CRANFIELD UNIVERSITY

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PLASMA HOMOCYSTEINE, MEASUREMENT AND CLINICAL APPLICATION.

INSTITUTE OF BIOSCIENCE AND ANALYTICAL TECHNOLOGY

THESIS SUBMITTED FOR THE
DEGREE OF PHD

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Abstract

Raised plasma homocysteine (Hcy) levels have been cited as a major risk factor for several vascular disorders. Yet hyperhomocysteinaemia is easily treated through dietary intervention and vitamin supplementation.

Commercial assays have facilitated routine plasma Hcy analysis. However, the problem faced by clinicians is stabilisation of Hcy in whole blood samples prior to delivery to the laboratory. Following blood collection, erythrocytes continue to produce and excrete Hcy increasing plasma concentrations by up to 10% per hour.

This thesis describes the investigation of stabilising plasma Hcy in whole blood, allowing wide scale screening for hyperhomocysteinaemia. The most effective method appears to be inhibition of the enzyme responsible for Hcy production, S-adenosylhomocysteine hydrolase (SAHH), using a competitive inhibitor 3-deazaadenosine (3DA).

Clinical trials were conducted on a pilot batch of evacuated blood tubes. Samples were stored in EDTA whole blood in the presence and absence of 3DA, at ambient temperatures (20 to 25ºC), and under refrigerated conditions (2 to 8ºC). Only samples that were collected into EDTA plus 3DA tubes and stored refrigerated showed stability over 72 hours ($p = 0.2761$).

For wide scale screening, samples must be stable under ambient conditions. As the structure of SAHH is known a molecular modelling approach was adopted in an attempt to identify other potential inhibitors from screened databases. Interference of SAHH, in an immunochemical method for Hcy, was to be utilised for *in vitro* screening before any further clinical trials were conducted.

The thesis also focuses on Hcy as a marker of vitamin deficiency and explores links between thiol metabolism and the development of cognitive decline eventually leading to dementia. Disruption of single carbon metabolism can lead to an increase in Hcy and a decrease in available methyl groups important in regulation of several metabolic pathways. Increased oxidative stress may also be a causative factor.

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Abbreviations

Aβ	Amyloid-β
ABD-F	4-(aminosulphonyl)-7-fluoro-2,1,3-benzodiazole
AD	Alzheimer's Disease
ANOVA	Analysis of variance
APP	Amyloid precursor protein
Asp	Aspartate
ATP	Adenosine triphosphate
C	Cytosine
CI	Confidence Interval
CBS	Cystathionine β-synthase
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Coefficient of Variation
Cys	Cysteine
3DA	3-deazaadenosine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
F→M	Female to male
FAD	Flavin adenine dinucleotide
FDA	Food and Drug Administration
Glu	Glutamate
GS-B12	Gluatathionylcobalamin
GTP	Guanosine triphosphate

Hcy	Homocysteine
His	Histidine
HPLC	High performance liquid chromatography
IFCC	International Federation of Clinical Chemistry
IQ	Intelligence Quotient
LDL	Low-density lipoprotein
Lys	Lysine
M→F	Male to female
Met	Methionine
5-MeTHF	5-methyltetrahydrofolate
5,10-MTHF	5,10-methylenetetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
MS	Methionine synthase
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards
NMDA	N-Methyl-D-Aspartate
NO	Nitric oxide
NTD	Neural tube defects
RNA	Ribonucleic acid
S-AdHcy	S-Adenosylhomocysteine
S-AdMet	S-Adenosylmethionine
SAHH	S-Adenosylhomocysteine hydrolase
SBD-F	ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate

SD	Standard deviation
SEM	Standard error of the mean
T	Thymine
TC	Transcobalamin
TCEP	Tris(2-carboxyethyl)phosphine
THF	Tetrahydrofolate
Thr	Threnonine
UK	United Kingdom
US	United States

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Plasma Homocysteine, Measurement and Clinical Application.

This thesis is based partly on unpublished practical work described in Chapters 2 and 3, and on the following papers (see Appendix A), referred to in the text by their roman numerals.

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XIII. McCaddon A, Hudson P, Hill D, Kenney A, Hughes A, Barber J *et al*. Hyperhomocysteinemia and oxidative stress - a pathogenic mechanism common to Alzheimer's and vascular disease? *Proceedings of the 3rd International Conference on Homocysteine Metabolism, July 1-5, 2001, Sorrento. Italy.*, p 131. Naples, Italy.: Edizioni Ziino, 2001.

Chapter 1 INTRODUCTION

1.1 Homocysteine Metabolism

Homocysteine (Hcy) is a naturally occurring amino acid. Although not found in protein, it can be found *in vivo* within the circulating plasma where it is usually present in trace amounts, such that the clinical reference ranges for a human adult are in the order of 5 to 15µmol/L[1].

In mammals Hcy is formed solely from the intracellular metabolism of methionine (Met)[2], via the pathway shown in Figure 1. The primary source of Met is dietary protein, in particular protein derived from animal products, however, Met may also be supplied from proteins within the body, due mainly to turnover of muscle mass.

In the first step of the cycle Met adenosyltransferase catalyses transfer of the adenosine moiety, from ATP to Met, yielding a high-energy compound, S-adenosylmethionine (S-AdMet). The methyl group can then be transferred to a wide range of acceptor compounds including, DNA, RNA, proteins and lipids. This reaction is key to several metabolic processes, in particular, the methylation of DNA, which is important in the regulation of gene expression.

Although over 100 methyltransferases exist most are subject to potent feedback inhibition by the product of methyl transfer, S-adenosylhomocysteine (S-AdHcy). It is important, therefore, that S-AdHcy is quickly removed, which is brought about by hydrolysis forming adenosine and Hcy. Under physiological conditions the enzyme responsible, S-AdHcy hydrolase (SAHH), actually favours the reverse, condensation reaction[3], however *in vivo* the reaction is driven forward to produce Hcy by effective removal of the other product of the reaction, adenosine, by adenosine kinase, and adenosine deaminase.

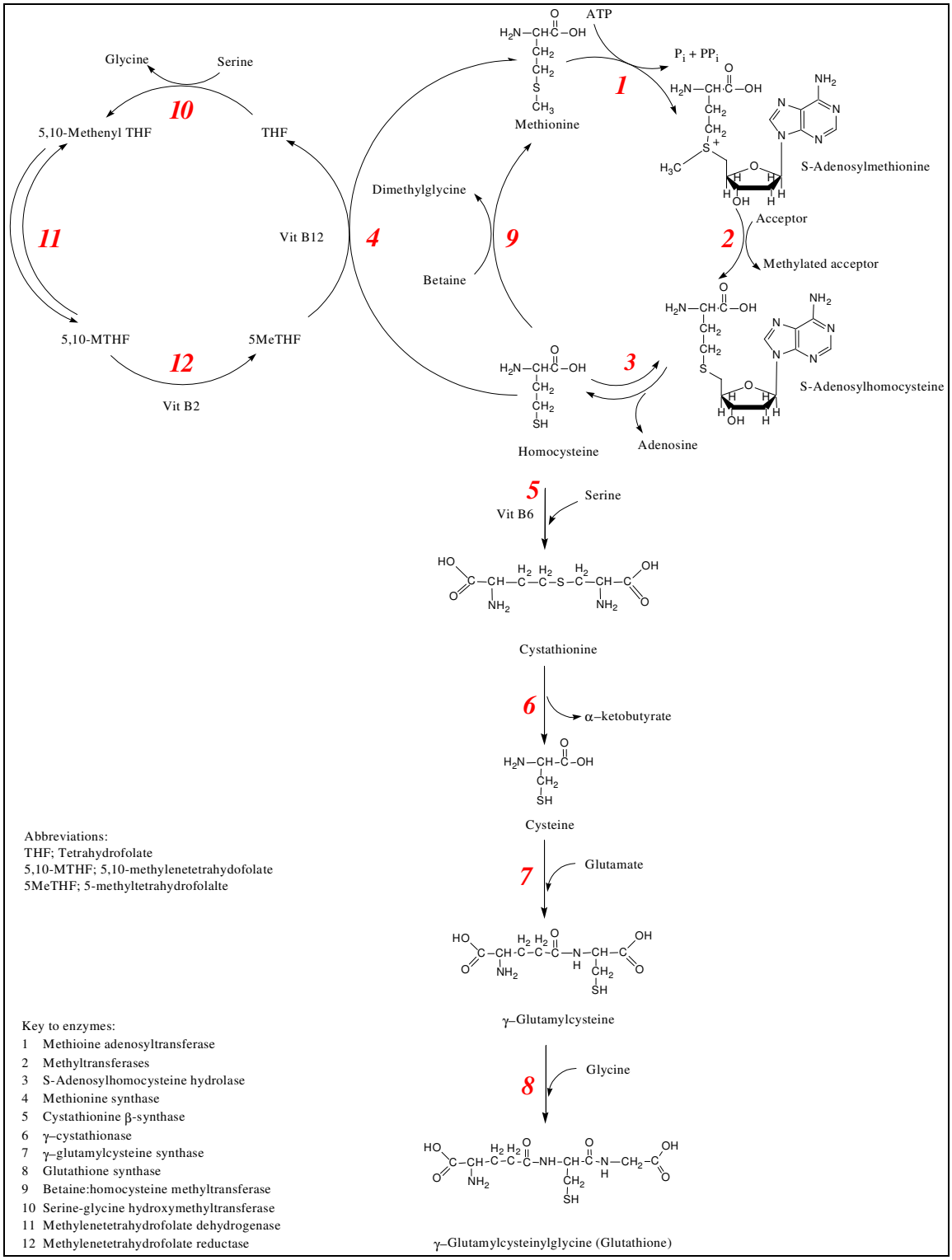

Figure 1 Metabolism of methionine and homocysteine.

Hcy then acts as a branch point in Met metabolism; it can either be used to synthesise cysteine (Cys) or to regenerate Met. Its route is determined by a number of nutritional factors including, vitamin status, and the oxidative environment within the cell.

Vitamins are important cofactors for several of the subsequent reactions in Hcy metabolism. Therefore deficiencies, due to poor diet or inadequate absorption from the gut, may cause disturbances within the pathway, which will be discussed in more detail later (see section 1.4.7). Provided that vitamin status is adequate, the fate of Hcy is governed by the availability of Met from the diet. Due to the importance of S-AdMet, if Met is scarce, the transfer of a methyl group from a donor compound brings about remethylation of Hcy, feeding the cycle in order to maintain the production of S-AdMet. In the majority of tissues this reaction involves 5-methyltetrahydrolfolate (5-MeTHF) as the donor molecule and vitamin B12 as cofactor for the enzyme Met synthase (MS). This enzyme is unique in its ability to metabolise the methyl group of 5-MeTHF, channelling one-carbon units derived from formate and amino acids such as serine, histidine and glycine into the methylation cycle, thus providing a methyl group for the synthesis of S-AdMet via Met and subsequently a methyl group that is used by many essential methyl transferases[4]. Serine-glycine hydroxymethyltransferase, methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate reductase (MTHFR) then serve to regenerate 5-MeTHF, from tetrahydrofolate (THF).

Most of the body's cells possess MS, however a small number tissues, including the liver and kidney, exhibit an alternative remethylation pathway which utilizes betaine (trimethylglycine) as a methyl donor in place of 5-MeTHF[2].

At times when Met is present in plentiful supply, Hcy enters the transsulphuration pathway where it undergoes a condensation reaction with serine to form cystathionine. In this case vitamin B6 acts as cofactor for the enzyme cystathionine β-synthase (CBS). Cys is formed through the hydrolysis of cystathionine by γ-cystathionase, the by-product of which is α-ketobutyrate. Cys may then be used for protein synthesis or the formation of glutathione, an important antioxidant. Alternatively, oxidation of the sulphur atom of Cys is responsible for the urinary excretion of about 70% of the sulphur of dietary Met as inorganic sulphate[4].

It is not the Met levels *per se* that regulate which pathway Hcy enters, but the levels of S-AdMet that acts upon key enzymes within the process. An abundance of S-AdMet promotes transsulphuration by its negative allosteric affect on MTHFR blocking remethylation, and a positive allosteric affect on CBS, thereby allowing the removal of excess Met and Hcy. If S-AdMet levels are low, allosteric regulation is removed, feeding the remethylation pathway and the regeneration of Met.

Flux through the remethylation or transsulphuration pathways, may also be influenced by redox status[4]. Both MS and CBS have redox-active cofactors. MS is dependent on vitamin B12 (cobalamin) as cofactor. The cobalt atom central to the structure of the vitamin can exist in three oxidation states (+1, +2 or +3). Cob(I)alamin, an intermediate in the transfer of a methyl group from 5-MeTHF to Hcy, is labile to oxidation leading to MS inactivity[5] (Figure 2), such that MS activity is favoured by a lower redox potential. As a result, MS requires periodic reactivation by MS reductase, which utilises S-AdMet to donate a methyl group to the inactive cob(II)alamin, to reform methylcobalamin. Within CBS, the oxidised ferric state of haem is more favourable to Hcy binding, possibly through conformational changes in the surrounding

protein. Therefore under conditions of oxidative stress, Hcy is channelled into the regeneration of depleted glutathione via the transsulphuration pathway.

Thus Hcy metabolism is complex, involving several enzymes, and B vitamins and folate as cofactors and substrates. Deficiencies in the supply or functioning of any of these compounds can have serious effects on homeostasis.

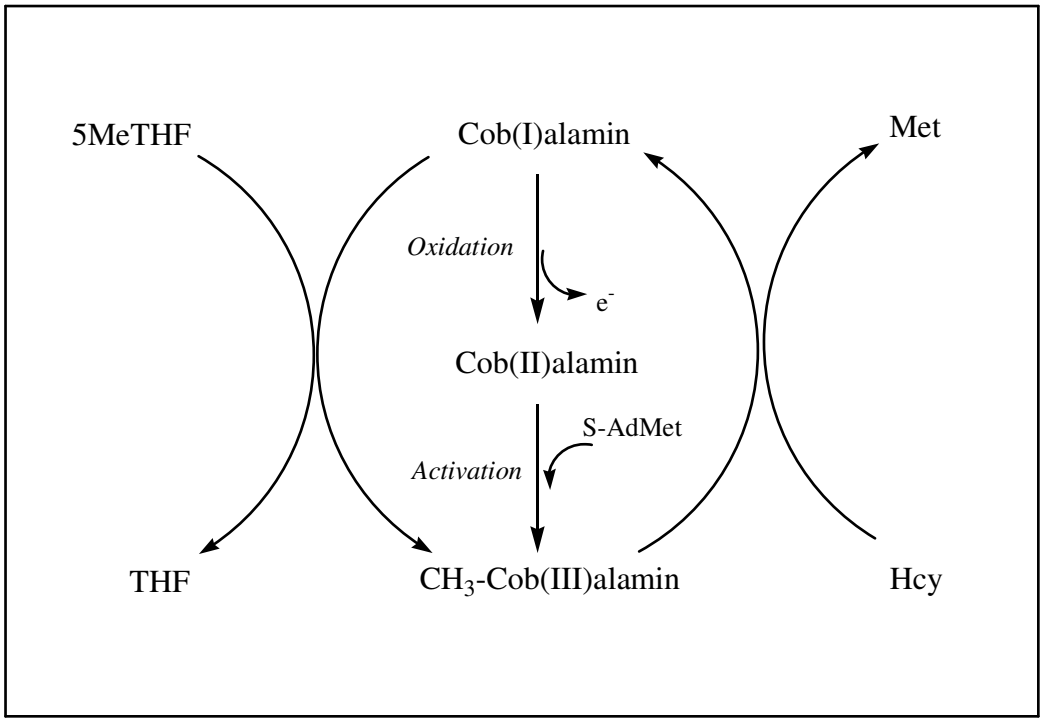

Figure 2 Vitamin B12 involvement in the action of methionine synthase.

1.2 Clinical Importance of Hcy

Although compared to the amino acids involved in protein synthesis Hcy is only present in small amounts in the plasma, it has recently provoked a huge amount of interest as can be seen in the explosion in Hcy related publications which took place from 1991 onwards[6].

1.2.1 Vascular disease

Diseases of the vascular system, including ischaemic heart disease, acute myocardial infarction, and stroke are responsible for more deaths per year, in the western world, than any other disorder. In England and Wales in the year 2003, there were a total number of 538254 deaths[7]. Of these deaths, ischaemic heart disease and cerebrovascular disease accounted for 99790 and 57808 respectively. In total, 29% of mortality from all causes was due to diseases of the circulatory system. In the US, where the numbers are treated slightly differently, statistics available for the same year listed diseases of the heart as the number one cause of death, cancer was the second highest, but cerebrovascular diseases were ranked third[8]. Yet, in the Framingham Heart Study, conducted on over 5000 participants in the US, established risk factors including cholesterol, smoking, and family history only accounted for about 50% of the risk of cardiovascular event[9;10].

The majority of cardiovascular disease cases present in an acute, traumatic way, such as myocardial infarction or sudden death[10], clearly this highlights the need to establish the risk factors involved in order to instigate risk factor reduction programmes, before the development of symptoms.

Hcy was first postulated as a causal factor in vascular disease some four decades ago. In 1959, in Northern Ireland, a family presented in which two young sisters were showing signs of mental retardation, and congenital anomalies[11]. Initial tests proved negative for any of the inborn errors of metabolism that were known about at that time. Amino acid analysis was still quite crude, but raised levels of a sulphur-containing compound believed to be homocystine were detected in urine samples taken from the siblings.

Three years later in Wisconsin an infant was discovered with a similar presentation, however improvements in the methodology meant that the presence of increased amounts of homocystine excreted into the urine could now be chemically proven[12]. Also of note was the detection of increased excretion of Met.

From these cases came the first clinical description of the syndrome referred to as homocystinuria, in which the clinical features of mental deficiency, fine, fair hair, ectopia lentis (dislocated ocular lenses), malar flush (red cheeks), peculiar gait, and genu valgum (knock knees) are relatively constant. Variable features may include pes cavus (high-arched) foot, long extremities and digits, convulsions, thrombotic incidents, cardiovascular disorders and fatty liver. Almost without exception, patients have elevated levels of Met and Hcy in plasma in addition to increased amounts of homocystine in the urine[13].

Laster, Finkelstein and Mudd[13;14] later mapped the underlying most frequently observed genetic cause of homocystinuria to a defect in the enzyme CBS, responsible for the removal of Hcy from the circulation via the transsulphuration pathway (Figure 1). A blockage in Met metabolism at this point leads to a build up of Hcy and its precursors, to a level where the renal threshold is exceeded causing homocystine to become detectable in the urine.

Through family studies they found that the disorder showed an autosomal recessive mode of inheritance[15], with heterozygotes possessing half the enzyme activity in cultured liver cells, but showing no clinical symptoms of the disease.

If left untreated, half the number of patients with homocystinuria due to CBS deficiency will experience a thromboembolic event before the age of 30 years[16]. It was plausible that either raised Met levels or raised Hcy levels observed in these cases may

be the causal factor of premature atherosclerosis. In order to shed further light on this issue, in 1969, McCully[17], took post mortem tissue samples from two different cases of disturbed Met metabolism and studied the similarities and differences between them. Arterial and venous specimens taken from a 7½ week old boy, who had died from the effects of an abnormality in vitamin B12 metabolism, were compared with samples from an 8 year old boy who had suffered a fatal thrombus in the carotid artery due to CBS deficiency. Both cases showed similar widespread arterial lesions, with fibrous plaques caused by damage to the intimal elastic lamina of the vessel walls. Since each child possessed a blockage in Met metabolism that occurred in a different location, the pattern of excess metabolites present in the plasma differed between the two.

In the younger child, a deficiency in vitamin B12 metabolism caused Hcy and cystathionine to be raised, whilst Met concentrations were lower than normal (Figure 1).

In CBS deficiency transsulphuration is blocked, Hcy accumulates in the plasma and feeds the remethylation pathway. In this way, cystathionine concentrations are low and Hcy and Met are raised.

These two cases, therefore, showed similar vascular pathology, which could only be linked to an accumulation of Hcy in the circulation.

McCully postulated that moderately elevated concentrations of Hcy, or one of its derivatives, might also be involved in the aetiology of atherosclerosis in individuals free from known enzyme defects. Together with his colleagues, he produced experimental evidence of this theory, by causing hyperhomocystinaemia in rabbits which led to the development of vascular lesions[18;19]. Furthermore, the addition of cholesterol to the diet resulted in lipid deposition in the aortic lesions, suggesting that the involvement of

cholesterol in arterial plaques is a secondary complication of pre-existing vascular injury.

1.2.1.1 Cardiovascular disease

If short term exposure to high levels of Hcy could lead to vascular disease in childhood, it followed that chronic exposure to milder elevations in plasma Hcy may be a risk factor for premature coronary disease in the general population. In 1976, Wilcken and Wilcken[20] showed that differences existed in Met metabolism between individuals of similar ages with and without coronary artery disease, suggesting a reduced ability to metabolise Hcy in the diseased group.

Between the late 1970's and early 1990's little was published on Hcy and vascular disease. A few scattered reports seemed to confirm that a link existed, but at the time attention was focused on the links between cholesterol and the development of atherosclerosis, with efforts being concentrated towards developing pharmacological treatments to lower plasma lipid levels.

Over the last two decades, there has been a shift in interest back towards Hcy, due in part to a publication by Clarke *et al*. in 1991[21], who recognised elevated Hcy as an independent risk factor for vascular disease. Within their study they found that hyperhomocysteinaemia was present in 30% of patients with premature vascular disease. Recent developments have also been facilitated by the emergence of various assays for Hcy measurement suitable to a clinical environment[22-30], which have been comprehensively reviewed by Rassmussen and Møller[31].

Twenty-seven of the early studies relating Hcy to arteriosclerotic vascular disease were reviewed by meta-analysis[32], a summary of those investigations which looked specifically at cardiovascular disease reported a graded risk for atherosclerosis of

cardiovascular vessels, such that a 5µmol/L increase in Hcy conferred an 80% increase in risk in women, odds ratio 1.8 (95%CI 1.3 to 1.9) and 60% increase in risk in men, odds ratio 1.6 (95% CI 1.4 to 1.7). However, the analysis included mainly cross sectional and case-controlled studies, which may be subject to bias compared to cohort studies, in particular selection of cases and controls. Therefore, a more recent meta-analysis, involving more than 38 studies of coronary heart disease and Hcy[33], attempted to update the data and divide the studies according to whether they were cohort, nested case-controlled, or case-controlled studies. They showed that whilst a stronger relationship was shown in case-controlled studies, with an odds ratio of 2.11 in women (95% CI 1.30 to 3.42), and 1.63 in men (95% CI 1.44 to 1.85), the link between Hcy levels and cardiovascular disease remained supported within the cohort studies. However, the association was weak with a 5µmol/L increase in Hcy conferring an increased risk of 6%: odds ratio 1.06 (95% CI 0.99 to 1.13).

While case-controlled studies may be subject to positive bias, cohort studies may show negative bias when assessing the links between Hcy and vascular disease. Many of these studies were initially designed to assess the degree by which well-established risk factors may be associated with the development of vascular disease. When the studies were initiated, and baseline samples were collected, Hcy may not have been considered for inclusion in the panel of blood tests to be conducted. Retrospectively, samples have been re-analysed as new risk factors are introduced. However, blood samples collected for Hcy analysis may be subject to false elevations if not handled under strict sampling conditions as will be discussed in Chapter 2. Therefore the boundaries between Hcy levels in those that subsequently become cases, and control individuals may not be clear-cut.

In both of the meta-analyses women showed a higher risk than men for cardiovascular disease, when exposed to a 5µmol/L increase in Hcy concentration. Although pre-menopausal women tend to have lower circulating levels of Hcy, they appear to be more sensitive to any increase in Hcy concentration, which may be due to increased sensitivity of the vascular walls to the damaging effects of Hcy.

As the evidence mounts, Hcy is now regarded by many as a significant risk factor in the development of cardiovascular disease. Yet, the story does not end here; Hcy is now being implicated as being central to the pathology of a multitude of other systemic disorders.

1.2.1.2 Cerebrovascular disease

Vascular disease is not only associated with heart disease, but also accounts for a large proportion of cerebral haemorrhage or stroke. Although fewer studies have looked independently at the risk of cerebrovascular disease, the link with Hcy appears to be stronger than that which exists between Hcy and cardiovascular disease. Meta-analysis[33] showed an odds ratio of 1.10 (95% CI 0.94 to 1.28) in 2 cohort studies associated with a 5µmol/L increase in Hcy, whereas a summary of 22 case controlled studies showed an odds ratio of 1.97 (95%CI 1.61 to 2.40). In contrast to the figures for cardiovascular disease, men, rather than women showed a higher risk of developing cerebrovascular disease, associated with elevated Hcy.

Elevation in Hcy again shows a linear and graded risk in association with cerebrovascular disease. In a prospective cohort study, involving almost two thousand participants the associated risk of subsequent stroke incidence increased with increasing quartile of baseline Hcy concentration[34].

1.2.1.3 Peripheral vascular disease

In addition to the vascular disease of major organs, increased Hcy shows a strong association with peripheral vascular disease[32]. Where peripheral disease is present, circulating Hcy concentrations may predict adverse outcome of surgical intervention, for example those with higher Hcy levels have a higher incidence of vein graft restenosis following lower extremity bypass.

1.2.2 Cognitive decline and Alzheimer's Disease

In recent years there has been increased interest in the possibility that Hcy metabolism is involved in cognitive decline, eventually leading the development of Alzheimer's Disease. Investigations into the metabolism and mechanisms involved form a major part of this thesis; as such this clinically important link will be discussed in depth later (see Chapter 4).

1.2.3 Adverse pregnancy outcomes

The Hordaland Homocysteine study was designed to investigate the relationships between established cardiovascular risk factors and plasma Hcy, however, within this fraction of the Norwegian population, high Hcy levels also correlated with a higher incidence of adverse outcomes in pregnancy, including neural tube defects (NTD)[35]. Pre-eclampsia, prematurity, low birth weight and increased frequency of miscarriage were also observed in women with Hcy concentrations in excess of 15µmol/L.

After cardiac defects, isolated, i.e. nonsyndromic, NTD are the most common congenital structural defects worldwide. The average prevalence is 1 in 500, although incidence is dependent on country, ethnicity, and socio-economic class. In Finland the incidence is relatively low at 1 in 2500, but in South Wales as many as 1 in 80 births

occur with some form of NTD. These rates, however, exclude spontaneous abortions, and it is estimated that less than 2% of NTD affected pregnancies will result in a live birth, with most (93%) not developing beyond the embryonic stage. The figures suggest that the actual incidence of NTD affected embryos may be as high as 1 in 10.

NTD and its association with folate and Hcy is thoroughly reviewed in a recent paper by van der Put *et al.*[4]. The link between folate, or at least nutritional adequacy and NTD, has been known about for many years, yet the mechanisms concerned have yet to be elucidated in full. However, as more is known about the involvement of folate in Hcy metabolism, theories may be established to explain the observed associations between folate intake and the development of NTD in pregnancy.

In studies of serum folate levels there appeared to be no difference between those women whose pregnancies were normal compared to those that went on to have a child with NTD[4]. However, in the first trimester, the levels of red cell folate, an indicator of folate stores, were lower, in the cases of NTD affected births. Therefore, although folate intake may be adequate during pregnancy it is more important to have an adequate store of folate prior to conception. Closure of the neural tube occurs towards the end of the first month of pregnancy, before most women are aware that conception has taken place, therefore it is recommended that folate is supplemented peri-conceptually. Studies have shown that such measures decrease the incidence of NTD, and it may be possible to prevent up to 70% of affected pregnancies.

