificities of >95% for the healthy volunteers, whether CDTect or AXIS %CDT is used. The specificity then decreases with increasing histological injury, so that the abstinent alcoholic cirrhotics have a specificity less than 65%, regardless of which equation is used. This is not surprising as the equations use AST which may often be increased in cirrhotic liver disease.

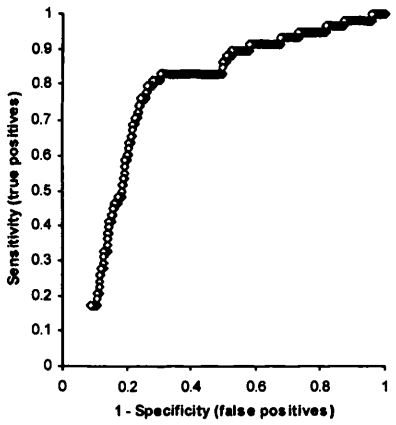
However, despite the fact that 95% specificity can be achieved in the healthy volunteers, and so it can be reasonably determined whether an individual is not an alcohol misuser, the sensitivity was lower. Overall for these equations, at these cut-offs, the sensitivity was between 71.8 and 83.1%.

The two equations were compared using the Chi-Square test. The only group where there was a significant difference between the two equations, for both CDTect and AXIS %CDT, was in the non-alcoholic liver disease group ($p<0.0001$). The equation using all the non-alcohol misusers as the control group was significantly higher for the non-cirrhotics ($p<0.0001$) and the cirrhotics ($p=0.002$ for CDTect and 0.006 for AXIS %CDT).

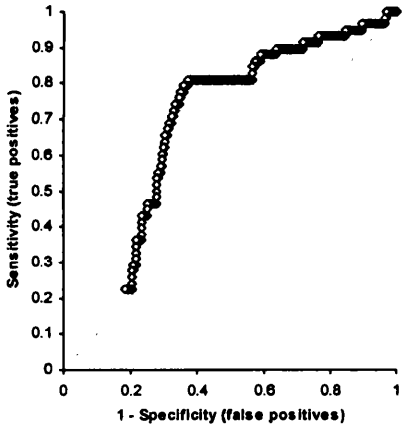
There was less than 95% sensitivity using a test cut-off determined using the mean + two standard deviations of the healthy volunteers to define a raised 'discriminant function' value, ROC curves were drawn to establish the best cut-off (Figure 9.6) in terms of both sensitivity and specificity.

Figure 9.6: ROC curves of the revised equations applied to the retrospective collection

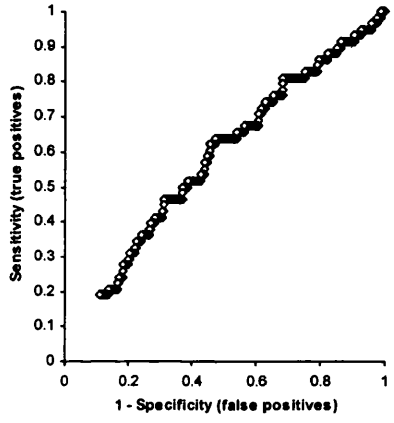
CDTect, non-alcoholics as controls



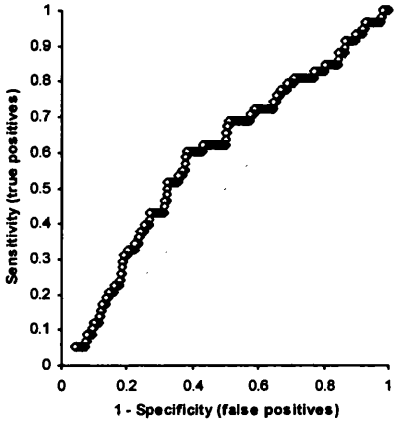
CDTect, healthy volunteers as controls



AXIS %CDT, non-alcoholics as controls



AXIS %CDT, healthy volunteers controls



The ROC curves plot the sensitivity, or the number of individuals correctly classified as drinkers, against 1-the specificity, or the number of individuals incorrectly classified as non-drinkers. The best discrimination is shown by a plot which would pass from the bottom left hand corner, vertically upwards and then straight across to the top right hand corner (area =1.0). No discrimination is shown when the plot is a diagonal from the bottom left hand corner straight across to the top right hand corner (area=0.5).

The ROC curves show that the AXIS test had a poor discriminating value, as the area under the curve was 0.57 when using all the non-drinkers as the control group and 0.59 when using the healthy volunteers as a control group. The CDTect had areas of 0.75 and 0.66 respectively.