The elevated Hcy levels observed in women with NTD pregnancies may be a consequence of a deficiency in folate, or in the enzymes involved in the remethylation cycle, which may be overcome by folate supplementation. Two common mutations in

the MTHFR gene are associated with an increased risk of NTD. In each case, the deficiency may be overcome by additional folate intake.

Although it is clear that folate supplementation reduces the risk of NTD, the reason is not fully understood. Folate is involved in DNA synthesis within the developing embryo both directly, acting as a substrate for the enzymes involved in the process, and indirectly through the regeneration of S-AdMet, via Met, which is used to methylate the developmental genes. Such methylation is partly responsible for the regulation of these transcription factors. Regulation which needs to be tightly controlled, particularly in the rapid changes involved in gene expression associated with neural tube closure. Therefore, a maternal disturbance in folate metabolism may result in low folate levels in the developing embryo, leading to defective DNA synthesis, or impaired transcription of genes involved in the closure of the neural tube.

Hcy may be acting purely as a marker of impaired folate metabolism. However, theory and evidence suggests that Hcy may also be directly involved in the mechanisms of defective tube closure. When Hcy was applied to chick embryos during the closure process, defects were induced suggesting a directly toxic effect. Increased Hcy leads to an increase in its precursor, S-AdHcy, which inhibits the action of methyltransferases, thereby mimicking the effects of S-AdMet depletion occurring in a deficiency of the folate cycle. In addition to DNA methylation, methylation involving S-AdMet is necessary for the proteins, actin and myosin filaments, required for contraction and folding in the process of neural tube closure.

One final theory is that in some cases, increased Hcy may be an indicator of reduced MS activity, which would also lead to a depletion of Met and S-AdMet. Removal of the negative allosteric effect of S-AdMet on MTHFR activity, leads to an increased

production of 5-MeTHF (Figure 1). This form of THF cannot be converted to the forms required for nucleic acid synthesis, a process commonly referred to as the "methyl trap hypothesis", which could lead to an insufficient supply of materials required to close the neural groove.

1.2.4 Cancer

Certain forms of cancer are frequently associated with high circulating levels of Hcy. In some cases this may be expected as several drugs used to treat cancer act through their anti-folate properties, thus inhibiting cell growth and division, however, in a case controlled study, women with Hcy levels in the highest three quartiles had statistically significant elevations in their risk of developing invasive cervical cancer, compared to women in the lowest quartile for Hcy[36]. None of the women involved in this study were on chemotherapy and their Hcy levels were not related to folate intake. What was more concerning was that women, whose Hcy level was ≥6.3µmol/L, showed risk factors of 2 to 3 times, those with values <6.3µmol/L, i.e. increased cancer risk was observed at the low end of what would be considered to be "normal" Hcy concentrations.

However, it is again unclear whether Hcy is the cause of the tumour or results from its presence. Disruption of one carbon metabolism may interfere with DNA synthesis, repair and methylation in a similar fashion to the mechanisms proposed in the development of NTD. In cancer, regulation over these processes is lost, leading to uncontrolled cell division and growth and subsequent tumour development.

In samples taken from cancer patients, plasma Hcy fluctuations coincided with fluctuations in tumour markers for breast, ovarian, colonic and pancreatic carcinoma[37]. Cultured cell lines taken from tumour cells released much more Hcy into the culture media than normal cells[37]. However, when expressed as Hcy concentration per cell

number the difference between tumour cell lines and normal cells was much less pronounced, therefore, Hcy production may simply be a marker of rapidly proliferating cells. More significantly perhaps, as the cells started to die, Hcy production declined whereas the concentration of tumour markers continued to rise. Hcy may therefore reflect the number of live viable cells, whilst tumour markers reflect the total cell burden (live and dead cells combined)[37]. Proliferating cells create a drain on folate stores as a result of DNA synthesis. This folate depletion causes increased Hcy, due to impaired remethylation and Hcy is therefore exported into the circulation.

Thus Hcy may be a much more sensitive marker of tumour activity, than those currently used, reflecting the number of surviving tumour cells, enabling monitoring of treatment with cytotoxic drugs providing that these do not interfere with folate metabolism.

1.2.5 Renal disease

Patients with chronic renal disease have plasma Hcy concentrations which are much higher than the moderate elevations commonly found in patients with atherothrombotic vascular disease[38-41], with some 75% of nephrology patients showing plasma Hcy concentrations of >13μmol/L[38]. Hcy is unlikely to be the cause of renal failure, but may be the consequence of reduced elimination of amino acids in the urine, in particular some sulphur containing amino acids. However, it is more likely that compromised Hcy metabolism within the kidney contributes to the elevation in plasma concentrations, as the kidney is one of only four tissues in mammals found to possess all the enzymes responsible for Hcy degradation through the transsulphuration pathway[42]. However, in a study conducted by Gale *et al*, although the severity of carotid atherosclerosis was

greatest in those with the poorest renal function, and those with the poorest renal function had the highest Hcy levels, the two relationships appeared to be independent[40].

1.3 Proposed mechanism of vascular damage

Associations between raised Hcy levels and several clinical disorders are well founded; however the finding of an unusual level of a compound in a disease state does not provide information or evidence as to whether the compound is the cause of the disease or an effect of disturbed metabolism caused by the disease itself. Some of the possible mechanisms by which raised Hcy may be involved in the development of disease have been discussed above; however, the absolute mechanisms whereby Hcy may cause vascular damage remain unclear.

Inborn errors of Met metabolism, due to CBS deficiency, or cobalamin C disease, share two significant features[16;17]. Firstly, if untreated, they all develop progressive and premature vascular disease in the form of atherosclerosis and thrombus formation in the arteries and veins of major organs. Secondly, in each case the disturbance in Met metabolism results in high levels of circulating Hcy. It is therefore plausible that Hcy, or an Hcy derivative is directly toxic to the vessel walls.

Several reviews summarise the experimental evidence to date[9;43-46]. The consensus opinion is that Hcy may be implicated as a participant in several processes, which may lead to vascular damage and dysfunction, resulting in atherosclerosis.

One barrier to developments in this field is the difficulty in mimicking slow atherosclerotic effects in short term *in vitro* studies. Therefore there is a tendency to use very high levels of Hcy compared to those present in the plasma of individuals with hyperhomocysteinaemia. Very few studies exist on *in vivo* models, however one study

used a primate model in which moderately raised Hcy levels were induced by dietary intervention, and found that upon relative stimuli, increased Hcy levels caused increased vasoconstriction or decreased vasodilation resulting in reduced blood flow to the limbs[47]. Hcy may produce these effects either through decreased responsiveness of the vascular smooth muscle to the endothelial derived relaxing factor, nitric oxide (NO), or through inactivation of NO.

Hcy may also be directly toxic to the endothelial cells. There is a growing body of evidence to suggest that Hcy promotes oxidative damage through the formation of free radical species. If Hcy metabolism is impaired, excess intracellular Hcy is exported into the circulation, where it rapidly undergoes auto-oxidation, resulting in the production of hydrogen peroxide, superoxide and hydroxyl radicals, which may be damaging to the vasculature. A chain reaction of events then occurs in an attempt to repair the damage. However, Hcy has also been shown to interfere, in several repair processes, in such a way as to cause fibrous plaques to develop, which may be followed by thrombosis and infarction.

Lesions in the elastic membrane of the vessel are followed by increased proliferation in the smooth muscle cells, which leads to intimal thickening and the development of fibrotic plaques. In the inborn errors of metabolism associated with high Hcy levels, these plaques differ from those seen in the vessels of elderly atherosclerotic vessels in their lack of deposited lipids, implying that the underlying vascular pathology is independent of circulating lipid levels.

By the age of 15 years almost 30% of untreated CBS deficiency cases will have suffered a thromboembolic event[16]. Hcy may predispose to thromboembolism through disruption of the components of the clotting cascade. Raised Hcy has been shown to

enhance the activities of factor XII and factor V, and depress the activation of protein C. Hcy also leads to decreased expression of the natural anticoagulant, heparin sulphate, and blocks tissue plasminogen activator binding to human endothelial cells. Combined these events may lead to inappropriate thrombus development and vascular occlusion.

Macroscopic changes, including the development of atherosclerotic plaques occur later in the progression of vascular disease. Cholesterol has now become widely recognised as a risk factor for heart disease yet on its own it possesses few atherogenic properties and requires oxidative modification before it becomes damaging to tissues. The development of free radicals caused by autooxidation of Hcy may lead to oxidative modification of low-density lipoprotein (LDL) enhancing LDL binding at the site of fibrous plaques through the binding of lipoprotein (a) to fibrin. Inflammation resulting from vascular damage attracts macrophages to the site which may take up the modified LDL, leading to the development of foam cells, and the accumulation of lipids within the pre-existing fibrous plaques, eventually leading to occlusion of the vessel.

Raised Hcy may therefore result in vascular disease through a combination of vascular injury, vascular dysfunction, and disturbance of antithrombotic activities predisposing to platelet adhesion and thrombus formation.

1.4 Determinants of Hcy

The complex metabolism of Met and Hcy is described in detail earlier (Section 1.1 and Figure 1). The pathway involves several enzymes, which require cofactors and substrates such as B vitamins and folic acid. Genetic or acquired abnormalities in many of the steps involved in the pathway may result in raised circulating Hcy levels. The

extent by which Hcy is elevated depends on the severity of the underlying defect, and in many cases can be overcome, or at least controlled by dietary intervention.

As a follow on to the initial cohort studies[34] which had their basis in determining risk factors for vascular disease, several second generation studies have been established, such as the Framingham offspring study, and the Hordaland Homocysteine Study, focussing initially on obtaining cross-sectional data, on the relationship between Hcy and life-style and risk factors related to chronic diseases, in particular cardiovascular disease [35;48-50]. In the longer term, these studies aim to relate Hcy to future all cause and coronary heart disease mortality and morbidity. In the mean time hyperhomocysteinaemia has been found to be associated with several physiologic and lifestyle factors, including age, gender, blood pressure, serum cholesterol, smoking, alcohol and coffee consumption, physical activity, diet and vitamin status.

Although genetic causes of disturbed Met metabolism may result in marked elevations in Hcy, in the general public a combination of acquired and environmental factors results in a wide range of observed Hcy concentrations.

1.4.1 Genetic variability

Classical homocystinuria is a deficiency in the CBS gene[14], so severe that Hcy levels exceed the renal threshold and homocystine becomes detectable in the urine[12]. Since the discovery of the initial cases, it has come to light that individuals with CBS deficiency are a heterogeneous population, with varying degrees of responsiveness to vitamin B6 therapy (vitamin B6 being the cofactor for the CBS enzyme). In some cases, individuals show no improvement in their Hcy levels when treated with vitamin B6, whereas in others a combination of Met reduction and vitamin B6 treatment can reduce Hcy levels to near normal. Therefore, in CBS deficiency, the age of onset of symptoms, and the

clinical severity of the disease, differs considerably between cases[16], being influenced by the nature of the defect within the coding gene.

Hcy usually exists in the circulation in very small amounts, and therefore most of the factors influencing Hcy levels are concerned with elevations above normal concentrations, however, an unusual situation occurs in Down's syndrome, or trimosy 21. As the CBS gene is located on chromosome 21, this group of individuals has a gene dosage of 150% of normal, consequently they clear Hcy, through the transsulphuration pathway, much more efficiently, leading to very low Hcy and Met concentrations, with increased levels of cystathionine and Cys[51]. However, as Hcy is not available for Met regeneration, a decrease in MS activity creates a "methyl trap" similar to that described in the case of NTD, whereby 5-MeTHF fails to be converted to THF. THF being the metabolically active form of folate required for *de novo* synthesis of nucleotides for RNA and DNA synthesis. Although the transsulphuration pathway is stimulated, glutathione levels are significantly lower in Down's syndrome than in control subjects. Glutathione may be depleted due to increased oxidative stress caused by over expression of the Cu-Zn superoxide dismutase gene that also resides on chromosome 21.

Patients with genetic defects affecting MS activity have also been reported. These may be related to defects in the enzyme itself, as appears to be the case in *cbl*G, or they can be defects in the redox proteins involved in the activation of MS as seen in *cbl*E patients[5] (see Figure 2). In both cases reduced MS activity leads to the same clinical and biochemical outcome, of megaloblastic anaemia, homocystinuria and hypomethioninaemia.

There have been conflicting reports about the association of several genetic polymorphisms in the enzymes involved in Met metabolism and their possible association with vascular disease. This inconsistency may lie in a failure to address the effects of combinations in genetic variability, and environmental factors. Mild genetic defects may, under normal circumstances, have no effect on the metabolism, however under stressed conditions, such as inadequate nutrition, or stress in the case of infection or ischemia, these mild defects may come to light through a temporary accumulation of metabolic intermediates. Should the stressed conditions be prolonged, or repetitive, elevated intermediate levels may result in the development of disease.

One such case is found in a commonly occurring polymorphism, which exists in the enzyme MTHFR, involved in Met regeneration from Hcy (Figure 1). A mutation of C $\rightarrow$ T at position 677 in the gene causes a deficiency in the protein, such that the enzyme becomes thermolabile. When folate status is adequate, the enzyme is capable of maintaining homeostasis. However, the enzyme possesses a low catalytic activity and reduced affinity for 5,10-MTHF and the flavin adenine dinucleotide (FAD) cofactor, such that when folate levels are only moderately reduced the enzyme becomes stressed leading to an increase in Hcy concentration. Therefore individuals possessing the TT genotype have an increased predisposition towards hyperhomocysteinaemia which is governed by lifestyle[35], which may explain the inconsistency of reports looking at MTHFR themoliability and Hcy concentration.

Several enzymes are involved in Hcy metabolism and it possible that mild defects in any of these may lead to alterations in Hcy levels, under stressed conditions.

1.4.2 Age and gender

Prior to puberty Hcy levels are very similar in boys and girls[52]. Although Hcy increases are observed within both sexes with increasing age, between the ages of puberty and the menopause, plasma Hcy concentrations are on average lower in females than in males[48]. This phenomenon may be related to higher circulating oestrogen levels in women, which is supported by the evidence that in the post menopausal age group, the differences between men and women become less pronounced[35].

In order to investigate this theory further, Giltay *et al*.[53], observed the effect on Hcy levels, caused by sex steroids used in the treatment of transsexuals. Prior to treatment, male to female (M→F) transsexuals had slightly higher Hcy levels at baseline than F→M, although this difference was not significant. However, following treatment with ethyl estradiol and antiandrogens, Hcy levels dropped from a mean of 8.2µmol/L to 5.7µmol/L, conversely, F→M transsexuals, treated with testosterone esters, showed an increase in Hcy from 7.7µmol/L to 9.0µmol/L.

These findings support the theory that sex steroids in some way may be responsible for the gender differences in circulating Hcy. Furthermore, when the results on all subjects were controlled for proportional change in plasma creatinine level, the effects of steroid treatment on Hcy levels disappeared, thus implying that the differences may be due to the anabolic or catabolic effects of steroids on muscle mass, and muscle turnover. Young weight lifters using additional androgenic steroids, in an attempt to improve their muscle mass and hence their performance, have been shown to develop premature myocardial infarction and stroke[54], which may be related to increased circulating Hcy.

Although some of the effect of steroids on Hcy levels may be due to muscle turnover a primary hormonal effect should not be excluded. In the study of Giltay *et al.*[53], M→F transsexuals who had no change in creatinine level, still showed a 20% decrease in Hcy, suggesting a direct effect of oestrogens on Hcy.

Increasing Hcy levels over the early years of development may therefore be due to increasing muscle mass, when boys reach the age of puberty they show a marked increase in Hcy, in conjunction with increased androgens[53]. However, the general tendency for Hcy levels to increase with increasing age in both genders may be due to declining nutritional status with increasing age. It has been shown that the mean Hcy levels can be decreased in an elderly population by an improved diet, however a decline in renal function, and impaired absorption of nutrients, from the intestine associated with age may also contribute to higher Hcy levels being observed in this age group[55].

1.4.3 Pregnancy

Substantial reductions in Hcy levels are observed during pregnancy[56], which appear to be independent of folate status and rapidly return to normal within two to four days post delivery. It is not clear what causes these changes but it may be an adaptation during pregnancy in order to maintain adequate placental circulation. It is also possible that for some reason the foetus is involved in the uptake of maternal Hcy.

1.4.4 Disease states

In some disorders raised Hcy is considered to be an effect rather than the cause of the underlying disease state. However, it may explain some of the vascular pathology, associated with these conditions. The association of hyperhomocysteinaemia, with

cancer and renal disease, and how in each case the disease may be both an effect and exacerbating cause has been discussed earlier in section 1.2.

Moderate elevations in plasma Hcy have also been shown in hypothyroidism, and hypothyroid patients have a high prevalence of coronary artery disease[57;58], however the reasons behind these observations are unclear. The effect of hypothyroid disease causing impaired renal function may be an explanation[57]. In addition hypothyroidism is associated with a decreased ability to convert riboflavin to FAD, the cofactor required for the action of MTHFR involved in Hcy remethylation. This may be due to decreased circulating thyroxine levels, since treatment with thyroxine, resulting in euthyroidism has been shown to reduce Hcy concentrations to within reference ranges[58].

1.4.5 Medications

Several therapeutic drugs interfere with Met metabolism in some way, leading to mild hyperhomocystinaemia. In most cases Hcy levels revert to normal upon cessation of treatment.

Methotrexate, a cytotoxic drug used in cancer chemotherapy, prevents cell proliferation through its interference in folate metabolism. However, depletion of folate leads to a decrease in the remethylation of Hcy.

Anticonvulsants, such as phenytion and carbamazepine, inhibit the polyglutamation of folate which is important for cellular retention and storage of folate in addition to playing an important role in the normal functioning and regulation of one-carbon metabolism[4].

Bile sequesterants, such as cholestyramine, prevent the reabsorption of bile from the gut and thereby lower cholesterol levels, through an increased demand for cholesterol in

the regeneration of bile. However, they may also interfere with folate and vitamin B12 absorption from the intestine.

Nitrous oxide, or laughing gas, may cause transient rises in Hcy levels, due to the irreversible oxidation of cob(I)alamin (Figure 2), and the inactivation of MS.

Theophylline, a bronchodilator used in the treatment of asthma, emphysema and bronchitis, may also cause disturbed Hcy metabolism, leading to transient, post-prandial increases in Hcy, as the drug impairs pyridoxal phosphate (vitamin B6) synthesis, by inhibiting pyridoxal kinase.

Cyclosporin A is used as an immunosuppressant following organ transplant, however treatment needs to be closely monitored as some of the metabolites are nephrotoxic. Impaired renal function may therefore explain the association between cyclosporin treatment and elevated Hcy levels.

Decreased Met and S-AdMet, and increased Hcy levels were seen in a group of patients treated with levodopa for the relief of Parkinson's disease[59]. Levodopa is administered with dopa decarboxylase inhibitors, in order to prevent its peripheral degradation. However, as a consequence, conversion of levodopa to 3-O-methyldopa, is increased which requires the transfer of a methyl group from S-AdMet, leading to mildly increased levels of Hcy (Figure 1).

Some sulphhydryl containing drugs such as D-penicillamine, and N-acetylcysteine, may actually decrease Hcy, which is possibly due to thiol disulphide exchange, which may enhance excretion, or lower plasma protein binding, altering the distribution of Hcy.

1.4.6 Blood pressure and cholesterol

In the Hordaland Homocysteine study a positive relationship between both diastolic and systolic blood pressure and plasma Hcy values was observed[35]. However, the correlation was weak and other studies have reported conflicting results and suggest that antihypertensive medication might be responsible for the observed associations[48]. A weak relationship was also observed between cholesterol levels and Hcy.

1.4.7 Diet

Diet has a large part to play in the control of Hcy levels. The primary source of Met is dietary protein, in particular protein derived from animal products. Affluent societies, which tend to have a high intake of meat, also show a higher incidence of cardiovascular disease compared to less affluent groups. Yet, strict vegetarians, who refrain from all foods of animal origin, have been shown to have higher Hcy levels, than those with an omnivorous diet[60]. Vitamin B12, important in the remethylation of Hcy to Met, is also typically found in foods of animal origin. Thus it appears that raised Hcy levels in individuals on a strict vegan diet can be explained by insufficient vitamin B12 consumption. Vegetarians who maintained an intake of egg and dairy products, showed only a slight rise in median Hcy level, compared to high meat eaters, which did not reach statistical significance.

It has been suggested that in the US, prior to the introduction of the folate supplementation program[61], two-thirds of hyperhomocysteinaemia was attributable to inadequate blood levels of one or more of the vitamins involved in Met metabolism, in particular, vitamin B12 and folate[55]. These two compounds are responsible for maintaining homeostasis, whereas vitamin B6 is involved in post-prandial control of

Hcy levels. Transient rises in plasma Hcy may be detected following a meal, or post Met load testing, but in the absence of a severe enzyme defect, fasting hyperhomocysteinaemia, usually indicates a deficiency in the cofactors involved in remethylation. Hcy is a sensitive indicator of vitamin deficiency and plasma levels start to rise as vitamin levels drop below mean values, well before signs of any clinical vitamin deficiency.

In the Hordaland Homocysteine study[35] folate derived from food showed a weaker negative correlation to Hcy levels, than folic acid taken in the form of vitamin supplements. These observations may be due to poor estimates of dietary intake, or due to a greater availability of metabolically active forms of folate being derived from supplements.

1.4.8 Smoking

In both the Caerphilly[50] and the Framingham Offspring[48] cohorts, smokers had significantly higher Hcy levels than ex-smokers or those who had never smoked. The Hordaland Homocysteine study[35] also showed a strong graded relationship between Hcy concentration and the number of cigarettes smoked per day. This was particularly true in women, where the estimated increase in Hcy corresponded to approximately 2% per cigarette per day. Following cessation of smoking habit, Hcy values usually return to normal within a short space of time.

Smokers tend to consume a less healthy diet than non-smokers, and smoking itself may effect the metabolism of B vitamins leading to altered Hcy metabolism[48;62].

1.4.9 Alcohol

Chronic alcoholism leads to increased plasma Hcy levels, relating to low levels of B vitamins[63]. The relative increase in Hcy and decrease in folate however appear not to be related to the intensity of alcoholism, the presence of liver disease or renal failure, but are in fact influenced by irregularity in feeding patterns. Moreover, hyperhomocysteinaemia is more intense in those patients who are homozygote for the TT polymorphism of MTHFR. Upon withdrawal, improved feeding habits in hospitalised patients can reduce Hcy levels by up to 40% over the course of couple of weeks.

Animal models and *in vitro* experiments however showed a direct relationship to increases in Hcy[64-66]. It is believed that acetaldehyde a metabolite of ethanol, forms covalent adducts with proteins that are inhibitory to MS activity. Folate deficiency due to irregular feeding habits may simply exacerbate the problem.

Mild alcohol consumption on the other hand, may help to reduce Hcy levels. A weak U-shaped relationship was observed in the Hordaland cohort[35], with a negative correlation between Hcy concentrations and the level of alcohol consumed up to 14 units per week. Beyond this point a positive relationship developed.

In the Caerphilly study[50], moderate alcohol intake was again negatively correlated with Hcy levels. However, it was noted that the cohort consisted of a large group of men in whom the preferred beverage was beer, which contains a large amount of folate.

A recent intervention trial showed that red wine and liquor consumption correlated with an increase in Hcy, but beer consumption did not[67]. These findings were confirmed in the Framingham Offspring Cohort[48]. Consumption of spirits also led to a decrease in folate levels, although this was not the case with red wine.

1.4.10 Caffeine

Heavy coffee consumption has been reported to increase Hcy levels[35]. However, this was not the case with decaffeinated coffee, implying that caffeine is in some way interfering with Met metabolism. This theory was strengthened in the Framingham Offspring study[48] where Hcy levels were also positively associated with consumption of soft beverages containing significant levels of caffeine.

1.4.11 Physical activity

Levels of physical activity have been shown to be inversely related to Hcy levels[35], however this may reflect a healthier attitude to lifestyle, which may be associated with a better diet.

1.4.12 Combined influences

Several factors affect Hcy levels, and these factors may combine, such that an individual who is a heavy drinker and smoker, who consumes a poor diet, may have much higher levels of Hcy, and therefore be more at risk of vascular disease, than a tea total, non smoker, who is well nourished.

In the Hordaland cohort[35], individuals with contrasting lifestyles of folate intake, caffeine consumption, and smoking showed 3-5µmol/L differences in their Hcy concentration. The total differences were greater than could be ascribed to each individual factor, therefore suggesting that each factor has its own individual mechanism for influencing Hcy concentrations.

Furthermore, such effects may be responsible for the skewed distribution towards higher Hcy concentrations that are observed upon definition of population based reference ranges[49]. Reference ranges calculated in populations with high folate intake,

low caffeine consumption, who are generally non-smokers form an essentially normal distribution.

1.5 Treatment of Hyperhomocysteinaemia

The association of Hcy with a number of diseases is well founded, and several factors may cause elevations in circulating concentrations of Hcy. So how can the clinician help his patient to reduce their Hcy level?

In homocystinuria, the first line of treatment is Met restriction in the diet. However, as Cys is produced from Met via Hcy, Cys then becomes an essential amino acid, requiring supplementation.

CBS deficiency is a heterogeneous disease, some individuals may respond to vitamin B6 supplementation to improve the function of CBS and thereby reduce Hcy levels through the transsulphuration pathway. However, in some cases vitamin B6 therapy is unable to induce the activity of CBS, and treatment with folic acid and vitamin B12 may be required to stimulate MS activity, and reduce Hcy levels through the regeneration of Met. Betaine therapy may also be useful to promote alternative remethylation pathways (Figure 1).

Since vitamin B6 responsive individuals have some residual CBS activity, if they are left untreated they tend to have a better prognosis than those who would not response to vitamin B6 therapy. Untreated, vitamin B6 non-responsive, cases have a much higher incidence of mortality in their early years. In an international study of homocystinuria, death was shown to occur before the age of 30, in 23% of non-responsive cases, versus 4% of vitamin B6 responsive cases[16]. Moreover, 50% of untreated cases were expected to have suffered a thromboemobolic event prior to the age of 30 years. Untreated

vitamin B6 non-responsive cases also show a much higher degree of mental retardation, than vitamin B6 responsives, with an average IQ of 57 versus 79. If detected early enough in new born screening programmes, vitamin B6 responsive individuals, who comply with treatment, may develop normal, or near normal intelligence levels. Upon treatment, it is possible to reduce Hcy levels dramatically, and vitamin B6 responsive cases may see their Hcy concentrations return to within population based reference ranges[68], thereby greatly reducing the prevalence of complications[69]. In the vitamin B6 non-responsive cases, where treatment is not as effective in reducing Hcy levels, complications may still occur, but compliance with the diet slows down the progression of the disease considerably, with patients showing improved IQ levels.

Even if homocystinuria remains undetected until the teenage years, commencement of dietary treatment at this late stage can increase the life expectancy[70].

Although homocystinuric patients clearly have a better prognosis if their Hcy levels can be reduced by dietary intervention, their pre-treatment levels are much higher than the mild elevations associated with an increased incidence of cardiovascular disease in the general population. Nevertheless, it may still be possible to reduce Hcy levels in the population as a whole, possibly leading to a reduction in the incidence of associated disease.

Eukaryotic cells are unable to synthesize folate *de novo*, therefore folate is an essential nutrient[4]. Natural sources include a wide variety of fruits and vegetables, particularly green leafy vegetables such as spinach or Brussel sprouts. Yeast, beans and organ foods such as liver are also rich in folate. Folic acid, the synthetic form of folate, is also present in several multivitamin preparations. Therefore improved diet and vitamin supplementation may help to lower Hcy levels, through stimulation of the

remethylation pathway (Figure 1). Even if plasma levels of vitamins B12 and B6, and folate are within the normal range, vitamin supplementation can effectively reduce Hcy levels[32].

Prior to government intervention in the US, two-thirds of the incidence of hyperhomocysteinaemia was attributable to inadequate B vitamin concentrations[55] and it was estimated that over 80% of adults consumed less than the recommended intake of 400µg / day of folate[32].

In 1993, the US government recommended that cereal products should be fortified with folic acid. The aim of this project was to increase folate intake by women of childbearing age in order to reduce the incidence of NTD, and fortification came into force by 1st January 1998[61]. However although the level of supplementation suggested in this program (140µg / 100g cereal grain products) may increase plasma folate levels there are conflicting results as to the effect that such supplementation may have on effectively reducing Hcy concentrations to levels which might reduce the incidence of cardiovascular disease[71-73].

In 1995, Boushey *et al.*[32], predicted that compulsory folic acid supplementation of flours and cereal products, could reduce the annual incidence of death due to heart disease by 30,000 men and 19,000 women per year, but this would require the introduction of a 350µg / 100g cereal grain products fortification scheme.

In 2002, a recommendation to follow the example of the US and introduce a folic acid supplementation program in the UK, was rejected by the Food Standards Agency[74], on the basis that folic acid supplementation may mask the anaemia whereby vitamin B12 deficiency usually presents. Progressive neurological damage may continue undetected, and may only present when severe damage has occurred to the spinal cord.

Therefore it may be wise to exclude vitamin B12 deficiency prior to folate fortification, or to introduce a combination of folate and vitamin B12 supplementation.

Additional supplementation with approximately 1mg of oral vitamin B12 per day is expected to avoid the theoretical risk of neuropathy due to unopposed folic acid therapy in patients deficient in vitamin B12, even in those with intrinsic factor deficiency or malabsorption states.

The minimum effective dose of any vitamin supplementation, of course, depends on the severity of the underlying deficiency. Whilst recommendations have been made for the reduction of Hcy levels in the population as a whole, hyperhomocystinaemia due to renal insufficiency, for example, may require much higher doses of folate.

Following initiation of treatment, Hcy levels rapidly drop. On average values return to normal within four to six weeks, but some may respond to therapy much faster, and Hcy may fall into the normal range within as little as two weeks.

The treatment of hyperhomocystinaemia is therefore, effective, inexpensive and relatively safe, although vitamin B12 supplementation may be recommended in addition to folic acid treatment.

1.6 Treatment Targets

Normal ranges, or more specifically "reference" ranges, will by the very nature of the way in which they are established, include a number of individuals with Hcy levels higher than might be desired. In Appendix A, a number of published poster abstracts and papers are included which make up a large part of the practical aspects of this thesis, Poster VI describes the establishment of a reference range for Hcy in accordance with IFCC guidelines[75] on 143 volunteers. The method used was the Drew DS30 Hcy

system (Drew Scientific Ltd., Barrow-in-Furness, UK), a HPLC system that will be described in more detail in Chapter 2. Exclusion criteria stated that no participant should have a prior history of cardiovascular disease, and that the volunteers should have been abstinent, for at least 6 months, from any medications known to affect Hcy levels. In this population a skewed distribution towards higher Hcy levels was observed in accordance with the findings of others[49]. Using a 95% interval the reference range was determined as 5.2 to 15.1µmol/L, which is also in line with other publications[76].

Hyperhomocysteinaemia therefore refers to a Hcy concentration above the defined reference range; moderate elevations are between 15 to 30µmol/L, intermediate increases as may be observed in heterozygotes for CBS deficiency are in the region of 31 to 100µmol/L, and anything over 100µmol/L must be described as severe hyperhomocysteinaemia, which is usually only present in those homozygote for an inborn error of metabolism[77].

Differences in reference ranges do occur however, and these may be due to differences in the performance of the methods used to analyse Hcy, or they may be due to true population differences. Therefore it is recommended that each laboratory should establish an appropriate reference range based on healthy individuals from the population under investigation.

Nevertheless, reference ranges are far from ideal in setting treatment targets. As Hcy concentration shows a graded association with disease risk, it may not be wise to think in terms of threshold values but the lower the level that can be achieved through intervention the better. A 5µmol/L reduction could potentially lessen the risk of vascular disease by as much as 80%[32].

Many studies have looked at different treatment regimes to assess the degree of Hcy reduction possible through intervention. These include studies on folate, vitamins B12 and B6 and combinations thereof. In an attempt to determine the optimal treatment regime, the Homocysteine Lowering Trialists' Collaboration, performed a meta-analysis on data from 1114 patients who participated in 12 trials covering periods of 3 to 12 weeks duration[78]. The aim was to evaluate the extent by which Hcy could effectively be lowered to assist in the development of protocols for future intervention trials. They found that a greater proportional and absolute reduction occurred in those with higher pre-treatment Hcy levels and lower pre-treatment folate levels, but no association was found with vitamin B12. Those within the highest quintile of pre-treatment Hcy values showed that a 39% reduction (95% CI 36% to 43%) in Hcy concentration was possible, whereas those in the bottom fifth, showed only a 16% reduction (95% CI 11% to 20%).

Standardising to a starting Hcy concentration of 12µmol/L and folate of 12nmol/L (approximately average concentrations for Western population), the meta-analysis estimated that supplementation of dietary folic acid may be capable of reducing Hcy levels by approximately 25% (95% CI 23 to 28%), with similar effects observed across a range of daily dosages (0.5mg to 5mg). Complementary vitamin B12, given as 0.02mg to 1mg daily (mean of 0.5mg), could lead to an additional reduction of 7% (95% CI 3 to 10%). Vitamin B6 (mean 16.5mg / day) was not particularly effective in reducing Hcy values as it is involved in post-prandial metabolism rather than in maintaining Met: Hcy homeostasis.

It is therefore predicted that dietary supplementation may reduce Hcy levels in a Western population by a quarter to one third of current values. Such an estimate corresponds to a reduction from 12µmol/L to approximately 8 or 9µmol/L.

Boushey *et al.*[32], indicated that a prolonged lowering of Hcy concentration by 1µmol/L was associated with a 10% reduction in risk, throughout the range of Hcy concentrations from 10 to 15µmol/L. If a 3 to 4µmol/L reduction in Hcy could be achieved by intervention, this could lead to a 30 to 40% reduction in the incidence of vascular disease.

1.7 The current position

Although it has been shown that dietary intervention is capable of reducing Hcy concentrations, the true effect that this could have on reducing the incidence of disease remains to be determined. Therefore, there is still some debate on the benefits of treatment[79].

It is hoped that treatment will at least retard, if not stop the progression of vascular disease. It may also be possible to reverse the damage that has already been done, through the regeneration of tissues and the shrinkage of vascular plaques.

To date a number of randomised control trials have shown that lowering Hcy improves surrogate markers of cardiovascular disease, for example vascular function may be improved[80-83] or the rate of restenosis following coronary angioplasty may be decreased[84-86]. However, many of the studies that have shown vascular improvements have used quite high doses of folic acid (~5mg / day) and the effects may be dose dependent, although folate supplementation of 400µg / day may be capable of reducing Hcy concentrations, at this dosage no improvement to endothelial function was noted[87] and the US supplementation program is using an even lower dose[61].

The addition of B vitamins to the treatments used in these studies complicates the interpretation. Where B12 and B6 were provided in the form of supplements, Hcy

reduction was greater than with folate alone, and in general more positive outcomes are observed, however Hcy reduction maybe a side effect and the surrogate markers of vascular function may be related to other mechanisms involving B vitamins. A true benefit of Hcy reduction could only be proved if a number of treatments leading to reduced Hcy levels produced the same degree of reduced risk of vascular events.

Although appropriate dosage regimes may improve vascular function, this tentative evidence of a potential benefit in Hcy reduction needs to be strengthened by demonstration in randomised control trials that lowering Hcy is followed by a significant reduction in the incidence of mortality and morbidity due to vascular disease. Within the last decade a number of large-scale prospective randomised clinical intervention trials have been initiated, and the results are starting to emerge. The details of nine of these studies are summarised in Table 1. Although each study is slightly different, they are all essentially designed to assess the effects of Hcy lowering on the prevention of vascular endpoints. Each trial will look at the potential benefits of folic acid supplementation versus placebo, and some will also include vitamin B12 and / or vitamin B6 in their assessments. In six of the trials detailed, the primary endpoint is coronary artery disease, whereas three trials will look at the possibility of reducing the incidence of stroke. We should expect to see preliminary results published from some of the studies over the course of the next couple of years. From these results it may be possible to determine an optimal treatment regime, for a reduction in the incidence of vascular disease as a whole. Of course it may be shown that Hcy is not directly causal but is a purely a marker of disease and that folate levels are directly associated with vascular disease.

Table 1 Ongoing intervention trials using folic acid to lower Hcy concentrations.

Primary End Point	Study Name	Base Country	Sample Size	Folic Acid dose (mg)
Coronary artery disease				
	Western Norway B Vitamin Trial (WENBIT)	Norway	2000	0.8
	Cambridge Heart Antioxidant Study (CHAOS-2)	UK	4000	5
	Norwegian Study of Homocysteine Lowering with B-vitamins in Myocardial Infarction (NORVIT)	Norway	3000	0.8
	Prevention with a Combined Inhibitor and Folate in Coronary Heart Disease (PACIFIC)	Australia	10 000	2
	Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH)	UK	12 000	2
	Women's Antioxidant and Cardiovascular Disease Study (WACS)	US	6000-8000	2.5
Stroke				
	Heart Outcomes Prevention Evaluation-2 (HOPE-2)	Canada	5000	2.5
	Vitamin Intervention for Stroke Prevention (VISP)	US	3600	2.5 vs. 0.2
	Vitamins to Prevent Strokes (VITATOPS)	Australia	8000	2

One difficulty faced by several of these trials is that they were designed based on estimates of achievable Hcy reduction compared to control groups prior to the mandatory folic acid fortification of cereal products[78], introduced in Northern America in 1998[61]. Although this ruling was introduced to prevent folic acid deficiency during pregnancy in order to reduce the number of NTD affected births, it is likely that it will also have the effect of reducing Hcy concentrations in the general public and may assist in reducing the incidence of vascular disease.

In a study of coronary angiograph patients pre- and post- fortification the ruling appeared to achieve a 1.5µmol/L reduction in median Hcy levels[88]. There was also a shift in the distribution of results with a significant reduction in the number of patients with Hcy >15µmol/L. Mortality risk was slightly reduced but the results were not significant. Although over 2000 patients were followed over 2 years a much larger and longer study would be required to detect if such a modest reduction would be beneficial in the population as a whole.

The consequence of mandatory folic acid fortification coinciding with the early phases of these large intervention trials, therefore, is that a reduction of ~1.5µmol/L Hcy may also be expected in control groups, and the predicted difference of 4 to 6µmol/L between treatment and control groups may not be feasible in individuals residing in Northern America.

Results are starting to emerge from some of the studies and it appears that the fears are being proved correct. Authors are suggesting that a failure to show a significant reduction in vascular events is due to underpowered studies, rather than a lack of association[89-92].

VISP, a US based study, was only able to achieve a Hcy reduction of 2µmol/L in a group of patients on high dose B vitamins compared to low dose[90;92-94]. Although a slight reduction in all cause mortality was observed this failed to reach significance[92]. In fact the selection criteria had to be adjusted to compensate for a shift in the expected Hcy distribution[89]. A further limitation was that the control group received the recommended daily intake of vitamin B12[92].

Both HOPE-2 and WACS, based in Canada and the US respectively, have also only managed to achieve meagre reductions in Hcy levels (approximately 1µmol/L)[89].

In the UK, where mandatory folic aid fortification has so far been rejected[74], CHAOS-2 achieved a 13% reduction in Hcy, but also failed to show a significant reduction in vascular events[89]. However in this study folic acid alone was used as treatment and B vitamins, in particular B12 may be required to achieve an adequate reduction in Hcy. A negative result may also be caused by a recent tendency for increased vitamin supplementation, and improved diet in the general population. Voluntary folic acid fortification is also becoming a popular practice with cereal manufacturers.

If treatment is inexpensive and harmless, the potential benefits should not be dismissed on the basis of one or two negative results from studies that may well be underpowered. More worrying was the recently announced results of the NORVIT study[95], which appeared to show that a combination of folate and B vitamins may actually increase the risk of cardiovascular disease. However an isolated result, in again what may be deemed an underpowered study, should not be enough to eliminate the theory that Hcy reduction is beneficial.

VITATOPS, based in Australia, is recruiting patients in 19 countries, most of which do not have mandatory folic acid fortification. In light of the findings from VISP, an early assessment of Hcy reduction was conducted at one centre in Perth, Australia. After 6 months of treatment, Hcy levels were 3.7µmol/L lower than levels in the placebo group[94], indicating that this study may prove to be one of the first that is adequately powered to measure the effect of Hcy reduction on the incidence of serious vascular events. However the authors warn that in Australia voluntary fortification of foods with folic acid is increasing. Luckily perhaps the study includes several centres based in the Far East, where nutritional status is expected to be lower and free from the effects of

supplementation. This is also a much larger study than some of the published ones as calculations of the number of participants required were based on the detection of more modest predictions of relative risk reductions[90].

While awaiting publication of the remaining intervention trials, clinicians will have to judge whether they believe the potential benefits of vitamin supplementation outweigh the potential risks and costs. Although most studies have so far failed to prove any benefit, slight reductions in Hcy levels and cardiovascular events have been seen in addition to those brought about by population-based increases in folate intake.

1.8 Summary

Hyperhomocysteinaemia is implicated as a risk factor for several diseases. Hcy is influenced by several factors and plasma levels are determined by a combination of acquired and lifestyle factors. Treatment to reduce Hcy levels is inexpensive and safe, however, there is still some debate as to whether raised Hcy is a cause or effect of disease. Therefore it is important that research in this field continues so that we may establish the mechanisms by which hyperhomocysteinaemia causes disease and the potential benefits of treatment.

Aims

The aim of this project was:

- To study the metabolism of Hcy in detail in order to develop a method of stabilising whole blood samples for plasma Hcy measurement.
- To study Hcy and its interaction with other aminothiol in patients with Alzheimer's Disease.

Objectives

The main objective in the first part of this project was to produce a commercially viable method of stabilising Hcy in whole blood samples.

In order to fulfil this objective the following targets should be met:

- Develop an understanding of Hcy metabolism, and the factors influencing Hcy concentrations both *in vitro* and *in vivo*.
- Determine the most suitable currently available method for Hcy stabilisation in whole blood samples and investigate its usage in a commercial product.
- Produce a non-invasive *in vitro* method for investigation of potential stabilising agents.
- Identify key enzyme(s) involved in Hcy production by erythrocytes as potential target(s) for inhibition. Develop an understanding of the reaction mechanisms, enzyme structure and key amino acids involved in substrate binding at the active site of the enzyme(s).
- Use molecular modelling techniques to identify a subset of enzyme inhibitors for further investigation using the *in vitro* assay.
- From the results of *in vitro* screening select one or two compounds for use in non-evacuated blood collection tubes in pilot studies.

- Select a single compound and produce a pilot batch of evacuated blood tubes.
- Conduct clinical trials and shelf life studies to assess the performance of a commercially viable product, and compare it with the most effective currently available method.

The objective within the second part of this thesis was to investigate plausible mechanisms linking increased plasma Hcy concentrations and the development of cognitive decline and Alzheimer's Disease. This objective may be met, through studies using patients with Alzheimer's Disease in the following way:

- Study the relationship between Hcy, vitamin B12, and transcobalamin.
- Investigate the theory that transcobalamin phenotype may determine Hcy concentrations.
- Conduct studies to support the theory that vitamin B12 is involved in the development of Alzheimer's Disease through a functional rather than a dietary deficiency.
- Determine how Hcy relates to concentrations of the other aminothiols involved in remethylation and transsulphuration.

Chapter 2 HCY MEASUREMENT

Hyperhomocysteinaemia has been associated with a number of disorders, particularly vascular disease. Expansion in this field of research has driven the requirement for fast and reliable Hcy analysis. Should prospective studies eventually provide confirmation of the beneficial effects of Hcy reduction on the incidence of vascular disease, then the establishment of widescale Hcy screening programs will increase the demand for accurate Hcy measurement within the clinical setting.

2.1 Total Hcy

The term "homocysteine" is often misused, it should relate to the free reduced form of the amino acid, but the term commonly refers to the sum of a number of compounds, including the free monomeric form, the oxidised dimer, and mixed disulphides. Disulphide links are formed with other thiols in the circulation, which are present either in the free thiol state or contained within protein structures. The methyl transfer reactions (Figure 1), which convert Met to Hcy, occur intracellularly throughout the body[42]. Although the thiol group of the Hcy is easily oxidised, the reducing potential of the intracellular environment maintains Hcy in its monomeric form. Upon export into the circulation, Hcy undergoes rapid oxidation to form disulphide bonds, the majority of which (~80%) are formed with cysteine residues within plasma proteins, in particular albumin, the principal protein present in the circulation. Another 5-10% of Hcy forms mixed disulphides with other plasma aminothiols, and 5-10% exists as the dimer, "homocystine", only a trace amount (<2%) remains in the free form. During storage of a processed plasma sample, redistribution occurs such that virtually all the Hcy becomes bound to plasma protein. Therefore when assessing risk of vascular disease it is important to measure all forms of Hcy. This is achieved through the use of reducing

agents that break the disulphide bonds and allow measurement of "total Hcy". Therefore, unless specifically stated analysis of plasma "homocysteine", refers to the measurement of the sum of all these forms.

2.2 Hcy Analysis

Although there are methods available for measuring the different forms of Hcy, many of these are only suitable for a research environment and to date there is little information about whether measuring free Hcy has any advantage over measuring the total amount present, therefore the routine clinical laboratory remains focussed on analysis of total Hcy.

There are currently a number of techniques available for Hcy measurement, many of which have been reviewed by Ueland *et al.*[76] and more recently by Rassmussen and Møller[31]. The majority of methods can be loosely divided into two groups, those that use chromatography[22-28], and those that are based on immunological techniques[29;30]. Other less popular methods include enzymatic colorimetric assays[96], molecular imprinting techniques[97] and substrate trapping using mutant enzymes[98].

2.2.1 Chromatographic techniques

Hcy analysis was originally performed by thin layer chromatography. This qualitative technique was not sensitive enough to detect plasma levels of Hcy, and as part of a general screen was used purely as a way of detecting abnormally high levels of the dimeric form of the amino acid present in the urine of patients with severe inborn errors of metabolism. Much more sensitive analysis, capable of detecting a few µmol/L is provided by high performance liquid chromatography (HPLC).

In order to determine total Hcy, a reduction step is necessary in order to free Hcy from its covalent attachment to other thiol compounds. Reduction agents vary from method to method, earlier methods used compounds such as tri-butyl phosphine [22;27;28], and dithiothreitol[23-25]. These reducing agents tend to have poor solubility and certainly have an unpleasant aroma. A much more desirable alternative was offered in the form of tris(2-carboxyethyl)phosphine (TCEP)[26] which is "nonvolatile, stable, and soluble in aqueous solution and thus is more suitable for routine use"[99].

Chromatography is used to separate out the different thiols present within plasma. In the plasma of a normal individual the most abundant total thiol is cysteine. Cysteinyl-glycine, a breakdown product of glutathione is the second most abundant, and Hcy and glutathionine are usually present in much smaller quantities. In very fresh plasma, it is sometimes possible to detect γ-glutamyl-cysteine, which is an alternative breakdown product of the tripeptide glutathione following a single peptide cleavage; however this thiol rapidly degrades to undetectable levels.

These compounds do not naturally absorb light or fluoresce, which are the easiest ways of detecting compounds emerging from a HPLC column, therefore some other method of detection is required.

Various detection techniques are used, which may involve pre- or post- column derivitisation. However most methods rely on derivatives using either ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F), or 4-(aminosulphonyl)-7-fluoro-2,1,3-benzodiazole (ABD-F) to attach to the sulphur of the thiol rendering the compound fluorescent.

Some methods use electrochemical detection, since the thiol group is capable of undergoing redox reactions. However, since plasma contains a number of compounds

that undergo redox reactions, these methods produce noisy traces, which can be difficult to interpret.

The advantage of chromatographic analysis is that it allows the simultaneous measurement of other thiol compounds within the plasma. Commercial kits based on HPLC methodology are currently available from several manufacturers.

One such kit is used for Hcy measurements throughout this thesis. The DS30 Hcy Assay System, manufactured by Drew Scientific Ltd. (Barrow-in-Furness, UK), is a dedicated reverse-phase HPLC system with assay kits capable of measuring total plasma Hcy in 30 samples per batch. For full details of the assay kit, see the product insert (Appendix B). In summary the method uses cysteamine as internal standard, TCEP to reduce all disulphide bonds, tricarboxylic acid to precipitate protein and SBD-F as a fluorescent tag. Correlation of the DS30 Hcy system against an in-house HPLC method using electrochemical detection is described in Poster VII.

Where other thiols were estimated these were run using a software adaptation to the commercial method to allow quantification of Cys, cysteinyl-glycine and glutathione.

2.2.2 Immunochemical techniques

The development of a monoclonal antibody against a small molecule such as Hcy, which is specific enough to allow it to be distinguished from other plasma thiols with minor structural differences, has so far not been possible. However, one group has produced an antibody against its precursor S-AdHcy[100]. This antibody forms the basis of several commercially available immunochemical techniques available for Hcy measurement[29;30]. All immunochemical methods require two initial steps. Firstly Hcy needs to be reduced, so that total Hcy may be measured in the monomeric form. Secondly, the enzyme, SAHH must be used in the reverse direction to form S-AdHcy.

The antibody is then employed in several different fashions, depending on the method, in order to determine the amount of S-AdHcy produced in the reaction, the concentration measured is used as an estimate of the total amount of Hcy originally present.

Although immunoassay only allows the measurement of a single analyte, it lends itself to the development of rapid and easy to use, automated analysis, requiring small sample volumes.

2.2.3 Other Hcy methods

In recent years new methodology has emerged with assays for Hcy based on enzymatic assay with fluorescent detection[96], molecular imprinting techniques[97], and substrate trapping enzyme technology[98]. However, to date, such methods have not found their way in to routine use.

2.3 Sampling Techniques

2.3.1 Sample type

Hcy is produced from the intracellular metabolism of Met. This process occurs throughout the body[42]. If Hcy exceeds the capacity for remethylation, it is exported into the circulation and transported to the liver, kidney, pancreas and small intestine for utilisation in the transsulphuration pathway, leading to the formation of Cys and glutathione[42]. Intracellular concentrations of Hcy are therefore very low, and hyperhomocysteinaemia represents an increase in plasma Hcy, i.e. extracellular concentrations.

The absence of tissues capable of the transsulphuration pathway *in vitro*, means that any Hcy produced in the erythrocytes[101], accumulates within the plasma. Plasma Hcy levels in whole blood samples have been observed to rise at a rate of some 10% per hour following venipuncture[22;101]. Since intracellular Hcy concentrations are some ten fold less than in the plasma, any rise in Hcy must be due to continued metabolism rather than cellular leakage.

The current recommendation is to store collected blood samples on ice until such time as centrifugation can be performed. Once the red blood cells have been removed plasma Hcy is stable for at least 24 hours at room temperature and for several months, if not years, when stored frozen[22].

Hcy may be measured in plasma or serum. Only in the rare genetic disorder of CBS deficiency, does the level of Hcy become so high that it is detectable in the urine, which gives this disorder its name "Homocystinuria". Hcy can be measured in serum, however, such samples are not ideal for Hcy measurement since significant rises in Hcy may be observed in the time required for adequate blood clotting to take place, therefore plasma is the sample of choice when screening for hyperhomocysteinaemia. EDTA is commonly used as anticoagulant, in addition to preventing blood clotting the chelation of divalent ions helps to prevent metal catalysed oxidation occurring within the sample during analysis. If the sample is not chilled and processed quickly, Hcy export from erythrocytes will cause rapid elevations in plasma Hcy concentrations[101].

If a 5µmol/L increase is associated with an 80% increased risk of vascular disease[32], artifactual elevations in plasma Hcy, caused by delays in sample processing could easily lead to false positive results being reported. Although the treatment for

hyperhomocysteinaemia is simple and non-invasive, proper risk assessment demands accurate data.

Several attempts have been made to modify collection procedures in order to prolong the stability of samples and avoid artifactual increases in plasma Hcy prior to sample processing in the laboratory.

2.3.2 Storage temperature

At present, the standard practise in the collection of samples for Hcy measurement is to put the whole blood sample on ice and centrifuge as soon as possible, preferably within 30 minutes to 1 hour post venipuncture. This protocol may allow plasma Hcy measurement to be undertaken within a small hospital with a fast and efficient portering system where one can guarantee that the samples will be received and processed within an hour of the sample being collected. However, as hospital resources are stretched increasingly, how confident can the clinician be that a sample has been correctly handled on its way to the laboratory, and that the Hcy values reported are an accurate assessment of the patient's Hcy status? Hcy measurement is not undertaken as part of an emergency situation, and is likely to be carried out as part of a screening programme, in risk assessment or in clinical studies. If Hcy measurement is to move into the community, an alternative method of sample collection and handling must clearly be developed.

Chilling samples may help to slow down the action of erythrocyte enzymes involved in the manufacture of Hcy. Alternatively, it may slow down the processes involved in Hcy export from the cells. To date, assessments into the stability provided by chilling samples[23;102-104] has shown that the maximum period of stability reported is 6 hours on "ice"[104]. Although this may be more practical within a hospital environment than the

current recommendations, it still does not open up Hcy screening into the community setting.

2.3.3 Acid citrate

If ice is unavailable, acid citrate may provide an alternative means of sample stability in the short term under ambient conditions. Again, however, the maximum stability reported is only 6 hours at "room temperature", and less if the temperature is higher, with significant changes being observed at 37ºC in under 4 hours[105]. Ducros *et al.*[103], found that sample stability is also dependent on the method of analysis. All the chromatographic methods investigated showed sample stability when collected into acid citrate and stored at room temperature up to at least 4 hours after collection, whereas when using an immunochemical method (fluorescence polarization immunoassay), a significant change was observed over the same time course. Similar observations were reported by Salazar *et al.*[106] and O'Broin *et al.*[107] who showed a significant rise after two hours, or a 10% rise after 6 hours respectively when using an immunochemical method to measure Hcy. Whereas chromatographic methods measure Hcy itself, or an Hcy derivative, immunochemical methods are indirect. Hcy is estimated by the amount of its metabolic precursor, S-AdHcy, formed during the assay using the reverse reaction of the enzyme SAHH. In acid citrate the low pH may prevent Hcy production by inhibiting SAHH, however precursor build up is not prevented, thereby giving the impression of sample instability. Where stability is reported, there are conflicting reports about whether acid citrate increases[105;107] or decreases[106] baseline Hcy compared to EDTA stored on ice. This confusion may be related to corrections for dilution due to the addition of acid citrate as a liquid, requiring haematocrit estimates. Either way it

appears that the use of acid citrate in blood collection tubes for Hcy measurement would require separate reference intervals for interpretation.

2.3.4 Cell lysis

An attractive alternative for sample collection for Hcy measurement appeared to be offered in the way of capillary whole blood lysates[108]. Sample collection is easy, and less invasive than venipuncture. The blood collection tube contains a non-ionic detergent (such as Non-idet P40), which lyses the blood cells and hence dilutes the enzymes and substrates required for Hcy production. This method has been reported to stabilise Hcy concentrations for at least 2 days under ambient conditions. However, a flaw with this approach is that, even following correction for dilution caused by the lysing agents, the results produced are lower than those in plasma due to further dilution by intracellular fluid, which is low in Hcy. Much of the literature on Hcy focuses on measurements made in the plasma, and hence if this approach were to be used new reference ranges would need to be established, with a much lower threshold value associated with increased risk of vascular disease. Even if the reference ranges were corrected for lysate measurements, this takes no account of an individuals' haematocrit and falsely high results may be observed in anaemic individuals with low haematocrit values. Further problems exist in relation to the assay used to measure Hcy, as greater method sensitivity would be required to differentiate between the lower concentrations obtained in blood lysates.