The ROC curves show that in order to achieve 95% sensitivity and specificity two different cut-offs must be used. The curves were then used to define cut-offs to gain 95% sensitivity and specificity (Table 9.4)

Table 9.4: Cut-offs used to achieve 95% sensitivity and specificity, calculated from the ROC curves

	Cut-off for 95% sensitivity	Cut-off for 95% specificity
CDTect, non-alcoholics as controls	-0.8	-2.2
CDTect, healthy volunteers as controls	0	-1.9
AXIS %CDT, non-alcoholics as controls	-1.0	-2.6
AXIS %CDT, healthy volunteers as controls	-0.1	-1.3

If these cut-offs were used, for CDTect at a sensitivity or specificity of >95%, the concomitant sensitivity and specificity with these would be <30%, and for AXIS %CDT <20%. In view of this these cut-offs are not as attractive as they may seem, and cannot be recommended for differentiating an alcohol misuser from a non-drinker in a non-selected population.

9.5.3 APPLICATION OF THE 'REVISED EQUATIONS' TO THE CONTEMPORARY COLLECTION

The revised equations were then tested in the contemporary collection. The sensitivity and specificity was then calculated as before (Table 9.5).

Table 9.5: Sensitivities and specificities of the revised 'discriminant function' equations when applied to the contemporary population

CDTect

Study subgroup	Using all the non-drinking groups as controls		Using the healthy volunteers as controls	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Healthy volunteers (n=48)		95.8		91.8
Hospital inpatients (n=50)		96.0		94.0
Non-alcoholic liver disease (n=74)		*70.3*		*54.1*
Non-cirrhotic (n=35)		*88.6*		*85.7*
Cirrhotic (n=39)		*56.4*		*25.6*
Abstinent alcohol misusers (n=95)		55.8		42.1
Non-cirrhotic (n=22)		*59.1*		*59.1*
Cirrhotic (n=73)		54.8		37.0
Drinking alcohol misusers (n=95)	70.5		87.4	
Non-cirrhotic (n=69)	*68.1*		*84.1*	
Cirrhotic (n=26)	*76.9*		*96.2*	
Total	**70.5**	**74.6**	**87.4**	**64.2**

AXIS %CDT

Study subgroup	Using all the non-drinking groups as controls		Using the healthy volunteers as controls	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Healthy volunteers (n=48)		95.8		95.8
Hospital inpatients (n=50)		92.0		92.0
Non-alcoholic liver disease (n=74)		68.9		60.8
Non-cirrhotic (n=35)		*91.4*		*88.6*
Cirrhotic (n=39)		*48.7*		*35.9*
Abstinent alcohol misusers (n=95)		56.8		48.4
Non-cirrhotic (n=22)		*68.2*		*59.1*
Cirrhotic (n=73)		*53.4*		*45.2*
Drinking alcohol misusers (n=95)	68.4		82.1	
Non-cirrhotic (n=69)	*63.8*		*78.3*	
Cirrhotic (n=26)	*80.8*		*92.3*	
Total	**64.4**	**73.5**	**82.1**	**68.3**

The healthy volunteers all have a specificity >95%, except using the CDTect (healthy volunteers as a control group) equation where the specificity is 91.8%. The specificity decreases with increasing histological injury, so that the lowest specificity is with the cirrhotic abstinent alcohol misusers.

The drinking alcohol misusers show a sensitivity overall of 68.4 to 87.4%. The cirrhotic population, unlike the retrospective collection, has a higher sensitivity than the non-cirrhotic population. This is as high as 96.2% using the CDTect, healthy volunteers as controls equations. This may be attributed, in part, to the fact that the cirrhotics may have had a higher AST due to the underlying liver disease.

The two equations for each marker were compared using the Chi-square test. In comparison between the equations using the non-drinking population as controls, and the healthy volunteers as controls, there were very few differences. The cirrhotic non-alcoholic liver disease group was significantly higher using the non-drinking group as controls than that using the healthy volunteers as controls (p=0.011). The sensitivity in the drinking group overall was significantly higher (p=0.006) in the equation using the healthy volunteers as the control group.

The CDTect and AXIS %CDT equations were compared and there were no differences between the two tests.

Finally the performance of the equations in the retrospective collection, from which they were derived, and in the contemporary collection, in which they were tested, were compared. Using the CDTect equations they only significant difference was in the cirrhotic non-alcoholic disease group in the equation where the healthy volunteers were used as the control group, the retrospective collection was significantly higher than in the contemporary collection (p=0.005).

Using the AXIS %CDT equations the only significant differences are in the non-alcoholic alcohol misusers where, overall, using the non-drinkers as the control group equation, the retrospective collection was significantly higher (p=0.007) and also in the cirrhotic part of this group (p=0.004). In the equation using the healthy volunteers as controls, the non-alcoholic liver disease cirrhotics were significantly higher (p=0.004) in the retrospective collection, and the alcohol misusers cirrhotics were significantly higher (p=0.02) in the contemporary collection.