2.3.5 Sodium fluoride

Sodium fluoride samples, at 2 to 3 hours post collection have shown similar Hcy concentrations to baseline EDTA values[109;110] and have therefore been recommended as

an alternative if there is to be a delay in sample collection. Yet on closer inspection, Hcy continued to rise over time. The fact that values at 2 to 3 hours post collection were similar to the baseline EDTA values was a result of an initial drop in concentration caused by the formation of hypertonic saline and consequential fluid shifts. This effect was confirmed by Hughes *et al.*[111], who saw a sodium fluoride concentration-dependent drop in haematocrit. The theory behind the use of sodium fluoride as a stabilizer in Hcy samples is related to the fact that the fluoride inhibits anaerobic glycolysis and therefore ATP production. Conversion of methionine to S-AdMet, the first step in the production of Hcy, requires ATP. Therefore sodium fluoride may indirectly prevent erythrocyte Hcy production by removing one the substrates required in the early stages of the pathway from Met to Hcy. However, the fact that Hcy values continue to rise in samples collected into sodium fluoride, may be attributed to cellular reserves of S-AdMet, first postulated by Andersson *et al.*[101]. In contrast Scheidhauer *et al.*[112] reported that following the initial drop in Hcy levels measured in plasma samples collected at 15 minutes, between 24 and 144 hours post collection Hcy levels remained stable.

2.3.6 Gel separator tubes

Within recent years the drive towards minimising infection risk through primary tube sampling has meant that the use of serum or plasma separator tubes has become widespread. These tubes contain a gel that, following centrifugation, keeps the red cells apart from the serum or plasma. Provided that Hcy cannot cross this gel membrane the measured total Hcy concentration should be unaffected by the continued production from red cells. Serum separator tubes were shown to keep Hcy concentrations within 4.5% of baseline levels over a 48hour period at room temperature[113]. Although this study showed only a small increase in Hcy levels, the tubes require time for the blood to

clot before centrifugation can take place and there is potential that at elevated ambient temperatures significant rises in Hcy may occur during this time. In a recent study heparinised gel tubes were used which could be centrifuged immediately after collection[114]. The samples were then stored at 2 to 8ºC prior to analysis. Heparinised plasma Hcy levels remained stable for up to 72 hours.

The downside of either of these types of tube is that they require a centrifuge at the site of blood collection prior to transport. Additionally EDTA is the recommended sample type for several methods and as yet no gel tubes containing EDTA have been evaluated for Hcy measurement. In some HPLC assays EDTA improves the fluorescent yield[56]; and in other assays its presence helps to prevent metal catalysed re-oxidation prior to derivitisation of the reduced disulphide bonds[76].

2.3.7 Enzyme inhibition using 3-deazaadenosine

Of the attempts made to stabilise Hcy concentrations in collected blood samples, none have produced a satisfactory approach to blood collection away from a centralised laboratory, without the requirement for considerable readjustment of literature reference ranges.

A more rational approach to the problem was taken by al Khafaji *et al.*[115]. They studied the pathway and decided to investigate the use of inhibitors to the enzymes and transport processes involved in the various steps of Hcy production. At 100µmol/L in EDTA whole blood, they reported that 3-deazaadenosine (3DA) stabilized plasma Hcy for up to 72 hours at "room temperature" prior to centrifugation. On the seven healthy volunteers used in their study, the mean Hcy concentration increased by 10% over 72 hours post collection, which was not shown to be statistically significant. 3DA prevents

the release of Hcy from red blood cells through competitive inhibition of the enzyme SAHH; the final enzyme in the pathway from Met to Hcy (Figure 1).

2.4 Pilot Study

Posters VIII and IX describe the results of a pilot study to evaluate the feasibility of developing the use of 3DA into a commercially available blood collection system. The ultimate goal was to produce an evacuated blood tube, containing 3DA at a final concentration identical to that used in the report of al Khafaji *et al.*[115], that would stabilize blood samples for up to three days under ambient conditions.

The tubes would be marketed for use in Hcy analysis, particularly in conjunction with the Drew DS30 Hcy system (Drew Scientific Ltd., Barrow-in-Furness, UK), which requires 200µL of plasma. Manufacture was to be contracted out to a company who were specialists in the production of blood collection tubes (L.I.P (Equipment and Services) Ltd., Shipley, UK). The smallest blood volume they could produce in an evacuated tube was 2.5mL, which would require 67µg per blood tube to give a final 3DA concentration of approximately 100µmol/L in the whole blood sample. For large scale manufacturing procedures this would have to be delivered to the tubes in the form of a liquid, however, in order to avoid dilution of the plasma, the liquid used for delivery should then be removed leaving 3DA behind as a solid. This process could be achieved by spraying the interior of the tubes with the appropriate volume of solution containing 67µg of 3DA, followed by a drying, evacuation and γ irradiation (used to sterilise the tubes). The liquid used to spray the tubes also contained the equivalent of 3.25 mg of dipotassium EDTA per tube. EDTA is used as an anticoagulant.

The intention of the pilot study was to establish whether the 100µmol/L 3DA, in whole blood collected into EDTA would ensure ambient temperature stability for at least three days to allow transit of samples through various delivery systems. However, as 3DA acts through competitive inhibition its effectiveness in the inhibition of Hcy production proved to be temperature dependent. Descriptions such as "ambient", or "room", temperature can cover quite a wide range of temperatures, controlling the storage of samples between 20 and 25ºC, the mean Hcy from four healthy volunteers rose significantly from 8.5µmol/L to 11.5µmol/L over 72 hours. This rise of 35%, conflicts with the 10% rise reported by al Khafaji *et al.*[115]. When samples containing 3DA were stored at 2 to 8ºC, over 72 hours, the mean Hcy dropped by a statistically insignificant 0.5µmol/L. As a control, whole blood samples collected into EDTA alone were also stored at 2 to 8ºC and these showed a significant rise from 8.8µmol/L to 16.7µmol/L over the course of three days. The increase in the mean Hcy at "time zero" was attributed to a rise in Hcy over the short time taken to centrifuge the samples and remove the plasma prior to refrigerating the whole blood.

To ensure that the spray drying process had not caused degradation of the 3DA, samples were collected from a further 5 volunteers in tubes containing liquid 3DA. Significant rises were again observed over 72hours. The spray drying process had no effect on the stability of the inhibitor as the mean increase in Hcy was very similar between the two processes (7.8µmol/L for liquid vs. 7.9µmol/L). Even with 3DA present at 200µmol/L the release of Hcy into the plasma could not be prevented. In blood collected from a single volunteer, the Hcy concentration rose by 1.5µmol/L over 72 hours at ambient temperature (Hill and Kenney unpublished results).

2.5 Clinical Validation - 3DA Blood Tubes

Despite the small number of volunteers used in the pilot studies, some important conclusions could be drawn. Firstly the use of 100µmol/L of 3DA cannot provide prolonged stability (up to three days) under ambient conditions. Doubling the concentration may reduce the amount of Hcy produced over time, however significant rises were still observed over two to three days storage. As 3DA is an expensive chemical any further increase in 3DA would not be cost effective in the development of a commercial product. Although not ideal, a combination approach of chilled storage at 2 to 8ºC, and the use of 100µmol/L 3DA showed some promise in allowing a delay between sample collection and processing. At worst the availability of such tubes would provide an interim solution for clinicians, enabling them to collect and store samples before having to send them to a centralised laboratory for processing. Therefore a trial batch of evacuated tubes was produced containing 3DA spray dried into the tubes in the presence of EDTA. Clinical validation of these blood tubes, conducted in such a way as to confirm whether a combined effect of low temperature and 3DA was sufficient to provide long-term stability of plasma Hcy in whole blood is described in Paper I. The study was conducted at two centres in order to collect samples from subjects spanning a large age range and from a range of initial plasma Hcy concentrations.

Most studies on sample stability have been conducted on apparently healthy populations. For a thorough clinical validation of the collection tubes, it was important to compare the stability of samples collected from a more diverse population, including patients with vascular disease and elderly patients, in addition to healthy volunteers.

Poster X describes the results from one of these centres where the volunteers were healthy individuals. The other centre recruited mainly aged patients with a tendency towards higher plasma Hcy levels.

DS30 Hcy Blood Collection Tubes (Drew Scientific Ltd., Barrow-in-Furness, UK), were manufactured for Drew Scientific Ltd. by LIP Ltd. (Shipley, U.K). The tubes contained 3.25mg of K2 / EDTA as anticoagulant, and 67µg of 3DA (100µmol/L final concentration) to inhibit Hcy production. Each tube was evacuated to collect 2.5mL (±0.25mL) of whole blood. 10mL K3 / EDTA Vacutainer™ tubes (Becton Dickinson, Cowley, UK) were used for comparison.

Hcy analysis was conducted using the DS30 Hcy analysis system (Drew Scientific Ltd., Barrow-in-Furness, UK) (Appendix B).

Only a combination of the DS30 Hcy blood tube and storage under refrigerated conditions offered stability of the plasma Hcy concentration in whole blood samples at all times up to 72 hours ($p=0.2761$). The changes in plasma Hcy over 72 hours under the three other storage conditions were all highly significant ($p<0.0001$). P values were calculated using one-way within-subject ANOVA (repeated measures).

Further analysis of the data showed that in EDTA alone the rise in Hcy concentration was significant before 3 hours had elapsed, regardless of whether the tubes were refrigerated or left at ambient temperature. Although long-term storage of blood in DS30 Hcy blood tubes requires that the tubes are kept at 2 to 8ºC, samples proved to be stable for up to 6 hours at 20 to 25ºC. A small subset of patients provided enough blood to show that whole blood stored in the DS30 Hcy collection tubes at 2 to 8ºC would maintain stable plasma Hcy concentrations for up to 1 week post venipuncture.

The study also highlighted the need for careful interpretation of published results regarding the stability of Hcy in collected blood samples. Many studies report their findings as percentage change over time, yet the rate of Hcy export from erythrocytes proved to be independent of initial plasma Hcy concentrations. This is clearly illustrated in Figure 3, where the percentage changes in the 0 to <5µmol/L and 5 to <10µmol/L bands over 72 hours differ significantly from any of the other groups. Beyond 10µmol/L percentage changes were not significantly different between the groups.

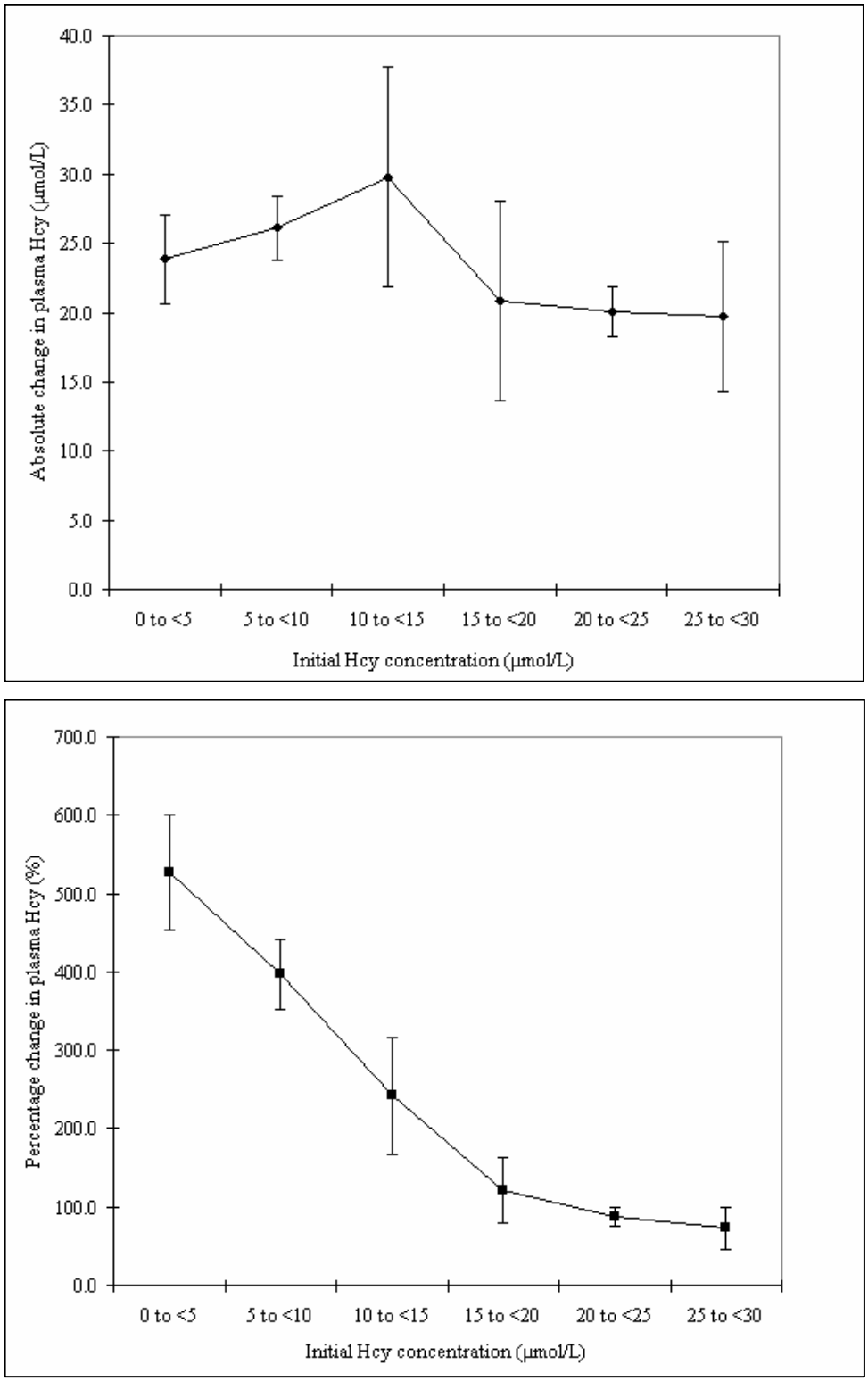

Figure 3 Absolute, and percentage changes in plasma Hcy after 72 hours storage of EDTA whole blood at 20 to 25ºC. Error bars show mean ±2SEM

Studies containing samples from individuals with moderately raised Hcy concentrations are therefore less likely to show a significant percentage rise over a given time frame. Greater credence should be given to reports of sample stability where the subjects have initial Hcy concentrations towards the lower end of the normal range, or studies that report absolute differences where Hcy is measured by a method with low imprecision.

In light of these findings, the data was reanalysed in two groups based on whether the initial Hcy was ≤10µmol/L or >10µmol/L. In each case the final result was the same after 3 days of storage, however, at 20 to 25ºC changes in Hcy became significant much earlier in the group with low baseline concentrations.

Within this study the sampling times were chosen to simulate different delivery scenarios. Current recommendations state that samples should be processed within 1 hour of collection. The initial sampling time of 3 hours was used to assess stability against baseline levels if delays occurred in sample delivery within the hospital setting. Sampling at 6 hours gave an indication of Hcy changes that might occur with same day delivery such as might be expected from clinics and doctors surgeries. A 24 hour delay may occur if samples are taken and stored overnight before delivery to the laboratory. Finally 72 hours allows enough time for delivery through postal services, as the ultimate aim of this project was to provide a means of allowing blood sampling to occur within the community with easy sample delivery to the laboratory under ambient conditions.

The results showed that if delays occurred within the hospital setting the samples must either be either chilled or contain a preservative in addition to EDTA. Chilling alone could only provide stability up to 3 hours, whereas 3DA at 20 to 25ºC stabilizes samples for 6 or even 24 hours, dependent on the initial Hcy concentration. This offers

advantages for samples collected near the site of processing, providing some relief in the requirement to have ice on hand and to get the sample delivered quickly to the laboratory. However, if there is a longer delay in transport, refrigerated storage in DS30 Hcy Blood Collection Tubes can offer prolonged stability for 72 hours and possibly up to a week.

From the results of this trial, product claims have been filed and approved, as part of FDA Section 510k notification for marketing in the US (510k No. 012152) (see Appendix C). The claims state that if whole blood is drawn into a Drew DS30 Hcy Blood Collection Tube and stored at 2 to 8ºC, plasma Hcy will remain stable for up to 72 hours. Although the data showed that samples may be stored up to 1 week under the same conditions, the numbers were limited, and 3 days storage should be adequate in most cases. The data also showed that the blood tubes might allow samples to be stored for up to 6 hours at 20 to 25ºC. Again no product claim was made to this effect as stability has been shown to be temperature dependent and it is difficult to ensure that the tubes are maintained at temperatures of <25ºC. Stability over 6 hours at room temperature, in vacutainers containing 100µmol/L 3DA, was however supported by further work done by two of the authors of the Al Khafaji paper, who in this case injected 40µL of a 3DA solution into 4mL vacutainers without loss of vacuum[116], they also showed very similar changes in plasma Hcy in tubes containing EDTA alone stored at room temperature (mean increase 3.2µmol/L over 6 hours).

During the clinical evaluation of evacuated blood tubes containing 3DA described above (Paper I), despite the wide range of initial Hcy values observed (3.9 to 28.7µmol/L), the absolute increase in Hcy in unpreserved samples showed very little difference after 72 hours storage. Consequentially, significant differences in the

percentage change over time were seen, with an inverse relationship to baseline Hcy. Similar phenomena have previously been noted over 24 hours by Fiskerstrand *et al.*[23] and more recently over 4 hours by Duarte *et al.*[117]. Poster XI describes further analysis of the data where the initial rates of Hcy production in blood samples were compared according to participants baseline plasma Hcy concentration. Median rates, measured over the first 3 hours post venipuncture, were 0.6µmol/L/h for those with Hcy values ≤10µmol/L and 0.7µmol//h where initial Hcy was >10µmol/L. These results were not significantly different (p=0.3890, Mann-Whitney U test). It follows, therefore, that the rate of Hcy production from erythrocytes *in vitro* appears independent of the *in vivo* mechanisms that cause moderate hyperhomocysteinaemia. For cellular survival a supply of S-AdMet must be maintained in order to repair cell proteins[118]. As described in Chapter 1, this can only be achieved if Hcy is removed, as the metabolic pathways involved are absent in erythrocytes[42] these cells must export Hcy into the plasma to maintain a source of methylated donor within the cell. Any excess in S-AdMet production would lead to a risk of uncontrolled methylation, therefore flux through the pathway is likely to be limited at the level of methyl adenosyltransferase[119] (see Figure 1). Plasma Hcy concentrations *in vitro* therefore are regulated by Hcy production and subsequent export from the cells.

Plasma total Hcy concentrations are much higher than intracellular levels, consequently any export of Hcy must occur against a concentration gradient. Within erythrocytes a high concentration of glutathione is required to prevent oxidative damage when carrying oxygen, the resultant reducing environment means that thiols are maintained in a reduced monomeric state. Upon leaving the cell Hcy is rapidly oxidised to form disulphides with other plasma thiols. In this way a gradient is established for the

reduced form favouring export. Blom[119] proposed a reduced Hcy carrier specifically involved in this mechanism; he also proposed that there must be a separate carrier for Hcy uptake which would recognise all forms of oxidised Hcy. This introduces the possibility of another level of control for intracellular Hcy concentrations, whereby the reduced Hcy carrier protein may be regulated to maintain a steady supply of S-AdMet. The results of the study presented in Poster XI support these theories in that plasma Hcy levels were found to increase at rates independent of baseline Hcy concentrations.

2.6 Shelf life Testing - 3DA Blood Tubes

The results of clinical validation of the DS30 Hcy Blood Collection Tubes were presented in Paper I. As a commercial product it is also necessary to establish a shelf life in order to quote expiry dates, in order to give the customer some guarantee that, providing the product has been stored under the recommended conditions, it will perform to the product specifications. This work is currently unpublished and therefore will be described in detail.

For the Drew DS30 Hcy Blood Collection Tube, the product specifications state that the blood tube should draw 2.5mL (± 0.25mL) of blood upon venipuncture. On the basis of the results of the clinical validation the specifications also state that plasma Hcy concentration will be stable for up to 3 days prior to centrifugation provided that the blood is maintained at 2 to 8ºC. The stability of both of these claims needed to be established in shelf life tests.

For ease of use by the customer, ambient storage conditions were chosen. The recommendation is to store unused tubes at 15 to 25ºC. Although real time ageing was

assessed up to 2 years under ambient conditions, accelerated aging tests were used in order to prevent delays in product launch.

Accelerated ageing is achieved by storage at elevated temperature[120]. Assumptions are made, based on the Arrhenius equation, which state that a 10ºC rise in temperature, will lead to an approximate doubling in the rate of chemical reaction. The theory then, is that doubling the rate of reaction, doubles the rate of decay. This process is subject to several errors and should be accompanied by real time testing, however, it can give an indication of the possibility of premature failure.

Shelf life testing of the DS30 Hcy Blood Collection Tubes, including assessment of fill volume and the functionality of the tubes, was conducted on the first batch of tubes manufactured following clinical trials. On subsequent batches it was decided that quality control testing would involve assessment of draw volume at the site of manufacture, and functionality testing (as outlined below) would be conducted as part of the goods inwards quality control upon receipt of each new batch before the product is released for sale to the customer.

2.6.1 Assessment of fill volume

2.6.1.1 Materials

A sample batch of DS30 Hcy Blood Collection Tubes (Drew Scientific Ltd., Barrow-in-Furness, UK), was used to assess fill volume. A Vacutainer ® Brand, Needle Holder and Precision Glide (Becton Dickinson Ltd, Cowley, UK), were used to draw water into the tubes. Weights were measured on an AT261 Analytical Balance (Mettler-Toledo Ltd., Beaumont Leys, UK).

2.6.1.2 Methods

Storage conditions

Sufficient tubes were stored to allow duplicate analysis to be performed if necessary at each of the time points described.

Accelerated shelf life tests were conducted on blood tubes stored at 40ºC for 6 months. Testing was performed as described below at 3 monthly intervals.

Real time data was collected at 6 monthly intervals on blood tubes stored under ambient conditions up to 24 months.

Fill volume

Due to changes in the density of water caused by atmospheric pressure and temperature, the density of water was estimated each time the assessment of fill volume was conducted. This was performed by recording the weight of 100mL of deionised water in a volumetric flask. The density, calculated in mg/mL, was then used to calculate the volume of water collected into each blood tube.

Fill volumes were assessed by determining the mass of water collected into each tube using a Vacutainer® Brand, Precision Glide and Needle Holder. At each time point 9 tubes were measured. The fill volume of each tube, the mean fill volume, and the percentage deviation of the fill volume of each tube from the mean were then used to assess the degradation of blood tube vacuum over time.

2.6.1.3 Results

Accelerated shelf life testing up to 6 months storage at 40ºC is presented below. Data was collected at the time of receipt post manufacture (Table 2), and at 3 and 6 months (Table 3). The same baseline results (Table 2) were used for real time assessment. Real

time data for 6, 12, 18 and 24 months of storage under ambient conditions are shown in Table 4.

Table 2 Baseline data on fill volumes for Drew DS30 Hcy Blood Collection Tubes.

Tube No.	Baseline data	
	Fill volume (mL)	Percentage deviation from the mean fill volume
1	2.62	0.43
2	2.62	0.20
3	2.61	-0.22
4	2.62	0.39
5	2.61	-0.07
6	2.62	0.16
7	2.60	-0.34
8	2.60	-0.68
9	2.62	0.12
Mean	2.61	

Table 3 Accelerated shelf life data on fill volumes for Drew DS30 Hcy Blood Collection Tubes.

Tube No.	3 months		6 months	
	Fill volume (mL)	Percentage deviation from the mean fill volume	Fill volume (mL)	Percentage deviation from the mean fill volume
1	2.36	0.53	2.25	1.89
2	2.28	-3.22	2.24	1.48
3	2.36	0.53	2.23	0.94
4	2.38	1.16	2.18	-1.60
5	2.39	1.82	2.20	-0.65
6	2.35	-0.07	2.20	-0.52
7	2.31	-1.76	2.15	-2.60
8	2.38	1.07	2.22	0.27
9	2.35	-0.07	2.23	0.80
Mean	2.35		2.21	

Tube No.	6 months		12 months		18 months		24 months	
	Fill Volume (mL)	Percentage deviation from the mean fill volume	Fill Volume (mL)	Percentage deviation from the mean fill volume	Fill Volume (mL)	Percentage deviation from the mean fill volume	Fill Volume (mL)	Percentage deviation from the mean fill volume
1	2.39	-0.48	2.17	-0.35	1.91	-2.19	2.04	0.43
2	2.44	1.47	2.15	-0.89	1.87	-4.22	2.05	1.02
3	2.36	-2.04	2.16	-0.63	1.84	-5.46	2.03	0.34
4	2.44	1.29	2.12	-2.58	2.08	6.86	2.01	-0.68
5	2.47	2.91	2.21	1.75	1.68	-13.96	2.02	-0.56
6	2.42	0.72	2.16	-0.59	2.10	7.53	2.03	0.08
7	2.32	-3.66	2.19	0.54	1.93	-0.78	2.02	-0.22
8	2.41	0.12	2.21	1.51	2.09	7.27	2.02	-0.20
9	2.40	-0.35	2.20	1.25	2.05	4.96	2.02	-0.21
Mean	2.40		2.17		1.95		2.03	

Table 4.Real time shelf life data on fill volumes for Drew DS30 Hcy Blood Collection Tubes.

Product specifications claim that the fill volume of each tube should lie within the range 2.5mL ±0.25mL. At baseline, all recorded fill volumes were within the specified range (Table 2). In accelerated tests, conducted at 40ºC (Table 3), the fill volumes of the sampled tubes were all within the range at 3 months, however, before 6 months had elapsed the volume of liquid drawn into each tube had fallen below the product specifications. In real time storage tests conducted under ambient conditions, the fill volumes were all within specifications at 6 months, however, after 12 months storage the volumes had dropped such that each tube fell below the specified lower limit (Table 4).

At each time point the fill volumes are measured on only a sample of the whole population. Assuming a normal distribution and using the product specifications as 3SD limits, to ensure that the fill volumes of the whole population fall within the specified range, the estimated mean from this sample of 9 blood tubes should lie within the range 2.38mL to 2.63mL. Under accelerated shelf life tests the mean fill volume at 3 months was just below this limit, indicating that although all the tubes measured lay within the range, some tubes from the whole population may draw less than the specified volume of blood. By 6 months storage at 40ºC, the sample population all lay below the specified range. In real time shelf life tests the indications were that the whole population of blood tubes would be acceptable at 6 months, however, by 12 months all fill volumes were below the product limits.

If failures were observed, percentage deviation from the mean was used to assess if the tubes were all degrading in a similar manner. Under each storage condition and at each time point, apart from 18 months, the measurements showed a tight grouping around the mean fill volume, with the maximum deviation from the mean being

–3.66%. At 18 months the results showed a wider spread around the mean, which was not the case at 24 months. It was felt that these results were due to experimental error. Perhaps the tray that these tubes were stored in had been mis-handled, or exposed to higher temperatures than the other tubes. In retrospect the 18 month testing should have been repeated on another tray of tubes but the error was only highlighted when a tight grouping, typical of the results at baseline, 3, 6, and 12 months was observed again at 24 months.

2.6.1.4 Discussion

Product specifications for the Drew DS30 Hcy Blood Collection Tubes state that the each tube will draw 2.50mL ± 0.25mL. For testing purposes it has been assumed that the volume of liquid drawn into the tube, upon breaking of the vacuum seal, is independent of liquid viscosity. Therefore water was used in place of blood to assess the draw volume of each tube. At the time of manufacture the evacuation procedure was set to produce tubes with a fill volume at the high end of the product specifications to allow for degradation upon storage under ambient conditions. As such the mean fill volume of the tested tubes was 2.61mL.