9.6 Discussion

In the first set of equations devised it is interesting that AST was not sufficiently discriminating in defining an alcohol misuser from an abstinent individual to be incorporated in the equations. It was not surprising as it is well recognised to be a source of false-positives in liver disease. However it was involved in the revised set of equations, where it may be useful in conjunction with other markers.

The equations were unable to separate drinkers from non-drinkers with >95% certainty using a single cut-off. This is likely to be a function of the large relatively diffuse non-drinking group in comparison to a much smaller drinking group in both populations in the first set of equations. Two different sets of equations were then devised in an attempt to test this, but it was still impossible to have a single cut-off. A fairer test may be to have tested healthy volunteers against alcohol misusers only. However, while this may have given some better results in terms of sensitivity and specificity, it would not have been applicable to the situation encountered in current medical practice. Here it would be particularly useful to distinguish an alcohol misuser from an individual with non-alcoholic liver disease. Unfortunately it was the non-alcoholic liver disease group where the specificity was particularly low, and this was seen in all the equations.

In terms of applicability, the second, revised set of equations was as effective in the test population as in the population in which it was devised. While there is no single cut-off for each equation to give 95% sensitivity and specificity, using two different cut-offs or indeed using the score obtained to indicate relative chances of detecting an alcohol misusers may yet prove useful.

10. SUMMARY AND CONCLUSIONS

Alcohol has been used in society over centuries and all the evidence we have indicates that, to society as a whole, the risks are heavily outweighed by the benefits and it is particularly expensive in health terms. A means to identify those at risk is required so that these individuals can be targeted for help. This in turn requires a means for monitoring. The means of detection should ideally detect alcohol misuse at a level at which damage occurs: 69 g/day for men and 40 g/day for women.

Histories and questionnaires are still the commonest initial means of detection of alcohol misuse. They are cheap, easily administered but are subjective. They still provide the 'gold-standard'. Biological markers have therefore been sought out and developed as they provide objective markers. The three commonest markers in current practice were GGT, AST and MCV, however these showed problems with detection, particularly in the context of liver disease. Serum CDT initially showed promise as having a high sensitivity and specificity and be ideally suited for both screening and monitoring. However following development of commercial assays (CDTect and AXIS %CDT) the sensitivity and specificity was not seen to be as promising as early work had suggested. There was a poorer performance in women and in those with liver disease.

Two series were evaluated in chapter 4 for sensitivity and specificity. Both confirmed a poorer performance in those with liver injury but a non-significant difference in gender performance. AXIS %CDT showed a better performance in the total population in terms of specificity. The performance of AXIS %CDT was superior to CDTect in those with non-alcoholic liver disease, in particular in the non-alcoholic cirrhotics. In liver injury the total transferrin levels were lower and the expression of CDT as a ratio to total transferrin (CDTect/transferrin and AXIS %CDT) should have taken this into account. However the performance in terms of sensitivity and specificity was similar whether CDT was expressed absolutely or as a ratio. There was no advantage in expressing CDTect as a proportion of total transferrin so that the advantages of AXIS %CDT over CDTect seem unlikely to be solely due to the percentage expression.

The high false positive rates in the non-drinking population and the high false negative rate in the drinking population was investigated. These were seen particularly in those with chronic liver injury. In chronic inflammation there is alteration in the ferritin and CDT was evaluated in three chronic disease states but there was no difference in the performance from that in

healthy volunteers. It is known that in iron deficiency there is an increase in CDT levels, and as there is alteration in iron status in alcohol misuse, this was explored further using soluble transferrin receptors. These are up-regulated in iron deficiency and were measured in both drinking and abstinent populations. Although they were up-regulated in drinkers there was no correlation with CDT. The reasons for high false positive and negative rates in significant liver injury did not seem to be a result of chronic inflammation or altered transferrin receptor status. They seemed more likely to be a result of liver disease *per se*, as transferrin is both synthesised and metabolised in the liver.

The overall poor sensitivity and specificity indicated that CDT did not seem to be suitable for general population or hospital screening. The performance was then evaluated on an individual level in the context of monitoring. In this context, again, it was not suitable to be recommended for monitoring all alcohol misusers. However in those who had a raised level during drinking and a fall to the reference range in abstinence ('responders') the performance was reliable and repeatable. This applied to 50% of individuals using CDTect and 30% using AXIS %CDT. The number of responders was higher in the non-cirrhotic male group using CDTect than in the female or cirrhotic groups. The performance was often superior in terms of sensitivity of response to other markers, particularly GGT. In malnutrition CDT was seen to be false negative, but reversible with improved nutritional state. In end-stage cirrhosis CDT was false positive, but reversed with transplantation.

HPLC was used to evaluate the isoform profiles of those followed serially. In those who were responders there were isoform differences between drinking and abstinence. In those who were false positive and negative there were no changes with drinking behaviour. Thus it would seem that in these individuals the sialic acid formation of transferrin was not altered by alteration in alcohol exposure. In the responders there were changes with drinking and abstinence with trisialotransferrin whereas in the non-responders these were not present. The effect of trisialotransferrin was possibly an explanation of the superior AXIS %CDT performance, as this assay incorporates half of trisialotransferrin.

Finally, as CDT had an insufficient performance to be recommended to be used alone, the combination of CDT, GGT, AST and MCV were combined in a statistical equation to maximise performance. Unfortunately there was no single cut-off that resulted with 95% sensitivity and specificity.

Serum CDT has a sensitivity and specificity equivalent to GGT. However both markers detect alcohol misusers in different individuals and are complimentary. In the future the best use of CDT is with other markers in both screening and monitoring. In monitoring a combination should be used initially and then the best marker selected to be used alone.

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12. APPENDIX

12.1 Development of Competitive Inhibition of Binding ELISA to Estimate Soluble Transferrin

Human transferrin is adsorbed onto wells of a 96-well plate. The principle of the assay is the interaction of a specific antibody with any transferrin present in samples that are added to the wells. This results in the removal of antibody from the system which is subsequently unable to bind to the immobilised transferrin on the wells. The antibody that does bind is detected by immunochemical techniques resulting in a coloured product that can be measured spectrophotometrically, the magnitude of which can be extrapolated to be directly proportional to the amount of transferrin in the sample.

Human transferrin, iron saturated, >98% purity was dissolved in 50mM carbonate buffer, pH 9.6 at a concentration of 5μg/ml. 100μl was dispensed into each well of a 96-well plate which was then covered and left for 24 hours at 4°C to allow maximum adsorption of transferrin to the wells. Each well of the plate was then washed four times with 350μl of 10mM phosphate buffered saline, pH 7.4 (PBS) to remove unabsorbed transferrin. Non-specific protein binding sites were blocked by the addition of 0.5% ovalbumin in PBS containing Tween20, 0.05% (PBS/T_{OAA}), 300 μl per well, for one hour at 37°C. The plate was then washed for a further 4 times with PBS containing Tween20, PBS/T, and allowed to dry thoroughly before use.

Samples to be assayed were dispensed to triplicate wells, 100μl per well, followed by 100μl of rabbit anti-human transferrin antibody. Blank wells were used where PBS/T_{OAA} was substituted for the antibody. A standard curve was also constructed for each experiment, where transferrin at a concentration of 100μg/ml was added to triplicated wells and 10 serial threefold dilutions were made in successive wells by diluting in PBS/T_{OAA}. The plate was incubated for 2 hours at 37°C with constant shaking and was then washed four times with PBS/T_{OAA}. The antibody that had bound to the immobilised transferrin was then visualised using a two-stage indirect immunochemical procedure. Biotinylated donkey anti-rabbit-IgG was added to each well, including blanks, 100μl per well, and the plate incubated for 1 hour at 37°C. Following another four-wash cycle as before, an Avidin-biotinylated horse radish peroxidase complex was added to each well, 100μl per well, and the plate incubated for a further one hour at 37°C. After a final four-cycle wash, the chromogen ortho-phenylenediamine tetrahydrochloride (Sigma),

dissolved in citrated PBS containing perborate as a source of hydrogen peroxide was added, 100µl per well, and colour reaction allowed to proceed. After about 5-15 minutes, the reaction was stopped by the addition of 50µl 4M H_2SO_4 and the plate read in a plate reader at 460nm.

The optical densities were used within the Excel™ software package. A standard curve was constructed from log/linear plots of the OD versus log concentrations of Transferrin used in the standard curves. The linear region of each curve was identified by eye and the values in this region used for linear regression analysis to produce the slope and intercept constants. Interpolation from this plot allowed the estimate of transferrin in the unknown samples.

Optimisation of Rabbit anti-Transferrin Titre.

Human transferrin was adsorbed onto the wells of a 96-well plate at a concentration of 5µg/ml. The first 3 columns of wells were used to determine the ability of various dilutions of the antibody to bind to the transferrin in the absence of any competing antigen. Into the first three wells were dispensed 150µl rabbit anti-transferrin at a dilution of 1:100. Seven serial three-fold dilutions were prepared in the remaining rows by removing 50µl and mixing with the 100µl aliquots of PBS/T_{OAA} dispensed in these wells. The dilutions of antibody used therefore covered the range 1:100 to 1:72900. The plate was incubated for 2 hours and the amount of rabbit antibody bound to the wells estimated as described above.