Accelerated aging tests were conducted in order to prevent delays in product launch. Accelerated ageing was conducted at 40ºC, assuming that a 10ºC rise in temperature, doubles the rate of decay[120], then 3 months storage at 40ºC gives an indication of the degradation expected after 9 months at 25ºC. If this relationship holds then the data from accelerated tests indicates that after 9 months storage at 25ºC a small percentage of tubes may have fill volumes of <2.25mL, lying below the product specifications. The

results of accelerated testing at 6 months indicate that the vacuum remaining in the tubes after 18 months storage at 25ºC will fail to meet the product specifications.

Although accelerated ageing tests help to prevent delays in product launch they are subject to error, since they make several assumptions about the nature of product failure and kinetics of any chemical reactions that may occur. Therefore accelerated testing should be backed up with real time ageing tests. After 6 months storage under ambient conditions the draw volumes of the tubes lie within product specifications, however the mean fill volume has dropped by 0.2mL from the time of manufacture, the maximum range allowed being 0.5mL over the shelf life of the product, based on a specification of 2.5mL ± 0.25mL. After 12 months storage under ambient conditions the tubes have failed to meet the product specifications. In this case the results of accelerated ageing tests, as an indication of the life of the vacuum within the tubes, were confirmed by real time testing. Accelerated tests indicated early signs of failure by 9 months storage at 25ºC, and complete failure before 18 months had elapsed; during real time testing product failure occurred between 6 and 12 months. Although the mean fill volume after 6 months storage at 40ºC should give an indication of the effect of 18 months storage at 25ºC, the mean fill volume after 12 months storage under ambient conditions showed greater degradation of the vacuum within the tubes. Ambient storage was not performed under any temperature control and it is possible that the tubes were exposed to temperatures in excess of 25ºC. In addition temperature cycling may produce a greater stress on the system.

The vacuum in the tubes degrades over time, such that the volume of blood drawn upon venipuncture decreases depending on how long the tubes have been stored between manufacture and use. The reason for the decrease in fill volume is gas transfer

over time through the rubber bung used to seal the tubes following the evacuation procedure. Using the current materials, the maximum shelf life that can be guaranteed to ensure that the product meets its fill volume specifications of 2.5mL ± 0.25mL is 6 months. A short shelf life such as this is impractical since this does not allow sufficient time for delays between manufacture, quality control testing, shipment to distributors, and supply to the end customer. Therefore for future production alterative materials will need to be investigated for the closure of the blood tubes. Shelf life testing on the fill volumes will need to be repeated should a suitable alterative be found. Unfortunately, changes to the product line of Drew Scientific have meant that this line of investigation was not pursued.

Although the tubes were found to fail the fill volume specifications before 12 months storage has elapsed under ambient conditions, the functionality of tubes in terms of the ability to maintain plasma Hcy concentrations in whole blood also needed to be assessed. This was especially true if an alternative blood tube closure was to be sourced as the functionality of the tubes may have a longer shelf life. Testing of this feature was to continue until either 2 years had elapsed or product failure was recorded in order to prevent this part of the work from having to be repeated should a suitable alternative closure have been sourced.

2.6.2 Functionality Testing

2.6.2.1 Materials

DS30 Hcy Blood Collection Tubes (Drew Scientific Ltd., Barrow-in-Furness, UK), were tested for their ability to stabilise plasma Hcy in whole blood. Hcy measurements

were performed using the Drew DS30 Hcy system (Drew Scientific Ltd, Barrow-in-Furness, UK) (Appendix B).

DS30 Hcy Quality Control material was obtained from Drew Scientific Ltd. (Barrow-in-Furness, UK).

A healthy volunteer, expected to have a low plasma Hcy concentration (≤8µmol/L), provided blood.

2.6.2.2 Methods

Storage conditions

Sufficient tubes were stored to allow duplicate analysis to be performed if necessary at each of the time points described.

Accelerated shelf life tests were conducted on blood tubes stored at 40ºC for 6 months. Testing was performed as described below at 3 monthly intervals.

Real time data was carried out at 6 monthly intervals on blood tubes stored under ambient conditions until 24 months had elapsed.

Functionality testing

For each time point and storage condition functionality testing was performed using the following protocol.

A trained phlebotomist was employed to collect blood into 6 blood collection tubes from 1 venipuncture on the healthy volunteer. Each tube was immediately mixed by inverting a few times to ensure that the contents of the tube had dissolved and were evenly distributed in the blood. Aliquots of 1mL of whole blood were removed from each tube and centrifuged for 10 minutes at 11,000 g. The plasma was collected and stored at –80ºC until analysis.

The original blood samples were stored within the temperature range 2 to 8ºC, for three days, at which time a second 1mL aliquot was removed from each sample and centrifuged for 10 minutes at 11,000 g. The plasma was again collected and stored at –80ºC until analysis.

Hcy was analysed on each sample using the DS30 Hcy system (Drew Scientific Ltd., Barrow-in-Furness, UK) (Appendix B). At each time point in the shelf life testing protocol, the plasma taken from the 6 tubes immediately after venipuncture and the plasma collected after 3 days storage of the blood at 2 to 8ºC was analysed within a single run. Thus between batch imprecision was avoided.

Two levels of DS30 Hcy Quality Control material (Drew Scientific Ltd., Barrow-in-Furness, UK) were run in each batch of samples to ensure that the assay was performing consistently.

Assuming that a shift in Hcy concentration of ±1µmol/L would be considered clinically relevant, the validity of the number of tubes used at each time of testing was assessed using the mean initial Hcy concentration, and information on the maximum coefficient of variation expected within a single batch. For a detailed explanation of these test criteria see section 2.6.2.4. Thus in terms of the ability of the blood tubes to stabilise plasma Hcy concentrations in whole blood the shelf life will be considered to have been exceeded should a shift in Hcy concentration of over 0.5µmol/L be observed.

2.6.2.3 Results

Results obtained on DS30 Hcy Quality Control material run in each batch of samples are shown, against the target values and acceptable ranges for the Drew DS30 Hcy assay, in Table 5. In each case the results were within the acceptable ranges. A single batch of QC material was used during shelf life testing up to12 months, however this

batch was no longer available at 18 and 24 months. Between batch imprecision therefore was assessed across the first 12 months. Both the low and high Quality Control material (mean Hcy concentrations of 13.3µmol/L, and 23.1µmol/L respectively) gave CV results of 3.9% across 4 time points.

Baseline results were collected at the time of receipt post manufacture (Table 6). Storage of the blood tubes at 40ºC allowed for accelerated shelf life testing which was conducted at 3 and 6 months (Table 7). The results of assessment of real time storage after 6, 12, 18 and 24 months under ambient conditions are shown in Table 8.

At each time point 6 blood tubes were used to collect blood from a single volunteer expected to have a Hcy concentration of <8µmol/L. The reasoning behind this protocol will be discussed in detail later (see section 2.6.2.4). In each case the baseline plasma Hcy was shown to be <8µmol/L.

Table 5 Results obtained from Quality Control samples during functionality shelf life testing of the DS30 Hcy Blood Collection Tubes. Results, Target Values and Ranges are shown as Hcy concentration in µmol/L.

Time Point (months)	Level 1 QC				Level 2 QC			
	Lot number	Result	Target	Range	Lot number	Result	Target	Range
0	MO-00103201	13.2	12.8	10.9 to 14.7	MO-0010302	23.9	22.4	19.2 to 25.6
3	MO-00103201	14.1	12.8	10.9 to 14.7	MO-0010302	23.3	22.4	19.2 to 25.6
6	MO-00103201	13.0	12.8	10.9 to 14.7	MO-0010302	21.8	22.4	19.2 to 25.6
12	MO-00103201	13.0	12.8	10.9 to 14.7	MO-0010302	23.4	22.4	19.2 to 25.6
	Mean	13.3			**Mean**	23.1		
	SD	0.5			**SD**	0.9		
	CV	3.9%			**CV**	3.9%		
Time Point (months)	**Level 1 QC**				**Level 2 QC**			
	Lot number	**Result**	**Target**	**Range**	**Lot number**	**Result**	**Target**	**Range**
18	99082302	9.5	9.4	8.1 to 10.9	99082303	21.5	21.3	17.7 to 25.1
24	MO-00143142	11.2	12.2	10.6 to 13.8	MO-00143143	21.3	22.4	19.6 to 25.3

Table 6 Baseline data on functionality for Drew DS30 Hcy Blood Collection Tubes.

Tube No.	Hcy Concentration (µmol/L)		
	Baseline Results		
	Day of venipuncture	3 days post venipuncture	Change
1	6.5	6.4	-0.1
2	6.5	6.7	0.2
3	6.3	6.2	-0.1
4	6.6	6.4	-0.2
5	6.7	6.5	-0.2
6	6.5	6.1	-0.4
Mean	6.5	6.4	-0.1

Table 7 Accelerated shelf life data on functionality testing for Drew DS30 Hcy Blood Collection Tubes.

Tube No.	Hcy Concentration (µmol/L)					
	3 months			6 months		
	Day of venipuncture	3 days post venipuncture	Change	Day of venipuncture	3 days post venipuncture	Change
1	5.3	4.8	-0.5	4.8	4.3	-0.5
2	5.3	4.9	-0.4	4.8	4.5	-0.3
3	5.2	4.8	-0.4	4.8	4.8	0.0
4	5.3	4.6	-0.7	4.9	4.5	-0.4
5	5.3	4.7	-0.6	4.9	4.7	-0.2
6	5.2	4.7	-0.5	5.1	4.9	-0.2
Mean	5.3	4.8	-0.5	4.9	4.6	-0.3

Tube No.	Hcy Concentration (µmol/L)											
	6 months			12 months			18 months			24 months		
	Day 0	Day 3	Change	Day 0	Day 3	Change	Day 0	Day 3	Change	Day 0	Day 3	Change
1	4.0	4.0	0.0	5.0	4.7	-0.3	5.4	4.9	-0.5	5.5	5.2	-0.3
2	3.9	4.0	0.1	4.9	4.6	-0.3	5.5	5.4	-0.1	5.5	5.3	-0.2
3	4.3	4.0	-0.3	4.9	4.4	-0.5	5.8	5.5	-0.3	5.2	4.9	-0.3
4	4.3	4.5	0.2	5.1	4.6	-0.5	5.7	5.4	-0.3	5.2	5.2	0.0
5	4.8	4.0	-0.8	4.8	4.8	0.0	6.1	5.8	-0.3	5.1	5.0	-0.1
6	4.0	4.8	0.8	5.0	4.8	-0.2	7.1	5.5	-1.6	6.1	5.3	-0.8
Mean	4.2	4.2	0.0	5.0	4.7	-0.3	5.9	5.4	-0.5	5.4	5.2	-0.3

Table 8 Real time shelf life data on functionality testing for Drew DS30 Hcy Blood Collection Tubes. Day 0, and Day 3 refer to the time elapsed post venipuncture.

The product specifications state that the Drew DS30 Hcy Blood Collection Tubes will stabilise plasma Hcy in whole blood for up to 3 days when stored at 2 to 8ºC. A shift in Hcy concentration of <0.5µmol/L was considered to ensure that a clinically relevant shift in plasma Hcy would not be observed when using the blood tubes correctly. Although in accelerated tests, after 3 months storage of the unused tubes, and at 18 months under ambient conditions, the mean shifts in plasma Hcy were borderline, the results after 6 months storage at 40ºC, and results of all real time ageing at 6, 12 and 24 months were within the limits of acceptable shift in plasma Hcy after 3 days storage of the whole blood at 2 to 8ºC.

2.6.2.4 Discussion

It would not be practical to perform full clinical testing on each batch of tubes manufactured, therefore the testing protocol was designed in such a manner, that shifts in plasma Hcy on a small number of blood samples collected from a single individual would represent a failure of the whole batch. Upon storage, whole blood samples collected from individuals with plasma Hcy concentrations at the lower end of the reference range (5.2 to 15.1µmol/L (Poster VI)) show more significant percentage increases in plasma Hcy concentration over time (Figure 3, Paper I and Poster XI). Knowledge of certain individuals' lifestyles meant that a volunteer could be selected whose Hcy was expected to be <8µmol/L. If a mean shift over 3 days of storage of <0.5µmol/L Hcy would ensure that a clinically significant shift in Hcy would not be observed when using the blood tubes correctly, it was important that a change of this magnitude would not be masked by the imprecision of the assay. Within batch imprecision tends to be lower than between batch, therefore at each time of testing the plasmas collected immediately after venipuncture and after 3 days storage at 2 to 8ºC

were measured within a single batch. Upon quality control testing of the DS30 instrument the coefficient of variation within batch must be <3%, in fact Zhang *et al.*[121] found that within batch imprecision was <2%. Nevertheless, working on a maximum Hcy concentration of 8µmol/L, and a coefficient of variation of 3%, the maximum standard deviation that would be observed within batch should be 0.24µmol/L. The number of tubes required can then be calculated by adapting criteria outlined in the NCCLS document for "Interference Testing in Clinical Chemistry"[122], although this document is designed for the assessment of significant interference in clinical assays the principles concerned also apply to this situation. A parameter d is calculated based on the effect that is regarded as a significant change in the result, and the standard deviation of the method, such that d = effect ÷ standard deviation. Within the limits of the testing criteria used the maximum value for d is therefore given by d = 0.5 ÷ 0.24 = 2.08. For 95% confidence in the results, comparing d to tables gives the maximum number of tubes required to be 6, thus a mean shift of >0.5µmol/L in plasma Hcy over 3 days of storage would be considered significant. Calculating 95% confidence intervals on a maximum mean shift of 0.5µmol/L Hcy gives a range for the mean of ±0.8µmol/L, which is within the clinically significant range of a shift of ±1µmol/L. During shelf life testing each set of data collected met the testing criteria described here.

Functionality testing follows the same procedure that will be used for quality control testing of subsequent batches prior to customer release. Therefore the baseline data show that the DS30 Hcy Blood Collection Tubes meet the product specifications, which state that the tubes are capable of stabilising plasma Hcy concentrations in whole blood for up to 3 days if stored at 2 to 8ºC. The results of accelerated ageing tests conducted at

40ºC indicated that this specification would hold if the unused tubes are stored for up to 18 months at <25ºC. This is based on the theory that increases of 10ºC will approximately double the rate of decay[120]. However, expiry dates should not be assessed on the results of accelerated testing alone and real time data should be collected to back up the results. In this case the results showed that under real time conditions the unused tubes were stable for at least 24 months if stored according to the recommendations (15 to 25ºC). Even up to 24 months there was no sign of deterioration of the ability of the ability of the tubes to maintain stable plasma Hcy concentrations in whole blood for up to 3 days under refrigerated conditions.

2.6.3 Summary – shelf life

Although the shelf life tests have shown that the fill volume of the blood tubes has a shelf life of only 6 months under ambient conditions, the tubes maintained their ability to stabilise plasma Hcy concentrations up to at least 24 months of storage of the unused tubes under ambient conditions. Nevertheless, if an alternative material had been found for the tube closure the shelf life of the draw volume would need to have been reassessed.

At any given stage in the shelf life testing, where a shift in the mean plasma Hcy concentration was observed between day 0 and day 3, the shift was downwards, however at no time did this shift become clinically significant.

Figure 4 shows the mean Hcy concentration, measured during functionality testing of the blood tubes, plotted against the fill volume at each time point during the shelf life assesment. At first glance there appears to be a trend within the data collected during the real time shelf life testing between 6 and 24 months, such that a decrease in fill volume is associated with an increase in Hcy concentration. However the time zero point, and

data collected during accelerated shelf life testing does not fit the same trend. If the trend were true it may imply that an increase in the concentration of 3DA in the collected plasma, due to a decreased fill volume, might interfere with the Drew DS30 Hcy assay. No such interference was observed during the studies conducted for Paper 1, 3DA interference in HPLC assays for Hcy has to date not been reported in the literature, and there is no chemical basis to believe that 3DA would interfere in the assay. Although the same individual was used to collect blood throughout the shelf life testing, it is impossible to control the Hcy concentration across 24 months. Within this study the measured Hcy varied within the range 4.2 to 6.5µmol/L. As described in detail in section 1.4, there are a number of factors that can influence an individual's Hcy concentration in particular the influence of diet, with transient increases in Hcy being observed following a meal. It is possible that the apparent association between fill volume and measured Hcy is a coincidental effect of a shift in the subject's Hcy concentration across the course of the study. Further shelf life testing on subsequent batches of tubes should shed light on this theory. In retrospect the vacuum could have been broken on a set of tubes allowing manual filling with a single collection of blood to varying fill volumes in order to assess if increasing the concentration of plasma 3DA would interfere in the Drew DS30 Hcy assay.

In summary the current shelf life of the DS30 Hcy Blood Collection Tubes containing 3DA and EDTA can only be guaranteed up to 6 months when stored at temperatures in the recommended range of 15 to 25ºC, based on the current product specifications.

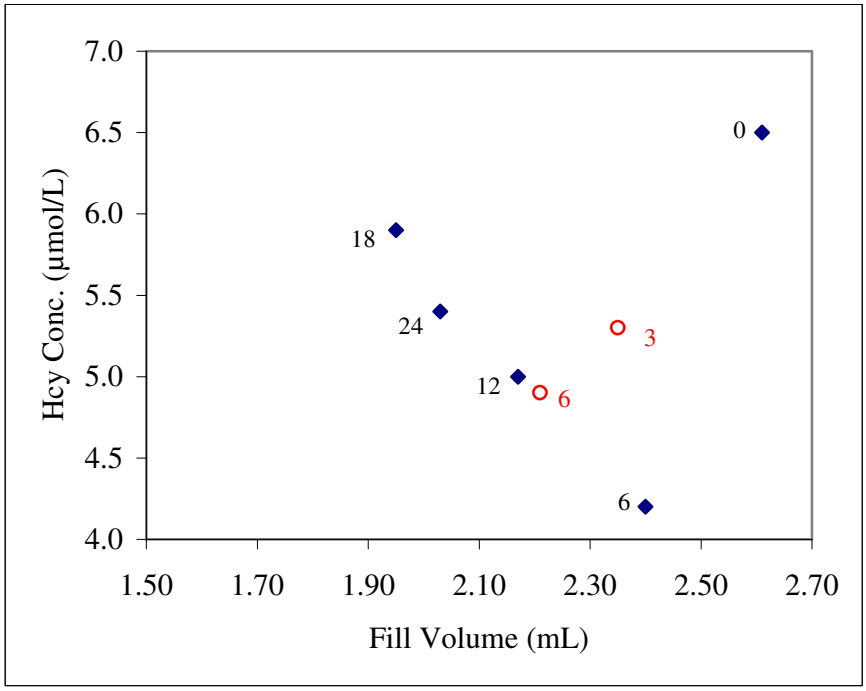

Figure 4 Hcy concentration against blood tube fill volume during shelf life testing of the Drew DS30 Hcy Blood Collection Tubes. (o = accelerated data, ♦ = real time data. Numbers by the data points represent storage time in months).

2.7 Conclusions

The initial aim of this thesis was to develop a commercially available blood collection system based on the best available knowledge, which would allow accurate plasma Hcy measurement on whole blood samples stored for up to 3 days prior to processing, thus enabling plasma Hcy measurement to move out of the hospital environment and into a community setting. The results of clinical validation of the DS30 Hcy Blood Collection Tubes show that such a product has been designed. However, whole blood must currently be stored at 2 to 8ºC prior to processing, which is far from ideal. Nevertheless, several samples may be collected and stored in doctors' surgeries, or in mobile clinical trial facilities, before they have to be transported to a centralized laboratory for processing, thereby facilitating wider population screening. If

refrigerated conditions are not readily available, the use of DS30 Hcy Blood Collection Tubes may offer sample stability over 6 hours, within a hospital environment, which maybe particularly useful in cardiovascular clinics. These tubes can be used with a range of methods, providing the technique does not rely on the action of SAHH[123], consequently tubes containing 3DA should not be used in some of the immunochemical methods currently available[124;125].

2.8 Further Work

Firstly the shelf life of the current design of DS30 Hcy Blood Collection Tubes has an inadequate shelf life. In any future developments alternative materials must be investigated for their ability to maintain a vacuum over the course of 2 years storage under ambient conditions. The results of shelf life tests, conducted on the fill volume of the tubes, indicate that accelerated aging may assist in this selection procedure.

Although the DS30 Hcy Blood Collection Tubes offer an improvement over the current recommendation for samples to be stored on ice and processed within 1 hour of venipuncture these tubes require that the whole blood is stored at 2 to 8ºC. The ultimate aim of this project is to develop a method of stabilising whole blood samples for plasma Hcy measurement for up 3 days under ambient storage conditions.

In mammals SAHH is the enzyme responsible for Hcy production (Figure 1). Inhibition of this enzyme has been shown to stabilise plasma Hcy, however the compound used is a competitive inhibitor and as such its efficiency is temperature sensitive. The structure of the enzyme and several of its inhibitors are currently known, therefore a molecular modelling approach based on the active site of SAHH was proposed to postulate suitable compounds for use in blood tubes for the stabilisation of

plasma Hcy concentrations. This approach would allow a short list of candidate compounds to be drawn up based upon the theoretical strength of binding within the enzyme.

Further selection should be based upon additional information on cost, toxicity, solubility, ease of manufacture, and stability. In fact this should include all factors concerned with their involvement in manufacturing processes and use for semi-invasive procedures in a clinical environment.

It was hoped that a group of compounds would be identified for use in pilot studies, and should a compound appear to offer ambient temperature stability of plasma Hcy in whole blood, full clinical trials would again be conducted before a commercial product was released.

The theories behind this alternative approach and results of the investigations that it was possible to conduct are discussed in the next chapter.

Chapter 3 AN ALTERNATIVE APPROACH TOWARDS IMPROVING SAMPLE STABILITY

This section of the thesis focuses on an alternative approach to discovering a system of inhibiting Hcy production in whole blood samples when stored at ambient temperatures. Due to financial and logistical problems encountered during the course of this project the practical work is limited, however the theory behind this proposed approach is described in detail.

3.1 Outline

To date, as described in Chapter 2, the most practical approach to blood collection for plasma Hcy measurement away from the hospital setting involves collection into tubes containing 3DA. Although the samples can be stored for up to 3 days, the requirement for refrigerated conditions prior to centrifugation is far from ideal and does not lend itself well to large-scale studies or population screening. This chapter discusses a radical approach that might enable the discovery of a compound, which could provide stabilisation of plasma Hcy in whole blood at ambient temperatures over the 3-day time period required.

3DA slows down Hcy production through inhibition of the enzyme SAHH, which is the final enzyme in the pathway from Met to Hcy (Figure 1). SAHH converts S-AdHcy into Hcy and adenosine. Physiologically, the reverse condensation reaction is favoured[3], however subsequent reaction of adenosine drives the reaction forward. The structural similarities between adenosine and 3DA (Figure 5), mean that 3DA binds to the enzyme trying to drive the condensation reaction, however its structural differences mean that the reaction can't take place and the enzyme is inhibited**.**

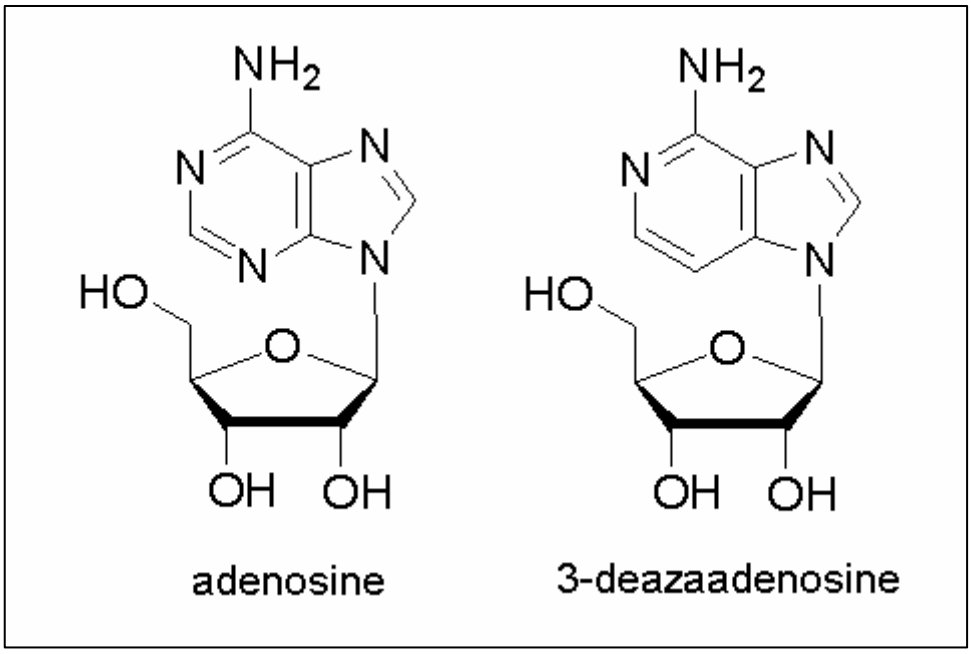

Figure 5 Structural similarity of adenosine and 3DA.
Diagram modified from http://www.sigmaaldrich.com

As shown in Posters VIII and XI, this competitive inhibition is temperature sensitive and can be overcome. If an alternative inhibitor for this enzyme could be found which has stronger binding characteristics this may prove to be suitable for use in Hcy blood collection tubes, yet for feasibility in a manufacturing process one must also take into account other factors such as price, solubility, and toxicity of any compounds selected. The idea was to use what is known about the structure of SAHH and its active site to formulate a computer model, which might allow rapid screening of chemical databases to produce a short list of compounds worthy of further testing. Scores based on strength of binding should assist selection.

This *in silico* approach would only provide a theoretical idea of whether or not a compound might inhibit SAHH and an *in vitro* method would also be required to allow screening of a short list of suitable compounds.

3.2 *In vitro* screening method

Molecular models can only provide a short list of compounds, which theoretically bind to the active site of SAHH, with perhaps some indication of the binding strength. In order to assess the true ability of these compounds to inhibit the enzyme an *in vitro* screening method would need to be developed which did not rely on the invasive procedures utilised in the pilot study and clinical trial described in Chapter 2 (Posters VII and XI and Paper I). This screening method could then be used to narrow down the short list further to 2 or 3 key inhibitors, which might be worthy of investigation in whole blood samples.

Both Wolterdorf *et al.*[125] and Dawling[124] have reported interference in the Abbott IMx method for Hcy analysis[30] due to the presence of 3DA. At final concentrations of 100µmol/L in plasma or whole blood they showed reductions in the measured Hcy concentrations by 13% and 8.8% respectively. Han *et al.*[123] showed that the interference is due to the fact that the method relies on conversion of Hcy to S-AdHcy, via SAHH, before reaction with an antibody against S-AdHcy. It may be possible to make use of this method interference to screen for other inhibitors of SAHH; however the Abbott method requires dedicated equipment. Axis-Shield also produce a commercial method which makes use of the same antibody against S-AdHcy[100] and also relies on SAHH to covert Hcy to S-AdHcy[29]. This method is produced in kit format and uses microtitre plates in a competitive immunoassay, which can be run in most laboratories, provided there is access to a suitable spectrophotometer. It was decided to try to make use of the Axis-Shield method as an *in vitro* screening procedure. For details of the assay kit see the product insert (Appendix D). Comparing the degree of interference in this method

compared to that seen with 3DA could provide an indication of how well a candidate compound may perform in stabilising whole blood for plasma Hcy measurement.

3.2.1 Materials

Axis® Homocysteine Enzyme Immunoassay Kits were purchased from Axis-Shield (Dundee, UK). 3DA came from Sigma-Aldrich Co. (Poole, UK). EDTA plasma from healthy donors was obtained from Biochemed Pharmacologicals, Inc. (Whinchester, USA). Where raised Hcy concentrations were used samples were spiked with homocystine (Sigma-Aldrich Co., Poole, UK).

Microtitre plate spectrophotometers used were a MRX Absorbance Reader (Dynex Technologies, supplied by Jencons-PLS Ltd. (Forest Row, UK), and a Double Strip MicroReader-1 fitted with a 450nm filter (Hyperion Inc. supplied by Wolf Laboratories Ltd., York, UK).

Microtitre plates were washed with either a multichannel pipette or a semi-automatic 8-manifold microtitre plate washer (Nalgene® Labware, supplied by Wolf Laboratories Ltd., York, UK).

Acid yellow 65 (Sigma-Aldrich Co., Poole, UK) was used for evaluation of the MicroReader-1 spectrophotometer.

3.2.2 Methods

3.2.2.1 Initial assessment of the screening method

Familiarisation with the kit, equipment and assay procedure included a quick assessment of the capability of 3DA to show adequate interference in the Axis-Shield Hcy method.

An EDTA plasma sample was chosen with a Hcy concentration at the lower end of the adult reference range (Poster VI), the plasma was then spiked with an aqueous solution of homocystine to produce a plasma sample with a slightly raised Hcy concentration. Stock solutions of 3DA were used to spike both plasma samples in the ratio of 1:9 (3DA solution: plasma). Samples contained final 3DA concentrations of 100, 500 and 1000µmol/L. To account for dilution an additional sample was produced in which deionised water was added to the plasma in place of the 3DA solution.

Quality control samples were also run to ensure the method was producing accurate results.

Homocysteine measurement was conducted according to the assay kit instructions. In the microtitre plate immunoassay steps reaction with the anti-S-AdHcy antibody the microtitre plates were washed manually using a multipipette. As in the method described by Frantzen *et al*.[29], all samples including the calibrators and quality control were run in duplicate.

Absorbance readings were taken using the MRX Absorbance Reader.

A four parameter logistic curve, of the type shown below, was fitted to the absorbance readings from the calibration wells using XLfit Excel Add-In Version 4.2.1 (ID Business Solutions Ltd., Guilford, UK).

$$y = \left(D + \frac{(A - D)}{1 + 10^{((\log(X) - \log(C)) * B)}} \right)$$

Hcy concentrations in the QC material and plasma samples were also calculated using XLfit Excel Add-In Version 4.2.1 based on the equation generated for the calibration curve.

Measured Hcy concentrations were plotted against the concentration of 3DA used to spike the plasma sample. An estimate of the IC_{50} for SAHH was determined as the concentration of 3DA that would result in a 50% reduction in measured Hcy result.

3.2.2.2 Initial evaluation of the Double Strip MicroReader-1

Initial assessment of the Axis-Shield Hcy method was performed on equipment available at Cranfield University. Continuation of this work off site resulted in the requirement to purchase a new microtitre plate spectrophotometer, a Double Strip MicroReader-1 fitted with a 450nm filter. This new spectrophotometer needed to be evaluated prior to use.

The Axis-Shield Hcy method states that absorbance readings should be conducted at 450nm. As acid yellow 65 will also absorb at this wavelength a solution was made and diluted to suitable concentrations to allow assessment of the imprecision and linearity of the absorbance readings taken using the Double Strip MicroReader-1.

The microtitre plates used were ThermoLife Sciences Combiplates 8 (Basingstoke, UK). Imprecision was assessed across 20 wells for blank readings, and at two concentrations of acid yellow 65 solution. Linearity was assessed and the working range confirmed by taking a saturated solution of acid yellow 65, centrifuging to remove any undissolved material, and then taking triplicate readings of 1 in 2 dilutions, until the 9^{th} dilution, at which point the readings were approaching zero.

3.2.2.3 Choice of plasma Hcy concentration & 3DA inhibition from 0 to 200µmol/L

This was the first run of the Axis-Shield Hcy method performed using the Double Strip MicroReader-1 to measure the final absorbance readings. Initial assessment of the reader has suggested that precision may be reduced compared to Dynex MRX

Absorbance Reader, however the data suggested that the equipment would be suitable for a comparative screening method.

The calibration curve obtained on the Dynex MRX Absorbance Reader showed absorbance readings ranging from 0.654 to 2.293. This range exceeds the quoted dynamic range of the MicroReader-1, however there is mention in the kit instructions of the possibility of reducing the incubation time during reaction with the second antibody / horse radish peroxidase conjugate, if there are "optical densities exceeding the measuring range of the microtitre plate reader". In this run the assay was performed following the instructions as written with a view to reducing the incubation time in further runs if required.

The results of the initial assessment of the *in vitro* screening method (see section 3.2.3.1) showed that in a plasma with a low starting Hcy concentration, large concentrations of 3DA would be required in order to detect a significant degree of interference in the method, yet at higher Hcy concentrations the precision of the measured results may be reduced since the nature of the calibration curve means that small changes in absorbance readings can lead to quite large difference in the reported Hcy concentration. As the precision of the assay was expected to be reduced using the MicroReader-1, results were compared on a sample containing a mean Hcy concentration reported as 35.3µmol/L with results on a sample containing 20.9µmol/L, which is closer to the centre of the calibration curve. Samples were spiked with 3DA solution in a 1:9 ratio as above. In this run, however, the aim was to evaluate inhibition at lower concentrations of 3DA. In the initial assessment the 3DA concentrations used were 100, 500 and 1000µmol/L; here the concentrations were 20, 50, 100 and 200µmol/L.

3.2.2.4 Further development of a potential in vitro screening method

Although the Axis-Shield Hcy method is intended for use with serum or plasma, it may be possible to use an aqueous solution of Hcy removing the need for the large amounts of plasma that would be required in screening. An aqueous solution of L-Hcy with a target value of approximately 30µmol/L was spiked with 3DA up to 200µmol/L. If it is not possible to use aqueous Hcy, it may at least be possible to produce a batch of plasma spiked with 3DA to use in comparison studies, which could be aliquotted and stored frozen. Aliquots of the 3DA-spiked plasma with an Hcy concentration of 35.3µmol/L used in section 3.2.2.3 had been stored at –80ºC for 4 days, these were assayed using the Axis-Shield Hcy method along side the aqueous Hcy solution and a freshly spiked plasma sample.

Due to an observation of high readings in end wells, all samples and calibrators were run in triplicate to allow any suspected falsely high readings to be omitted.

3.2.2.5 Further evaluation of the Double Strip MicroReader-1

Although initial evaluation of the MicroReader-1 had not highlighted a problem with readings taken from end wells, the evaluation had been performed using microtitre plates from ThermoLife Sciences and absorbance readings were measured on an acid yellow 65 solution.

It was plausible that the high readings were connected with using the microtitre plates supplied in the kit or were perhaps a feature of the coloured end product of the assay. In order to test these theories the resultant solution at the end of the assay was collected from several wells and pooled together. 200µL aliquots were dispensed into three strips of ThermoLife Sciences microtitre wells, and three strips of washed wells

taken from an Axis-Shield assay kit. Absorbance readings were taken at 450nm using the MicroReader-1.

To further elucidate sources of variability in the assay, CV estimates of absorbance readings were assessed across strips of 8 wells for plasma samples containing 0 and 100µmol/L 3DA replicated throughout the whole assay, compared with the same plasma samples in which a single aliquot was taken through the "sample pre-treatment procedure" and only dispensed into 8 aliquots for the "microtitre plate procedure".

The "sample pre-treatment procedure" involves reduction of disulphide bonds to convert all forms of Hcy to the monomer, followed by reaction with SAHH to convert Hcy to S-AdHcy. The final step in this procedure involves removal of excess adenosine, which can interfere with the antibody reaction, using adenosine deaminase. The "microtitre plate procedure", involves a competitive immunoassay between S-AdHcy which coats the microtitre plates and S-AdHcy produced in the "sample pre-treatment procedure". After washing away antibody bound to this later source S-AdHcy, a second antibody / horse radish peroxidase conjugate binds to any of the first antibody bound to the plate. Any colour produced using horse radish peroxidase is then inversely proportional to the concentration of Hcy in the original sample (provided there are no interfering substances present).

3.2.3 Results

3.2.3.1 Initial testing

The calibration curve generated on initial testing of an Axis® Homocysteine Enzyme Immunoassay Kit is shown in Figure 6.

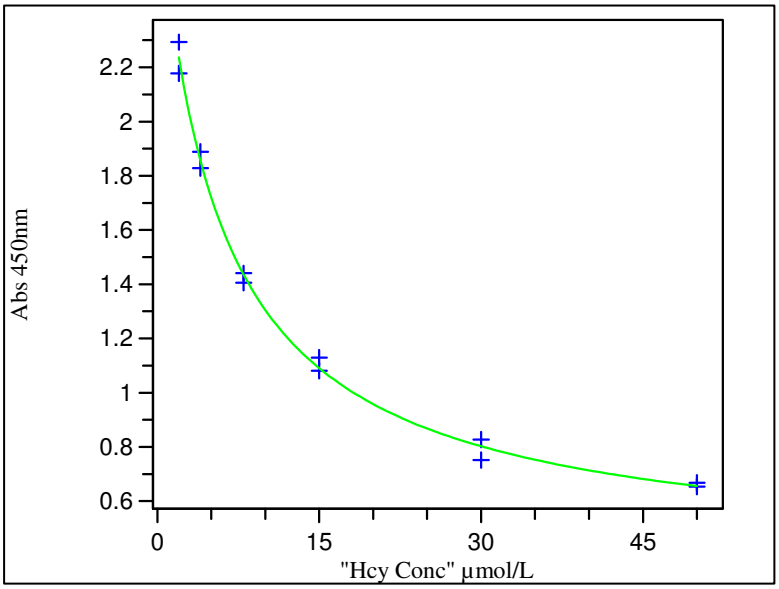

Figure 6 Calibration curve from initial testing of Axis Hcy Enzyme Immunoassay Kit.

QC results were slightly low on Level 1 compared to ranges established for the DS30 Hcy Assay kit; duplicate results were 11.0µmol/L and 11.2µmol/L (Target Range 11.5 to 14.5 µmol/L). Level 2 QC results, 21.1µmol/L and 20.8µmol/L were within the target range (19.0 to 27.0 µmol/L).

The measured Hcy results in two plasma samples with mean Hcy concentrations of 4.6µmol/L and 17.8µmol/L, when spiked with water, are shown in Figure 7 plotted against the final concentration of 3DA present. A progressive interference from 3DA was observed in both plasma samples, however this was more apparent in the sample with the higher initial Hcy concentration. In this sample the IC_{50} was estimated at around 400µmol/L 3DA.

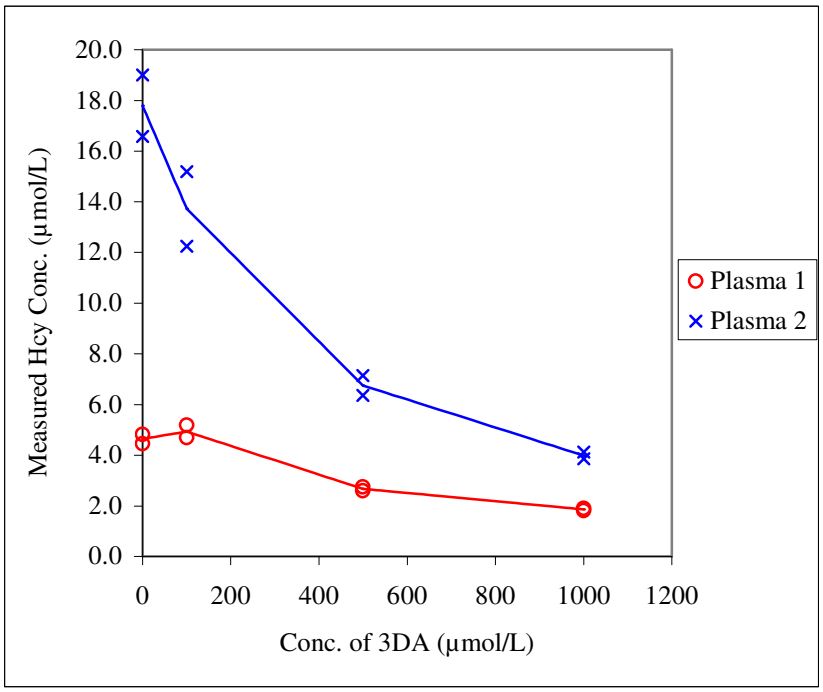

Figure 7 Hcy concentrations measured on the Axis Hcy Enzyme Immunoassay Kit, in two plasma samples spiked with 3DA.

3.2.3.2 Evaluation of the dual strip MicroReader-1

The results of imprecision assessment on blank wells and at two absorbance readings within the manufacturers quoted dynamic range of 0.000 to 2.000 are shown in Table 9. A CV estimate is obviously not an appropriate measure on a zero reading, as division by a mean reading of 0.000 would result in an infinite CV (CV = 100*(SD/Mean)). The instrument was blanked before taking 20 absorbance readings from empty wells; the SD from these readings was 0.001 (mean = 0.001), which is within the manufacturers quoted performance of <0.005. At mean absorbance readings of 0.459 and 0.711, the CV estimated from 20 readings of acid yellow 65 solutions were 0.4% and 0.3% respectively. Again these readings are within the quoted performance specifications of <1.5%. For comparison, when using the MRX Absorbance Reader, Dynex Technologies quote a dynamic range of –0.100 to +4.000, with imprecision estimates

based on 20 readings of: 0.2% CV or 0.005 whichever is the greater for readings between 0.000 and 2.000, 0.6% CV for readings between 2.001 and 3.000, and 1.0% CV for readings between 3.001 and 3.500.

Table 9 Imprecision assessment of the MicroReader-1 spectrophotometer.

Reading No.	Readings taken on empty wells.	Acid Yellow 65	
		Solution 1	Solution 2
1	0.001	0.453	0.705
2	0.002	0.457	0.707
3	0.001	0.458	0.710
4	0.000	0.459	0.710
5	0.001	0.458	0.710
6	0.002	0.459	0.710
7	0.001	0.459	0.711
8	0.000	0.460	0.711
9	0.000	0.460	0.711
10	0.001	0.461	0.711
11	0.000	0.461	0.710
12	0.001	0.459	0.712
13	0.000	0.460	0.712
14	0.001	0.460	0.713
15	0.001	0.459	0.712
16	0.002	0.460	0.712
17	0.000	0.460	0.712
18	0.001	0.459	0.712
19	0.000	0.459	0.712
20	0.000	0.459	0.712
Mean	0.001	0.459	0.711
SD	0.001	0.002	0.002
CV	N/A	0.4%	0.3%
n	20	20	20

Figure 8 shows that the absorbance readings on the MicroReader-1 are linear across the working range ($y=0.1457x - 0.0235$, $R^2 = 0.9999$).

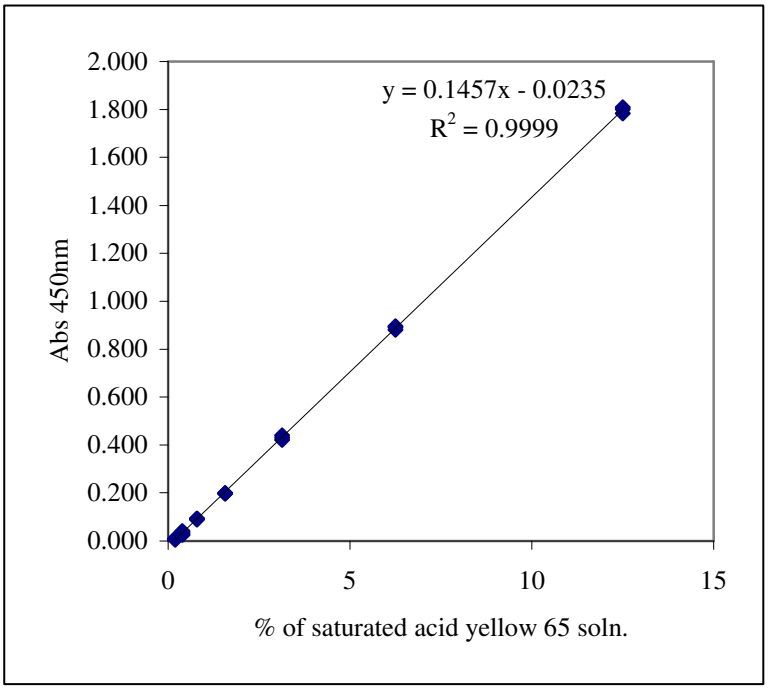

Figure 8 Assessment of linearity of the MicroReader-1 using acid yellow 65.

3.2.3.3 Choice of plasma Hcy concentration & 3DA inhibition from 0 to 200µmol/L

Calibration produced a curve similar to that shown in Figure 6; however the absorbance readings were in the range 0.290 to 1.457. As these results are within the dynamic range of the MicroReader-1 the method can be followed as per the kit instructions without the need to reduce one of the incubation steps.

During this run there was a tendency for the first well in each strip of the microtitre plate to show higher absorbance readings than its duplicate sample. This could be due to a number of practical causes. As it was not observed during evaluation of the MicroReader-1 it may be due to a fault in the assay kit plates, as highlighted by the large error in the estimate of the mean at 50µmol/l 3DA in plasma 1 seen in Figure 9. Further investigation was required.

The measured Hcy results from the two plasma samples are shown in Figure 9 plotted against the final concentration of 3DA. From these readings, compared to those seen in Figure 7, it becomes apparent that interference due to 3DA is insignificant between 0 and 50µmol/L. The results also show that for screening of potential inhibitors of SAHH it may be best to use a plasma sample with an initial Hcy concentration in the region of 30µmol/L and that due to variability between runs and poor precision, comparative assays should be performed to narrow down the selection of candidate compounds.

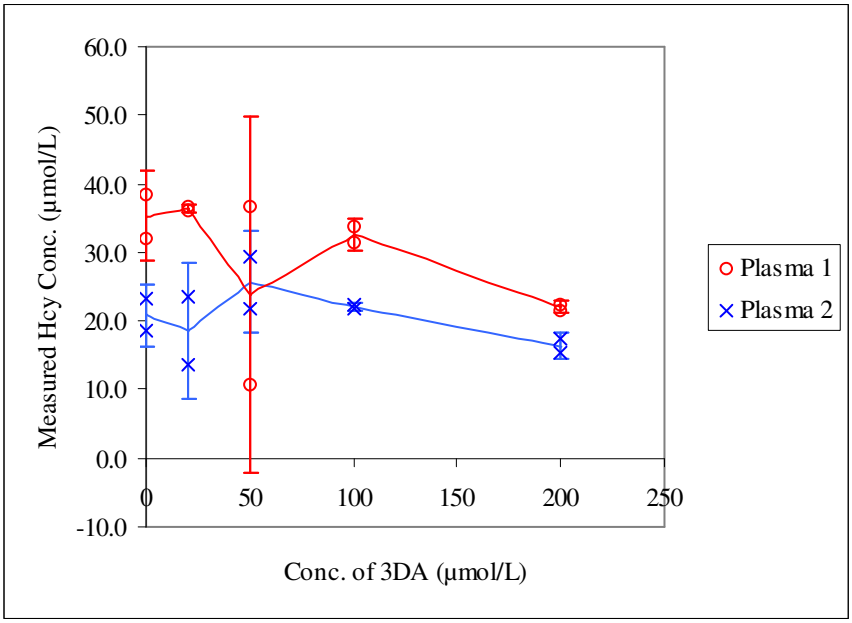

Figure 9 Hcy concentrations measured on the Axis Hcy Enzyme Immunoassay Kit, in two samples spiked with between 0 and 100µmol/L 3DA. Error bars show ±2SEM.

3.2.3.4 Further development of a potential in vitro screening method

Unfortunately the calibration and results during this run were poor (not shown) CVs were over 12% on some triplicate readings. Omitting high readings from end wells brought the CVs down to around 6%, but all Hcy results, including the plasma assayed

in section 3.2.2.3, were higher than expected and approached the limits of the dynamic range. The progressive interference from 3DA observed in previous runs was not so apparent in this run.

Results were no worse for frozen plasma or aqueous Hcy solution and these samples would warrant further investigation.

3.2.3.5 Further evaluation of the Double Strip MicroReader-1

The results of absorbance readings taken on pooled reaction product in microtitre wells from ThermoLife Sciences and washed wells from the Axis-Shield Hcy assay kit are shown in Table 10. Imprecision estimates are worse than those measured with acid yellow 65 solution (see Table 9). Using the ThermoLife Sciences wells the CV estimate at a mean absorbance of 0.714 was 1.8% exceeding the manufacturers specifications (<1.5%), compared with a CV of 0.3% at a mean absorbance of 0.711 when using acid yellow. In the Axis-Shield wells the same solution gave a lower mean absorbance of 0.600, with a CV on 24 readings of 4.6%. One result stood out as high, but even removing this, the CV was still 2.5%. In any case the high results observed during assay runs in the end wells were not observed with pooled reaction product.

Table 11 shows the absorbance readings measured on replicate samples in the microtitre plate procedure alone, compared with replicates taken through the whole assay procedure. In the microtitre plate procedure alone there was no difference in the imprecision estimated in the presence and absence of 3DA (CV values of 8.0% in each case), indicating that 3DA does not cause variability in the antibody reactions.

Table 10 Absorbance readings measured on the MicroReader-1 using pooled reaction product from the Axis-Shield Hcy assay.

Strip	Well	Absorbance 450nm	
		ThermoLife Sciences	Axis-Shield
1	A	0.742	0.618
	B	0.751	0.710
	C	0.716	0.607
	D	0.726	0.603
	E	0.722	0.608
	F	0.721	0.614
	G	0.709	0.610
	H	0.715	0.620
2	A	0.710	0.576
	B	0.710	0.598
	C	0.710	0.571
	D	0.709	0.577
	E	0.704	0.577
	F	0.698	0.582
	G	0.715	0.613
	H	0.718	0.595
3	A	0.703	0.598
	B	0.703	0.593
	C	0.703	0.583
	D	0.716	0.591
	E	0.704	0.580
	F	0.709	0.583
	G	0.719	0.590
	H	0.693	0.596
Mean		0.714	0.600
SD		0.013	0.028
CV		1.8%	4.6%
n		24	24

When adding in the pre-treatment steps, imprecision is increased as might be expected. In the absence of 3DA, additional pre-treatment steps only increased the imprecision by 3.1% (11.1% CV compared to 8.0% in the microtitre plate procedure alone), implying that the greatest source of variability was in the antibody reactions. However, when the plasma contained 100µmol/L 3DA, the additional pre-treatment steps caused the imprecision to increase by 8.3%, indicating a high degree of variability

attributable to sample to sample differences in the amount of inhibition caused by 3DA on the action of SAHH.

Table 11 Absorbance readings for replicates in the "microtitre plate procedure" compared with the whole assay in the presence and absence of 100µmol/L 3DA.

Well	Absorbance 450nm			
	Replicates in microtitre plate procedure alone		Replicates across the whole assay	
	No 3DA	100µmol/L 3DA	No 3DA	100µmol/L 3DA
A	0.835	0.860	0.833	0.838
B	0.747	0.826	0.704	0.887
C	0.743	0.783	0.710	0.909
D	0.673	0.712	0.644	0.677
E	0.674	0.734	0.628	0.627
F	0.664	0.733	0.634	0.630
G	0.697	0.699	0.602	0.645
H	0.696	0.703	0.634	0.679
Mean	0.716	0.756	0.674	0.737
SD	0.057	0.060	0.075	0.120
CV	8.0%	8.0%	11.1%	16.3%
n	8	8	8	8

Figure 10 shows the reading plotted in the order that they were read, again a trend towards higher readings at one end of the plate was observed which does not appear to be related to the order in which samples are pipetted. Since the trend is not apparent with pooled reaction products measured in either ThermoLife Science or washed Axis-Shield microtitre plate strips, it does not appear to be related to use of the MicroReader-1 and could be a result of the coating process during kit manufacture. If this is the case then the trend may disappear when a new batch of kits is used.

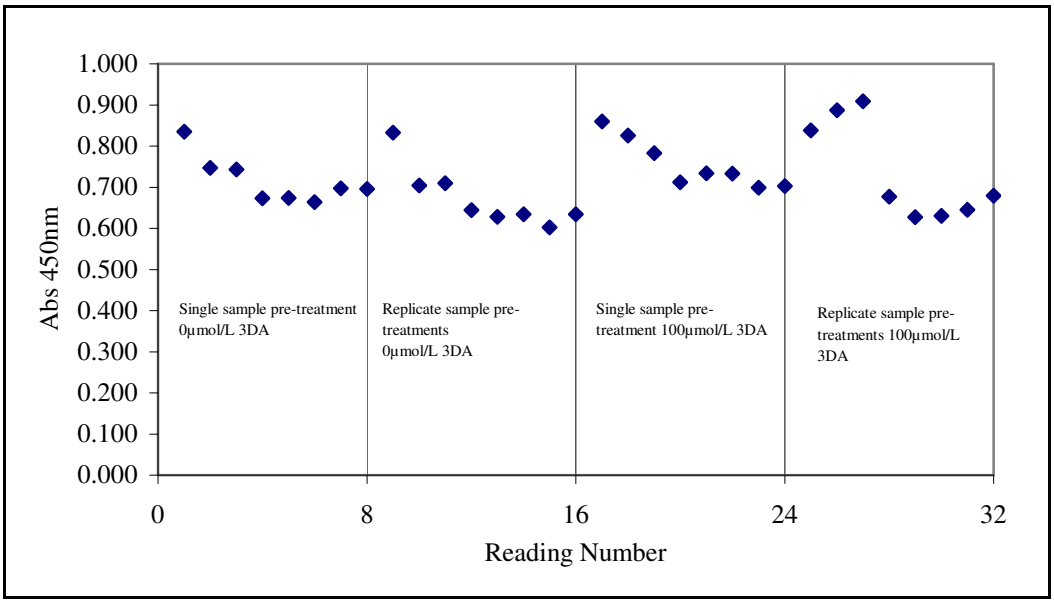

Figure 10 Trends in absorbance readings on replicates measured in the Axis-Shield Hcy assay kit.

3.2.4 Discussion

Previously authors have reported that 3DA causes interference in the Abbott IMx immunoassay for Hcy[124;125], Han *et al*. showed that interference was due to inhibition of SAHH, however to date there have been no published reports of 3DA causing interference in the Axis-Shield Hcy Enzyme Immunoassay. The results show that at 100µmol/L 3DA and above significant and progressive interference is observed in line with the reports on the Abbott method. Although only a quick estimate of IC_{50} was conducted at 400µmol/L this is somewhat lower then that of 1500µmol/L reported by Dawling on the Abbott IMx[124], however it should be noted that these values may be dependent on the assay conditions including the concentrations of enzyme and substrates.

Although QC results on a sample in the reference range were reported as lower than the target values, the ranges were established using the Drew DS30 Hcy HPLC method and new ranges should be established for each new method used.

As time progressed the precision of results worsened. Evaluation of the new equipment purchased for work off site indicated that only a small degree of worsened imprecision could be assigned to use of the MicroReader-1. It was noted that the reagents in the kits have short shelf lives and there are several warnings in the kit insert regarding careful maintenance of the enzyme containing reagents and the microtitre strips. Increasing imprecision over time may, therefore, be due to degradation of the assay kits. If the screening method were to be employed careful planning would be needed to ensure that fresh kits were used to their full potential to avoid kits being exposed to fluctuating temperatures and humidity conditions.

Due to time and financial constraints full development of the *in vitro* screening method was not possible within this thesis. The aim of the screening method is to enable selection of one or two compounds selected from a short list generated by molecular modelling techniques that may be capable of stabilizing whole blood samples for Hcy measurement at ambient temperatures. Initial testing indicated that use of a 30µmol/L Hcy sample spiked with 400µmol/L of inhibitor measured using the Axis-Shield Hcy Enzyme Immunoassay, would provide a suitable screen providing the results were compared to samples spiked with water or 400µmol/L 3DA run in the same assay. Similar results may be possible through spiking an aqueous solution of L-Hcy and this is worthy of further investigation. Nevertheless use of plasma samples in this method offers advantages versus trying to assess potential inhibitors in whole blood samples as fresh whole blood would be required, whereas it is possible to purchase larger volumes

of plasma from single donors from companies that supply pharmaceutical manufacturers. Plasma samples offer greater stability compared to having to assess Hcy production from viable red blood cells as the plasma can be stored frozen for long periods of time.

One word of caution is that kit calibration is performed on aqueous samples of S-AdHcy, which assumes that the SAHH conversion step works to completion. This is why quality control samples should be run in order to test that the whole assay is working correctly in the absence of inhibitor, or a comparative sample spiked with 3DA should be used to prevent false positive results in this screening method.

A further caution, although interference is seen in the Abbott method and believed to due to SAHH interference it is possible that 3DA may also interfere with the antibody reaction due to its structural similarity with S-AdHcy. In fact in the Axis-Shield method adenosine deaminase is used to remove excess adenosine "to avoid interference by Ado [adenosine] in the succeeding [immunoassay] step"[29]. Han *et al.*, however assessed 3DA interference on SAHH in the conversion of S-AdHcy to Hcy in the absence of an immunoassay step[123]. Their results clearly indicated that 3DA interferes with the enzyme-catalysed reaction.

In conclusion, if fresh kits are used the Axis-Shield Hcy Enzyme Immunoassay kits used in conjunction with samples containing 30µmol/L Hcy, spiked with 400µmol/L of potential SAHH inhibitor would provide a suitable screening method for candidate stabilisers of whole blood Hcy samples.

3.3 *In silico* screening method

3.3.1 Theory

Although 3DA inhibits SAHH, it is limited in its use for stabilising whole blood samples for Hcy measurement. Section 3.2 describes how a short list of candidate compounds may be experimentally screened but the problem that remains is how to create that short list. Once a compound has been identified as a ligand or inhibitor for a particular enzyme, with a little knowledge of the mechanism of the reaction and details of the active site it is relatively easy to explain why that compound is active, but with the vast multitude of potential molecules available how does one go about selecting which molecules should be investigated? It is this very frustration that led to the development of computational tools explicitly designed to have as their output a list of molecules, which may be active against a receptor, based on three-dimensional structural information. These tools are primarily used in drug design, but the theory can be adapted for other problems such as the task in hand.

A number of terms have been used to describe this practice, including molecular modelling, computer aided drug design, virtual screening, computational chemistry and *in silico* screening, but they all basically cover the same ground. Of course the complex dynamics describing the macromolecules concerned requires large resources in computing power. Until very recently molecular modelling had to be conducted on dedicated computers, which were traditionally designed as graphics workstations with very fast processors, and it is only during the course of this study that personal computers have come onto the market with enough processing power to handle such computing programs. Where Unix was required as an operating system, there are now

Windows® based programs available which opens up this previously restricted field to a much wider group of research scientists.

Any molecular modelling study must start with the model itself. The Research Collaboratory for Structural Bioinformatics operate an international repository for the processing and distribution of experimentally determined three-dimensional macromolecular structure data; this service is referred to as the Protein Data Bank (formally operating as the Brookhaven Database) and is available at http://www.pdb.org. Data is submitted from experimental results such as X-ray crystallography, NMR or from fellow molecular modellers. Once the data has been published the Data Bank will release the structure, which then becomes freely available. From their website information on a number of proteins with experimentally determined structures can then be downloaded in a format which contains details of the relative locations of all the atoms with that protein. These files can then be used to enter the given starting model into the molecular modelling software. In many instances though the structure may not yet have been determined, in these cases receptor models may be built based on inference using homologous structures. Of course virtual screening is most successful in an information rich environment[126], involving not only knowledge of the structure of the target site but also based on knowledge of ligand-protein interactions that are essential for binding and activity.

A molecular model consists of spherical atoms joined together with springs that represent the bonds. Employing mathematical functions to describe Newtonian mechanics within the system, the molecular force field i.e. the total energy of a macromolecule associated with shifting conformers or with binding between ligand and protein can be estimated. This total energy can be expressed as the sum of the potential

energy functions created by interactions between each of the individual atoms. Knowing, or theoretically estimating, the force constants associated with each of the energy terms, the total energy of a molecule can be estimated based on the sum of several thousand individual contributions involving van der Waals, electrostatic and hydrogen interactions, together with stretching, bending and angular interactions between molecules that are covalently bonded[127].

Energy minimisation programs using molecular mechanics involves modifying the parameters governing the geometry in small increments in a number of successive iterative computations[127]. Comparison with the total energy of the previous conformer and acceptance or rejection based on whether the total energy is reduced or increased allows the approximation of the most stable system or "ideal geometry". Minimum energy structures are found by reducing distortions and unfavourable interactions such as van der Waals forces, whilst optimising favourable interactions such as hydrogen bonding. Looking for minima in this way though, there is no guarantee that the absolute lowest energy structure, the "global minimum" will be found, it is possible that a system can fall into a local minima with a strained conformation. The introduction of molecular dynamics allows small barriers to be breached dependent on the temperature associated with the simulation. Following internal motions by computational simulation it is also possible to sample the relevant behaviour of a given system[128]. It should be noted however that the global minimum of a protein in isolation does not necessarily represent the receptor bound conformation, in which case it is wise to perform minimisations with a substrate in place[127].

In drug design two approaches are commonly used[127]. In the first type of screening compounds are matched against a template compound and similarities in electrostatic

potential, molecular surface, or molecular shape are searched for. In the second approach a more physical method is adopted where candidate molecules are forced to interact with a common binding sites defined by hypothetical points of interaction, rather than forcing them to directly superimpose, in this case the degree of interaction between the model and each candidate is assessed. This latter approach is referred to as "docking". The challenge then is in constructing the "query", i.e. the defined criteria or points of interaction that must be satisfied by a candidate drug[129].

The lock-and-key model suggested by Emil Fischer in 1894 as the origin of biological specificity unfortunately continues to dominate the thinking of many drug designers[129]. This model implies that for a molecule to be active it has to have steric characteristics that perfectly match the shape of the receptor. Although shape and steric interferences play a large part it is probably more important to think of the functional groups involved in specific binding to key regions of the receptor. Following on from this idea, one can think of two-dimensional constraints as the functional group specificity, three-dimensional constraints as the steric or geometric interactions and a finally a group of constraints that should not be forgotten in drug design, and for which the term one-dimensional constraints has been adopted, are the practical constraints such as cost and ease of manufacture and perhaps even the amount of compound in stock if rapid screening is to be conducted[129].

In setting up a query, a few factors should be remembered. Hydrophobic interactions are usually the major driving force for binding, whilst hydrogen-bonding and electrostatic interactions primarily provide specificity[127]. Van der Waals forces may also play a major part, and an extra radius across the surface of the receptor site atoms should be taken into account. Following docking of a given molecule energy

minimisation should again be conducted which will give a good indication of the plausibility of the model and a rough estimate of the relative interaction enthalpy of the candidate drug. Poor models will usually fall apart during this process. VanDrie[129] states that the use of two functional groups and one distance constraint seems to be highly effective in identifying active compounds. With a little experience it becomes clear that certain interactions contribute much more than others to the binding affinity.

Queries may also be based on, or at least use information gathered from the literature on key amino acids present in the active site that are important in binding. This is where point mutation studies are particularly helpful[126]. The orientation of groups such as lone pairs or side chains relative to the target compound may also provide useful information, and these can be fixed in setting up the criteria for a given query. Such knowledge is not essential and docking techniques may be employed with a loose query, but a more specific approach will help to narrow down the search and increase the accuracy of the results.

Once a query is defined databases containing large numbers of candidate compounds are screened to see which compounds may match the criteria, those that do are termed "hits".

Early screening methods relied on docking single conformers of each ligand, but as it was realised that the molecules may be under tension in the bound state, multi-conformational or flexible docking was introduced[126]. Ideally movement within the receptor should also be allowed, although for ease of rapid screening the receptor is frequently treated as a rigid structure.

For a thorough test of a given molecular model and query, a number of known inhibitors and substrates should be tested to see if they will be returned as hits, whilst

compounds that are inactive should be rejected. If the query fails to identify known inhibitors and substrates, then the factors defining the query should be modified until these compounds are returned as hits.

In a successful modelling study a small, manageable, list of candidate compounds will be produced. Synthesis of these compounds followed by *in vitro* screening can be used to refine the model[127].

It might be assumed that a maximally diverse database, or library of compounds would be ideal for the identification of novel active compounds. In reality this concept is just not practical, as screening would take far too long. Databases should be focussed on specific types of problem, contain compounds which are drug-like, i.e. small not too lipophilic, and can be synthesized at large yield with reagents that can be bought or made at a reasonable cost[126].

Once a database has been searched and a number of hits identified a scoring system is required based on the strength of the receptor-ligand interaction in order to prioritise synthetic and *in vitro* screening efforts. A number of scoring systems have been developed, but several have limited results. Accurate affinity calculations rely on fast quantum calculations, taking into account the full flexibility of the molecules, solvation and entropy. In principle free energy, as opposed to binding enthalpies are more accurate methods of scoring ligand-receptor interactions, but these methods are processor intensive[126]. General purpose scoring functions may be more useful in the early stages of screening; one such scheme was designed by Boehm and includes terms of surface area, hydrogen bonds and ionic interactions, as well as a penalty for the number of rotatable bonds[126]. Each pose – i.e. a snapshot of a flexible ligand interacting with a flexible receptor, returns a number indicating the likelihood that the pose

represents a favourable binding interaction a low (negative) energy indicates a stable system and thus a likely binding interaction.

One cannot, however, rely on the results of *in silico* screening methods alone as a large number of false positives will be included in the candidate short-list, to exclude these *in vitro* methods are required. Furthermore, sometimes human experience and intuition is required as compounds yielding poor scores may turn out to be false negatives and be highly active.

3.3.2 Structure of SAHH

Cells from several species have been tested and found to contain SAHH, the few exceptions include *Escherichia coli* and certain related bacteria where two enzymes are required for the hydrolysis of S-AdHcy to Hcy and adenosine[130]. The gene for human SAHH is located on the long arm of chromosome 20 and comprises of 1299 nucleotides, resulting in a protein of 432 amino-acids with a molecular weight of 47660[131]. The sequence shares a 97% amino-acid identity with that of the rat enzyme (91.5% match in the coding sequence); there is even 75% identity with slime mould *Dictyostelium*[131]. A 51-kDa enzyme obtained from *Rhodobacter captulus* also shares 64% homology with its human counterpart[130]. This high degree of homology between diverse species, representing years of evolutionary development, is truly remarkable and may represent one of the highest levels of conservation that has been observed between a human and a prokaryotic enzyme; highlighting the importance of SAHH in cellular metabolism.

Nicotinamide adenine dinucleotide (NAD^+) acts as cofactor for SAHH, and the domain that incorporates its binding region is particularly well conserved between species. Several NAD-dependant dehydrogenases have been shown to possess a similar

3D structure in their NAD^+ binding domains. Since SAHH performs a similar function to NAD^+ dehydrogenases in terms of redox interconversion of NAD^+ to NADH, it was believed that SAHH would also show structural similarities in the active site to NAD-dependant dehydrogenases. Therefore, prior to the elucidation of an X-ray crystallographic structure of SAHH, Yeh *et al.*[132] proposed a molecular model for the active site of SAHH based on the X-ray crystal structure of dogfish lactate dehydrogenase and the primary sequence of rat liver SAHH.

As X-ray crystallography became possible for SAHH, Turner *et al.*[133], were able to perform a detailed analysis on the structure of human SAHH, shown in Figure 11, this was followed a year later by another group who extracted the enzyme from rat liver[134]. The structure of the tetrameric rat liver enzyme is shown in Figure 12. Both these structures are filed in the Protein Data Bank under the file names 1A7A and 1B3R. Much of the following discussion on the structure of the protein comes from these two papers, however, it should be noted that there are significant differences in the spatial arrangement of the catalytic and cofactor binding domains between the two structures. In the file for human SAHH (1A7A) the enzyme was studied in the presence of inhibitor, whereas the rat liver enzyme is presented in the substrate free form. The effect that the presence of substrate or inhibitor has on the structure of the protein will be discussed below.

The enzyme comprises of four identical subunits, each approximately 48,000 in molecular weight. Within each subunit a single molecule of NAD^+ cofactor remains tightly associated with the enzyme. Since this domain is extremely well conserved between species [130;131] it may indicate the importance of enzyme structure within this region on the metabolic functioning of the enzyme.

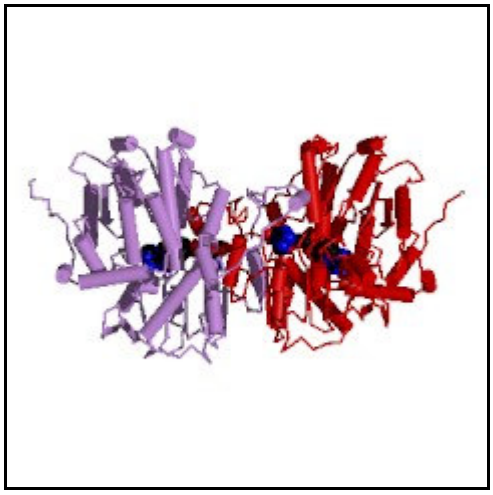

Figure 11 Bottom view of the dimeric structure of human placental SAHH (1A7A). Reproduced from: http://www.ebi.ac.uk

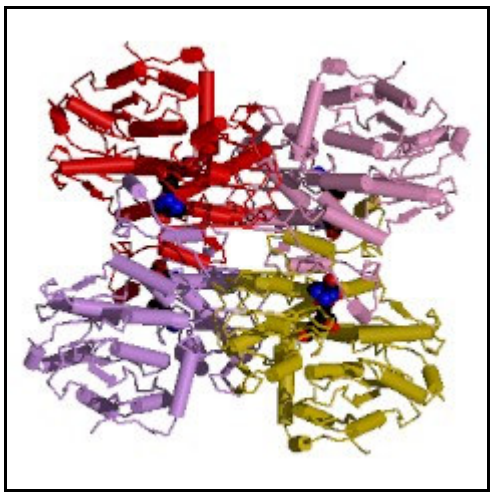

Figure 12 Bottom view of the tetrameric structure of rat liver SAHH (1B3R), showing the channel that runs down the centre of the enzyme.
Reproduced from: http://www.ebi.ac.uk

Each subunit of the protein contains two or three key domains. The catalytic and NAD^+ binding domains are folded into a structure reminiscent of NAD-dependent dehydrogenases, whilst a third domain comprises of the C-terminal region.

The catalytic domain of human SAHH comprises of residues 1-190 and 355-432, compared to residues 1-181 and 352-402 in the rat, which are folded into a pattern similar to many methyltransferases. The crevice that holds S-AdHcy has a hydrophobic pocket at one end in which the adenine moiety is held, whilst Hcy and ribose are exposed to the solvent. The folding pattern of the whole catalytic domain implies that S-AdHcy can travel freely in and out of this enzyme and other methyltransferases allowing it to properly regulate methyltransferase activity[133;134]. The C terminal domain exists far from the body of the main enzyme and extends into the opposite subunit of a dimeric structure[134]. Two main functions are served by the remaining amino acids which make up the middle portion of the protein chain, firstly they are responsible for binding the cofactor, and secondly they form the structural core of the tetrameric structure through numerous interactions with the three other subunits.

In NAD^+-dependent dehydrogenases, a single protein chain usually holds each molecule of NAD^+. SAHH differs in that the cofactor is held in a region, which exists between the two units of a dimer, requiring contributions from both subunits. Although each NAD^+ molecule is predominately held by one of the subunits, two amino acids from the C-terminal domain of the other subunit are also involved binding NAD^+ through hydrogen bonding interactions. The molecule is thus held with its nicotinamide moiety close to, and adenine moiety far from, the catalytic domain. In all other ways NAD^+ binding by SAHH is largely typical of NAD^+-dependent dehydrogenases.

As a dimer, SAHH is capable of binding all the substrates and cofactors required for the catalysed reaction, however the dimeric structure does not form a strong enough rigid core framework. Formation of a tetramer provides this strength in addition to allowing flexibility of the catalytic sites. The four units are brought together through interactions between two regions of α helices within each subunit. In addition, interactions occur between the catalytic domains that are located on the outside of the quaternary structure.

The NAD^+ and substrate binding sites are held in a cleft between the two main domains of each subunit. This cleft forms a channel from the protein core to the exterior, which allows entry of the substrates into the catalytic domains of the protein. The coming together of the dimer places two NAD^+ molecules at each end of a channel that passes through the centre of the tetramer, a channel that is unique to SAHH, the function of which is as yet unclear. Hu *et al.*[134] however, identified twenty-four well defined water molecules within the channel.

In the absence of substrate the catalytic and co-enzyme binding domains are physically remote, such that enzyme appears to have an "open" conformation[134;135]. Two conformational changes occur upon ligand binding[136], the first of these will occur in the absence of cofactor, and therefore appears to be important in the formation of the enzyme-ligand complex. On binding of S-AdHcy, a flexible hinge element between the catalytic and NAD^+ binding domains presumably allows the protein to shift such that the ribose moiety of S-AdHcy is brought into close proximity with the region in which the nicotinamide moiety of NAD^+ would be held. A second conformational change then occurs as NAD^+ is reduced to NADH, which, upon oxidation, becomes trapped in a "closed" form of the enzyme. Until recently, it was unclear which of the two processes

caused the formation of the closed enzyme. However, Yin *et al.*[135] have studied these conformational changes in detail and it appears that the initial substrate binding is largely responsible for the enzyme shifting into a closed conformation, such that the overall radius of the protein dramatically reduced. In contrast, subsequent reduction of the coenzyme produces only a small change in the protein structure.

The NAD^+-binding domains form the core of the tetrameric enzyme, held together by both polar and non-polar interactions. These co-enzyme binding domains have much lower temperature factors than the catalytic domains and therefore contribute largely in determining the structure of the protein[134]. As the co-enzyme binding domains are attached together at the centre of the protein, entry of the substrate, and subsequent closing of the of the clefts which exist between the catalytic and co-enzyme binding domains must cause the catalytic domains to move closer to the core of the tetrameric protein, this flexibility is facilitated by the channel that exists through the centre of the protein.

3.3.3 Preparation of the SAHH computer model

Although there is a high degree of homology between rat and human SAHH, the Protein Data Bank file 1A7A contains the structure of the human placental enzyme in the presence of an inhibitor and cofactor. As discussed earlier the binding of a substrate has a significant effect on the overall structure of the protein. Any docking procedures are therefore likely to be more successful using the active, closed conformation.

One potential downside to using the structure from the 1A7A file is that it only shows the structure of two subunits from the tetrameric protein, referred to as the A chain and the B chain. Formation of a tetramer adds strength to the structure of the protein; however as the dimer is capable of binding all substrates and cofactors required

there should be very little difference in the structure of the active site where docking of potential inhibitors will be conducted.

The Protein Data Bank file 1A7A was therefore downloaded and visualised using the molecular modelling software, Sybyl 6.7 (Tripos UK Ltd, Milton-Keynes, UK).

A number of modifications to the downloaded file were required to prepare the structure prior to database screening using a docking program. Firstly some of the atom types and valances where incorrectly identified by Sybyl and these needed to be modified. More importantly, during the X-ray crystallography procedure, Turner *et al.*[133] had expressed the protein in the presence of selenomethionine. The presence of these groups in place of Met may have a significant effect on any minimisation conducted, and as Met groups are present in the active site it was also important to modify these groups back to Met residues prior to any docking procedures. Fourteen Met groups were replaced in each chain, and the structure at each of these locations was taken from the original paper of Turner *et al.*[133].

Before any screening was conducted the modified enzyme structure needed to be optimised. Initial optimisation was conducted using simplex minimisation, which was followed by the Powell method. The initial total energy of the dimer was over 14000000kcals/mol. Following minimisation, using a termination gradient of 0.02kcals/mol, over 21000 iterations had been conducted which resulted in the total energy being reduced to –2259.3kcals/mol. The model was then ready for use in determining a query for the docking procedures.

3.3.4 Active site and docking query

If the active site within a protein has not been identified, then there are programs that can locate potential binding pockets, however, as the file for 1A7A contains a bound

inhibitor the active site is known allowing selection of the pocket where docking should take place. Key residues within the protein can be identified from their proximity to important groups within the ligand requiring knowledge of the reaction mechanism in order to set up the docking query.

The following work was conducted using the protein model prepared in section 3.3.3. When the whole protein is displayed there is too much information on screen to be able to locate the residues of interest. In order to focus on the active site, a "cavity" set of amino acids was created for display, which contained all residues within a 15Å sphere from the centre of the ligand contained within the A chain. The size of this cavity is user defined; when a 20Å area was initially tried this produced a set which contained 330 monomers, which was considered too large a set for dynamic docking since the time required to dock each compound increased based on the number of energy interactions that need to be minimised. Reducing the set to a 15Å sphere around the bound inhibitor meant that the number of included residues was reduced to 208.

A "shell" set was then defined containing all atoms lying within 5Å of the ligand. This set was used to display amino acid residues that may be involved in hydrogen bonding interactions with the substrate or inhibitor.

Although the ligand is used to define each group of amino acids it was removed before each set was saved. The cofactor and any residues present from the B chain were retained.

The reaction mechanism for the reversible hydrolysis of S-AdHcy, shown in Figure 13, was reported by Palmer and Abeles[137]. In either direction, the first step is oxidation of the 3'-hydroxyl group of the adenosine ring by NAD^+ to produce a 3'-keto derivative. This facilitates abstraction of the 4'-proton by an enzyme base and the

resulting carbanion eliminates the 5'-substituent (either Hcy or water) to give 3'-keto-4'-5'-dehydroadenosine. Addition of water or Hcy in a Michael type addition reaction, followed by reduction of the 3'-keto group by NADH, results in formation of the final product.

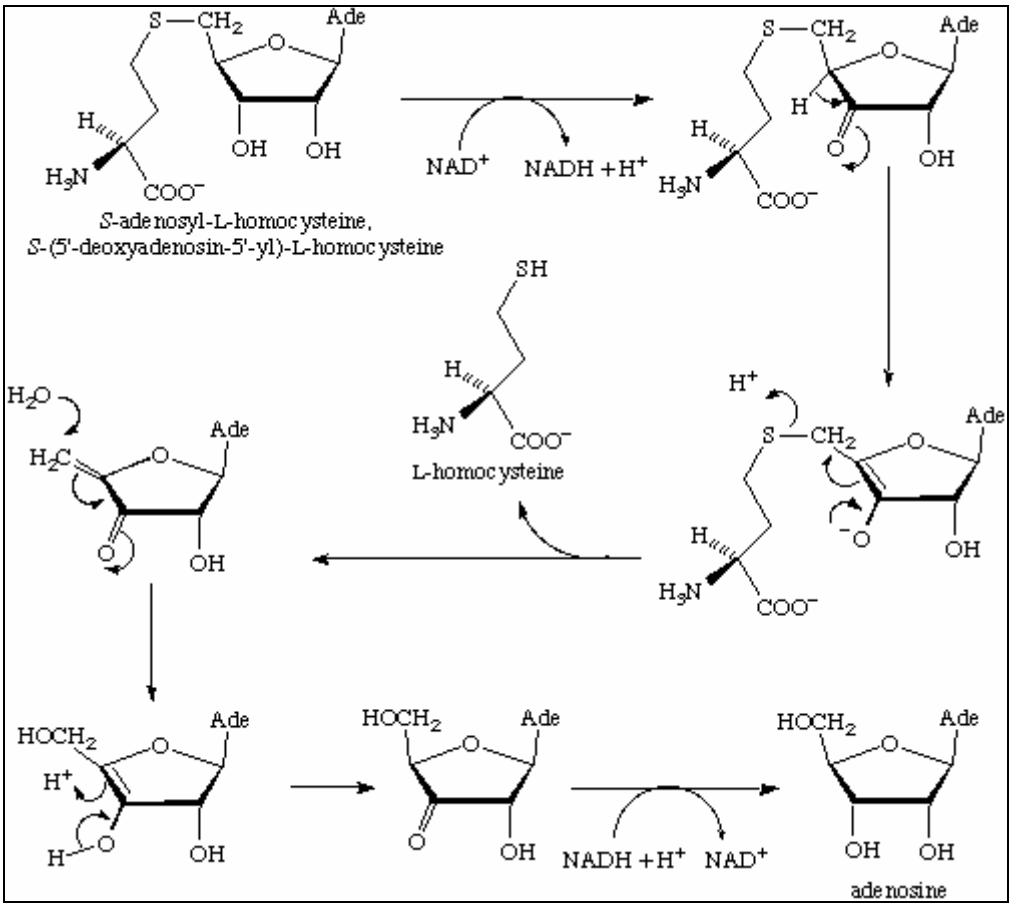

Figure 13 The reaction mechanism of SAHH proposed by Palmer and Abeles[137]. Reproduced by kind permission of Dr. G.P. Moss from: http://www.chem.qmul.ac.uk/iumb

A number of authors have proposed key amino acid residues within SAHH which may be involved in substrate binding and the reaction mechanism itself, although the

protein is highly conserved, some care must be taken with regards to the sequence as the papers often refer to different species and the residue numbering may differ.

Chemical modification of three accessible Cys residues in the human placental SAHH (Cys 113, Cys 195, and Cys 421) led to complete inactivation of the enzyme. However, site directed mutagenesis of each of these residues in turn showed that only one, Cys 195, is important in the reaction mechanism of SAHH[138]. Comparison of the substrate binding energies between the wild type and mutant enzymes, together with the fact that the mutant enzyme was able to catalyse the 3'-oxidative and 5'-hydrolytic reactions, yet severely impaired when it came to the 3'-reduction indicated that Cys 195 is involved in the catalytic centre and may play an important role in maintaining the 3'-reduction potential for the effective release of the reaction products and regeneration of the active form of the cofactor (NAD^+). The importance of Cys 195 in the function of SAHH is supported by the fact that this particular residue is highly conserved throughout evolution[130;131].

When looking for a potential inhibitor, however, clearly the release of the reaction products is not important and the focus must be on residues involved in the recognition of substrates. If irreversible inhibitors are to be found it may be more important to identify residues that allow binding of substrates, which can undergo the early stages of the reaction mechanism, but fail to be released.

Lysine (Lys) 426 may be involved in 5'-hydrolytic activity and may also required for subunit interaction[139], and is therefore not a good candidate. Glutamate (Glu) 155, Lys 185, or Threonine (Thr) 157, have been cited as possible proton acceptors in the formation of the 3'-keto group[134;140-143], while Histidine (His) 54 may act as a general acid-base catalyst[134;143]. Aspartate (Asp) 130 has been cited as being potentially

involved in the extraction of the 4'-proton resulting in the formation of the carbanion[140;142;143].

By evaluating the binding specificity of substructures of the substrate, Hu *et al.*[140] looked at the amino acid residues important in recognising and holding the substrate while the reaction takes places. They reported that binding of the substrate with the closed form of SAHH was highly specific, with the main recognition factor being the adenine moiety of the substrate, whilst the major role of the ribose moiety is not in recognition but is its involvement in the oxidation- reduction reaction. In this model Thr 57, Glu 59, Met 351, and His 353 are important in binding the adenine ring. These residues are identical to those found in contact with the adenine fragment in the crystal structure[133]. In the open form of the enzyme, docking was non-specific and only Thr 57 and Glu 59 were maintained in the model[140]. Computational mutation of the residues identified in the closed model, followed by redocking of the inhibitor found in 1A7A, confirmed that Thr 57 and Glu 59 are particularly important in substrate recognition[140].

Through interaction with Thr 57 and Glu 59, the adenine moiety resides in the deepest pocket within the active site cleft; adenine may act as the anchor locking the two hinged domains of each subunit into the closed conformation[144]. The side chains of Thr 57 and Glu 59 are believed to make contact with N6 and N1 of the adenine ring respectively[133;140;144].

When Hu *et al.*[140] attempted to dock the complete substrate S-AdHcy, they found they needed to apply weak constraints in order to restrain the adenine ring in the general area of the binding site. In this model the substrate binds in a strained conformation. This straining may be important for the catalytic function of SAHH, as it may lower the energy barrier for the elimination reaction to take place[140].

With the above information in mind an initial docking query was defined. The cavity set containing all amino acids within a 15Å radius from the centre of the ligand was displayed in Sybyl. Using the three-dimensional database searching application UNITY (version 4.3), N1 and N6 of the adenine ring of the bound ligand were defined as hydrogen bond donor atoms, corresponding to acceptor sites in the hydroxyl groups of the side chains of Glu 59 and Thr 57 respectively. Atoms within the shell set, in close contact with the ligand were used to define a receptor site constraint, with a van der Waals scaling factor of 0.5. A hydrophobic feature based on the 6 membered ring of the adenine fragment was also added to the query. Once all the features and constraints were defined the structure of the ligand was removed from the query to allow docking of alternative structures. The whole query was then saved before any database searching was conducted to allow it to be used as a base should any refinements be required.

3.3.5 Database screening

Having defined the query, UNITY was used to conduct a flexible search of the available databases. During this process a two-dimensional screen, looking for structural chemical features, is followed by a three-dimensional dynamic search, which allows flexing of the stored chemical structures in order to find a conformation that may match the active site constraints. In this case flexing of the structures is particularly important as Hu *et al.*[140] reported that the substrate binds in a strained conformation, which is unlikely to match a database structure that may have undergone energy minimisation prior to storing.

At the time of this investigation only two databases of chemicals were available for searching: the Maybridge Database, from the Maybridge Chemical Company (Tintagel, UK) containing approximately 61000 compounds, and the National Cancer Institute

Database, constructed from their Developmental Therapeutic Program containing over 235000 compounds. Using the query as defined in section 3.3.4, the two dimensional screen reduced the number of compounds suitable in each database to 37, and 3806 respectively. However, when followed by a flexible search none of the structures matched the active site constraints.

Due to this lack of hits, the initial query was rechecked, at this point it was discovered that no spatial tolerances had been given for the donor atoms and acceptor sites, as such the three-dimensional database search would have tried to match the exact positions given for the original ligand.

A second query was defined with less features and constraints than the original. Glu 59, and N6 of the adenine moiety were maintained, this time with spatial tolerances of 1Å and 3Å respectively. The receptor site constraint was tightened to a 3Å sphere around the ligand, again with a van der Waals scaling factor of 0.5. When used to screen the Maybridge database, the search was abandoned when only 10% complete when it was discovered that approximately 10% of the structures were identified as hits. Where the original query had produced no hits, this second query was producing too many.

Unfortunately it was not possible to conduct any further work on database screening, but, as adenine is so important in substrate recognition, the next option would have been to add back into the query a hydrophobic feature, which would search the database for 5 or 6 membered ring substructures.

Should an appropriate query have been defined which would return a manageable number of hits from chemical database searches, these hits would then have been further screened in a docking approach using the FlexX module of Sybyl. In this program, a binding site needs to be redefined into which a number of potential ligands, i.e. the hits

obtained from database searching, are docked. FlexX uses an enhanced version of the scoring function of Boehm[126], to estimate the strength of binding energy and to rank the different docking conformations for each ligand. Scoring can also be used to identify the best candidates for further investigation, based on their interaction with the protein. Comparison with the scores obtained whilst docking 3DA might also provide an indication of the suitability of potential inhibitors.

3.3.6 Discussion

Although a computer model for *in silico* screening was created, database screening for potential ligands was unsuccessful. Time and finances were limited and since hits were possible with the databases it is believed that the query could have been modified to produce a manageable number of hits. The two databases available at the time may not have been the best choice from which to identify potential inhibitors for SAHH. 3DA, has been used as an antiviral agent[145;146] and databases containing drugs of this type may have been more suitable.

In retrospect the suitability of the database query and model could have been tested using the ligand contained in the 1A7A crystal structure. If this did not produce a hit then it would have been clear that there was an error in the definition of the query. Docking the same compound using FlexX would provide a method of verifying the docking procedure when compared with the docked structure defined by Turner et al. [133;144].

It is possible that potential inhibitors would be discarded due to high binding energies which may come from binding in a strained conformation as was observed with S-AdHcy[140]. In all these procedures there is a trade off between wanting to make

sure that suitable compounds aren't missed, and the chances of increasing the number of false positives.

In a similar manner to the validation of their docking model conducted by Hu *et al.*[140], computational mutagenesis of potentially important amino acid residues within the active site could have been performed, and the resulting binding energies generated in FlexX whilst docking the given ligand, used as an indictor of their suitability for defining a database search query. Key residues are also likely to be highly conserved across a number of species.

3.4 Conclusion

Although unable to complete the objectives of this part of the thesis, an outline as to how one might be able to use a molecular model of the active site of SAHH to identify agents capable of stabilising whole blood samples for plasma Hcy measurement has been proposed. Through scoring methods one might be able to short list a group of candidate compounds for further investigation in the *in vitro* screening method described above. Comparison of the results of *in vitro* testing with the theoretical binding energies should give an indication of one or two compounds that are likely to produce better results in stabilising plasma Hcy in whole blood than 3DA. It must be remembered that the enzyme used in the *in vitro* method is of bovine origin, however due to the fact that SAHH is highly conserved across species, it is fair to assume that the results would be very similar if a human enzyme had been used.

One or two compounds would then be used in pilot studies similar to those described in Chapter 2, before full-scale clinical trials are conducted on one key inhibitor. Within the pilot studies other factors must be considered for suitability in a commercial

product. Such factors include the safety of production staff exposed to the chemicals, the cost of manufacture, and the solubility of the compound if it is to be spray dried into the blood tubes prior to evacuation. Another factor to be considered, which would not be accounted for in the *in vitro* screening method is the ability of the compound to cross the plasma membrane and enter the red blood cell to come into contact with SAHH, which is obviously crucial if the inhibitor is to be active in whole blood samples.

Chapter 4 HOMOCYSTEINE, B12 AND DEMENTIA

This section of the thesis focuses on work conducted in conjunction with the COBALZ Research Team: a group originally founded to investigate the links between vitamin B12 (**COB**alamin) and **ALZ**heimer's Disease. The work looked at Hcy as a marker of vitamin deficiency and explored possible mechanisms linking thiol metabolism into the development of cognitive decline eventually leading to dementia.

4.1 Alzheimer's Disease – History, Diagnosis and Pathology

In 1906 Alois Alzheimer presented the case of a woman, who at the age of 51, was admitted to a state asylum with progressive cognitive and language impairment, hallucinations, delusions and psychosocial incompetence. The mental regression advanced steadily until, after suffering with the illness for four and half years, she died. On post mortem the brain was evenly affected with fibrils in the cortical neurones, and deposits of peculiar substance in the cerebral cortex, which was later determined to be amyloid plaque, otherwise known as senile plaque. Alzheimer also found ateriosclerotic changes, however these came to be ignored over the course of time as it was felt that the reports focussed on the novel factors in this case[147;148]. Thus the finding of neurofibrillary tangles and amyloid plaques has become known as the hallmark pattern in the brains of patients with a disease named Alzheimer's Disease (AD).

The disease itself may be described as the end point of a steady decline in cognitive and psychosocial function[149]. The process begins with a gradual loss of recent memory[150]. Although memory loss is a common complaint in the aging population, in AD and other forms of dementia related to vascular disease, including dementia caused by stoke, the degree of cognitive decline is extreme and could never be classed as

"normal"[151]. Other symptoms include confusion, impaired judgment, personality changes, disorientation, and loss of language skills.

Age is considered to be the single most important risk factor in the development of dementia[152;153]. As the number of people surviving into their elderly years is rising, there is a growing burden on society caused by increasing numbers of individuals suffering from senile dementia. At the turn of the millennium it was estimated that the fraction of people who were older than 85 years and suffering from AD was almost 50%[154]. It is estimated that there are currently 18 million people worldwide with Alzheimer's disease[155]. In the UK, combined data from the Office for National Statistics and the Alzheimer's Society predicts that the number of people living into old age is increasing significantly such that by 2010 the UK will be caring for an estimated 840,000 people with dementia[156]. In every nation where life expectancy has increased, so has the incidence of AD. Predictions suggest that unless checked the burden in North America will triple, to around 14 million over the next 50 years[153;155;157]. These patients will obviously need medical care and may require possible institutionalisation putting a huge strain on the health systems of the Western world.

Dementia is defined as significant memory impairment, and loss of intellectual functions. There are several forms of dementia, with possibly up to 70 different disorders being causal[157], however dementia of the Alzheimer's type accounts for the majority of cases (70%), whilst the second most frequent cause is vascular dementia, sometimes referred to as multiinfarct dementia, since in these cases cerebral damage is caused by repeated stroke attacks. Vascular dementia accounts for approximately 15% of dementia cases[152;154].

Early diagnosis of AD is hindered by the fact that family members frequently fail to recognise the mild dementia that occurs in the initial stages, often assuming that the changes are simply a symptom of the normal aging process. An additional hindrance is that one of the criteria used in clinical diagnosis is the measurement of a gradual cognitive decline over an extended period[157]. Unfortunately to date the "gold standard" for a definitive diagnosis of AD remains the neuropathological findings at autopsy[158], but even here there remains differences in opinion as to how to interpret the findings when there is more than one disease present[157].

As described by Alzheimer[148], at post mortem the brain is found to contain extracellular amyloid plaques, and neurofibrillary tangles. Cortical atrophy is significant with widespread loss of neurones and synapses[158].

As yet there are no definite tests or biological markers of AD[158], thus, in the living, AD diagnosis is guided by clinical criteria; a diagnosis of probable AD is made in the absence of other disorders that might contribute to dementia, whereas a diagnosis of possible AD is made when there may be other contributing causes for the dementia.

Intellectual decline is well recognised as a key feature in AD, whereas behavioural disturbances are often overlooked. Current clinical diagnosis of AD is based upon a patient history, mental status testing, physical and neurological examination, a panel of conventional laboratory tests and neuroimaging to rule out other causes of dementia. Where there is an unusual case, additional laboratory testing and detailed psychometric testing may be conducted to increase the confidence of the diagnosis[157]. A number of examinations can be used to assess whether an individual has a lowered cognitive ability, and to provide a measure of disease severity, these include the Mini-Mental State Examination (MMSE), The Alzheimer's Disease Assessment Scale (ADAS), and

the Cambridge Cognitive Examination (CAMCOG). Each measures a variety of cognitive functions, such as orientation, concentration, and memory. From these tests scoring schemes have been devised so that on initial examination a poor score may mean a diagnosis of dementia. On follow up, repeat testing allows a way of assessing the extent of disease progression based on the degree of declining scores over a given time period. An additional tool for monitoring disease progression is the extent of atrophy of the medial temporal lobe[159].

As new therapies, both of a pharmacological and non-pharmacological nature, become available for the treatment of AD, it is important to be able to diagnose the condition early and accurately in order to prevent further deterioration and ideally to bring about a reversal in the process. If the clinician has to wait until (s)he is able to detect a decline in cognition this is clearly not good enough as it may by then be too late to reverse the process and maintain normal cognitive function. A number of markers have been proposed including ApoE, presenilin (PS) and amyloid precursor protein (APP) genotyping[157]. The relevance of each of these markers will become clear in later discussions regarding the possible pathological mechanisms behind the disease. Recent progress in this field has focussed on analytes present in the cerebrospinal fluid (CSF). Tau, a microtubule associated protein is the primary constituent of the neurofibrillary tangles reported by Alzheimer in 1906[148] whilst the insoluble amyloid-β_{42} ($A\beta_{42}$) forms the plaque deposits.

Vascular dementia may be diagnosed on basis of computerised tomography (CT) or magnetic resonance imaging (MRI) showing subcortical lacunes and/ or multiple involvement of the inner white matter, whilst cerebral CT or MRI in probable AD shows cortical atrophy and normal aspects of the white matter[160].

Although the gross pathological features of AD and vascular dementia are quite different, AD is often associated with atherosclerosis and cerebral microvascular abnormalities, in fact AD commonly co-occurs with stroke, leading many to believe that there may be vascular involvement in the aetiology of both forms[149;161], it is perhaps for this reason that attention has shifted to a possible link between Hcy and AD.

4.2 Possible links between AD, Hcy and Vitamin B12

A possible link between low levels of serum vitamin B12 and AD was first reported in the mid 1980s[162], however it was not until some ten years later, following a resurgence in interest in Hcy, that links with this aminothiol were investigated.

Conflicting results have been published when looking at the levels of B12 in patients with AD. In humans only two enzyme reactions are known to depend on vitamin B12[163]. The action of MS in the metabolism of Hcy had already been discussed in Chapter 1. The other pathway, involves the enzyme methylmalonyl-CoA mutase in the conversion of methylmalonic acid to succinyl-CoA, which in turn is involved in energy production and the synthesis of porphyrins. Conversion is rapid such that methylmalonic acid levels within the plasma are usually very low, however B12 deficiency, in addition to causing Hcy accumulation can lead to increased levels of methylmalonic acid (MMA), and a subsequent porphyrin deficiency leading to haematological symptoms[163]. Therefore Hcy and MMA, may be sensitive markers of subclinical B12 deficiencies. Further reasoning behind investigations of Hcy levels in patients with AD comes from the suggestion that there may be vascular involvement in the development of AD, coupled with the association of Hcy and vascular disease (see Chapter 1).

One possible factor in interpreting the results of these studies is the effect of institutionalisation on nutritional status. Joosten *et al.*[164] compared patients with AD and two elderly controls groups, one being nondemented hospitalised patients and the other control group being healthy elderly subjects living independently at home. They found that although Hcy levels were significantly higher in AD, there was no difference in the B12 and folate levels across the three groups. They did however, find that MMA was raised in AD patients compared to the healthy controls living at home, but not compared to the hospitalised controls. These results highlight the need for careful selection of control groups and suggest that a subtle nutritional B12 deficiency may have been partly to blame for increased Hcy levels in hospitalised AD cases. However the difference between Hcy levels in AD and hospitalised controls indicates that there may be additional factors involved.

In the COBALZ study, designed to investigate the relationship between cobalamin (or vitamin B12) and AD, higher levels of plasma Hcy were reported in individuals with senile dementia of Alzheimer's type compared to age matched controls[165]. Scores of cognitive function were also inversely related to Hcy concentration. In this group hyperhomocystinaemia was independent of nutritional status (assessed by measurement of retinol binding protein concentrations), despite previous reports showing poor nutrition and vitamin B12 deficiency to be associated with AD.

In trying to establish whether there is a true link between AD and any biochemical marker one of the major problems is establishing a correct diagnosis of the patient. Clarke et al.[159] took a group of patients with Alzheimer's type dementia and again looked at B12, Hcy and folate levels compared to controls. The difference in this study was that in almost half of the patient group, histological confirmation of the diagnosis

had been performed. Both Hcy and folate were significantly reduced in patients, and this association became stronger in the confirmed cases. Although clinically diagnosed patients had lower B12 levels than controls the levels failed to reach statistical significance, however in patients with a confirmed diagnosis B12 levels were significantly reduced. Two other important results from this study were that Hcy did not show escalating levels with increasing duration of symptoms and that over a three year follow up those with highest levels of Hcy at entry showed the worst radiological evidence of disease progression. These results argue against an explanation of increased Hcy, and reduced folate and B12 levels being a consequence of the disease. In contrast, Malaguarnera *et al.*[160] interpreted the findings of raised Hcy with a low folate in both vascular dementia and AD, with B12 levels only being reduced in the AD cases as suggesting that Hcy damage to neurones could lead to a secondary B12 deficiency.

Although low levels of B12 vitamins and high levels of Hcy may be associated with AD, these findings are also common in healthy elderly individuals. In one study low B12, folate, and B6 levels, and elevated Hcy were found in 6%, 5%, 9% and 30% of healthy elderly subjects compared to corresponding figures of 5%, 19%, 51% and 51% of AD patients. Due to the high prevalence of dementia and poor vitamin status in the elderly it is not unreasonable to expect that a combination of disorders is common. Alternatively low levels of vitamins and high Hcy levels may be an early, preclinical, indictor of the potential to develop the disease.

McCaddon *et al.*[166] followed up the control subjects from the original COBALZ study[165] and showed that Hcy levels were correlated with cognitive decline over a 5 year period. Cognitive decline occurs with age, but it appears that increased Hcy concentrations may be responsible for accelerating the rate of deterioration.

The Framingham study was initially designed to identify common factors or characteristics that contribute to cardiovascular disease, however after following the enrolled cohort for over 50 years several additional observations have been worthy of publication. In a recent paper, a sub-cohort of over a thousand elderly individuals were selected from those originally recruited; in this group the Hcy levels measured at baseline predicted the development of dementia and AD, over the following 8 years[167]. In the majority of subjects, they were also able to measure Hcy concentrations on samples taken 8 years prior to baseline. The fact that elevated Hcy levels were also found in these samples on the patients that later went on to develop dementia excluded the possibility that subclinical dementia may have been present in an apparently healthy population. Raised Hcy clearly preceded the development of disease. Although earlier studies have shown that elevated Hcy may accelerate cognitive decline[166], this was the first prospective study to show a strong graded risk of the development of dementia associated with Hcy concentration. A 5µmol/L increase in Hcy level was associated with 40% increase in the risk of developing dementia. Hcy levels over 14µmol/L, or in the upper quartile of Hcy level based on 5-year age brackets, showed twice the likelihood of developing dementia.

Further studies continue to add weight to a link between Hcy and AD[168-171], and few would now dispute that a patient with AD is likely to have a raised Hcy. However the picture is not as clear when it comes to B12. Some studies report reduced levels of B12 in AD[159;160;162;172], whilst other studies failed to find any difference between AD cases and the control group[164;165;168;169], however the majority of studies show a tendency towards reduced B12 levels in the patient group which fails to reach clinical or statistical significance. This may reflect the difficulties seen in diagnosis, the controls

groups chosen, or the methodology when measuring B12 levels. It may also be that a reduction in B12 levels is secondary to damage caused by increased Hcy, such that the difference between studies is caused by the patient groups showing differing lengths of duration of the illness.

A paradoxical relationship was seen in the original COBALZ study were AD patients showed decreasing cognition scores with increasing B12[165]. Where Hcy was concerned the relationship was as expected with increasing levels associated with declining cognition. The absence of such findings in the control group implies metabolic significance.

Total vitamin B12 is a measure of cobalamin bound to both haptocorrin and transcobalamin (TC). Whilst haptocorrin binds the majority of serum B12, only the later is involved in transporting the vitamin to metabolically active tissues, with some 5 to 30% of the total B12 bound as holoTC[171;173;174]. Decreasing cognitive scores with increasing "total" serum B12 could therefore be explained by aberrant tissue delivery in AD[165;175]. If this were true, in AD patients one would expect to find decreased holoTC levels, with holo-haptocorrin levels increased, leaving total B12 unchanged or perhaps even elevated. Recent developments have enabled routine measurement of holoTC[174;176;177]. As predicted, where total B12 levels were not found to be significantly low in AD patients compared to control groups, holoTC levels were reduced[171].

Paper II reports investigation of Hcy concentrations in an elderly population including 51 patients with dementia and 60 controls. HoloTC concentrations were found to show a significant and independent relationship to Hcy whereas total serum B12 levels failed to show any association, proving that TC saturation may be a more reliable indicator of cobalamin status.

In humans TC has four common phenotypes as determined by their isoelectric points. Namour *et al.*[178] suggested that a polymorphism at codon 259, where a neutral proline reside is replaced by arginine, could account for this phenotypic variability. They also showed that the polymorphism was a determinant of apoTC, i.e. unsaturated, with homozygotes 259-P having the highest concentration. Therefore the polymorphism may affect B12 cellular availability and hence Hcy concentrations. Strangely though, heterozygotes (R/P) showed significantly higher Hcy concentrations than either homozygote. No association was shown between genotype and total B12 levels.

In Paper III and Poster XII, codon 259 was confirmed as a major determinant of TC phenotype. An apparent trend existed for increasing Hcy in the presence of a 259-R allele, which would fit with the findings of Namour *et al.* where homozygotes 259-R had the lowest apo-TC concentrations, however this was not found to be statistically significant. In response Namour and Guéant[179] provided data which suggested that an increase in Hcy in the elderly population may have masked the difference between the genotypes in our papers. They confirmed that heterozygotes showed increased Hcy levels compared to homozygotes in younger adults, but the difference was not significant in an older subgroup. They proposed that a dimeric TC receptor would have a lower affinity for a heterodimer of Pro/Arg isoforms.

Unfortunately holo-TC was not measured in these studies due to the high variability seen between methods available at the time.

If, in AD patients, holo-TC levels are lowered and holo-haptocorrin levels increased, what brings about the shift in distribution leading to a tissue deficiency in B12? Cobalamin "analogues" resemble cobalamin in structure but are functionally inactive. The concentration of these analogues correlated positively with serum Hcy and

negatively with CSF B12[172]. In this study patients with AD had lower levels of active B12 than controls, and a higher ratio of analogue to active forms. Interestingly holohaptocorrin and haptocorrin saturation levels were significantly reduced in AD patients compared with controls, with slightly lower holo-TC and TC saturation levels failing to reach significance. When broken down by age, concentration of analogues and transcobalamin levels, two distinct groups emerged, thus suggesting that two separate mechanisms might exist leading to B12 deficiency in AD, although both could involve a disruption of the balance between selective assimilation of B12 and elimination of analogues. The distinction between the groups appeared to be related to age. The majority of patients had a high ratio of analogue to active forms of B12, with a low TC saturation; a small minority, who tended to be younger, showed a low analogue to active ratio and a high TC saturation. These patients may have a genetic predisposition causing them to develop cerebral B12 deficiency. This may be a defect in TC resulting in impaired ability to release B12 to the tissues, or a dysfunctional receptor. Despite the confusing results in this study, in patients with AD, increasing age and analogue concentrations were found to be factor influencing a reduction in holoTC, TC/haptocorrin ratio and TC saturation implying a shift in binding between the carrier proteins and reduced delivery to the tissues. This shift in binding may be caused by the analogues displacing active cobalamin from TC to haptocorrin. A similar age related decrease in holo-TC levels was reported in a later study[173]. The source of cobalamin analogues is largely unknown but they may be either the by-products of oxidative stress associated with AD, or originate from the diet or the enterohepatic circulation which should serve to remove them from the body.

Most studies seem to agree that mean holo-TC levels are reduced in AD; however caution should be used in interpreting the results for any individual. Gender, diurnal variation, and fasting status may all influence the concentration measured[173], additionally analytical variation should be considered. As such holo-TC reference intervals may be of limited use. A change within an individual is probably more significant than the absolute concentration. Monitoring would therefore be required in high-risk individuals in order to use holo-TC as an early marker of change in B12 status. Results of one particular study however indicated that values would need to change by nearly 50% in order to be 95% certain that the change was significant[173].

The mechanism behind an increase in B12 analogues and a shift in binding from TC to haptocorrin is unclear. One possible explanation is that B12 analogues are generated through damage caused by oxidative stress. Oxidative stress has been proposed as a mechanism leading to both hyperhomocysteinaemia and vascular disease [180]. Poster XIII reports that although Hcy is a determinant of the presence of vascular disease, both are independently associated with a diagnosis of AD. Although the results fail to show a definitive link, there are clearly a number of associations here and it is possible that hyperhomocysteinaemia might reflect a common pathogenic mechanism in the development of vascular disease and AD. The influence of redox status on key enzymes in Hcy metabolism along with the sensitivity of vascular endothelium and brain tissue to oxidative stress, could explain the observed relationships.

McCaddon *et al.*[181], proposed a model which would complement the theory that Hcy potentiates vascular and neurological oxidative injury in both vascular dementia and AD, but also explaining how oxidative stress might cause hyperhomocysteinaemia in

the first place. This model accounts how B12 deficiency might be involved in AD in the absence of macrocytic anaemia[162;165].

As reported earlier age is perhaps the biggest risk factor in the development of AD [152;153]; aging is also associated with increased production of oxygen-derived free radicals and decrease in related cellular defences. Yet in AD the evidence of oxidative damage is more severe than in the average elderly brain[182]. Whether the oxidative stress is the cause of the AD or a secondary event, it is still an important neurodegenerative element in pathogenesis of the disease, and there may be benefits from treatment with antioxidants[158]. Increased levels of redox-active iron, catalysing hydroxyl radical formation from hydrogen peroxide, together with increased production of superoxide anions, from activated microglial cells surrounding the amyloid plaques, and a direct implication of Aβ in the formation of free radicals have all been cited as causes of oxidative stress in AD[182]. Furthermore, aluminium, which accumulates in neurones containing neurofibrillary tangles, may have an effect on cellular redox potential by stimulating iron-induced lipid peroxidation[182;183].

Under normal conditions the body is protected from damage due to oxygen radicals by an array of antioxidant systems that show a large degree of redundancy, for example the simultaneous metabolism of hydrogen peroxide by both catalase and glutathione peroxidase. In pathological conditions the balance between antioxidant and oxidant is disturbed, which may lead to damage. In order to locate the site where oxidative species were generated Smith *et al.*[182] looked at a marker of primary attack (8-hydroxyguanosine) and found that this was located in the cytoplasm of vulnerable neurones, whereas those affected by neurofibrillary tangles had low levels of this marker, despite evidence of previous oxidative damage. Furthermore cases of AD with

the most extensive Aβ deposits also showed lower levels of this short-lived marker of oxidative attack. These results suggest that senile plaques and neurofibrillary tangles may be cellular compensations for oxidative stress and may have some protective anti-oxidant function. These results are supported in studies of Down's syndrome and AD where levels of 8-hydroxyguanosine are increased prior to Aβ deposition, with levels declining after the plaques have formed.

On release from cells Hcy is rapidly oxidised to form disulphide bonds with either other free thiols or proteins in the surrounding tissues and plasma. This oxidation may also result in the generation of free radicals, which may in themselves contribute to tissue damage. If hyperhomocysteinaemia is both a cause and a consequence of oxidative stress, its generation may lead to a harmful feed-forward cascade.

Cysteine formed in the transsulphuration pathway (see Figure 1) is the rate limiting precursor for the synthesis of glutathione, an important intracellular antioxidant. In the brain S-AdMet, formed in the alternative pathway for Hcy, is the sole methyl donor for numerous reactions involving nucleoproteins, proteins, membrane phospholipids, and neurotransmitters. The relative fate of Hcy is determined by a number of factors including the maintenance of adequate S-AdMet levels, and the cellular redox state. These factors are described in more detail in Chapter 1, but in essence, S-AdMet is a key regulatory element. When in abundance S-AdMet increases CBS activity and inhibits MTHFR, such that 5-MeTHF is not available for remethylation of Hcy through MS, and flux through the transsulphuration pathway is enhanced. When S-AdMet levels are depleted, Hcy is conserved within the Met cycle.

As discussed in Chapter 1 (see Figure 2) the intermediate form of B12, cob(I)alamin, involved in the transfer of a methyl group from 5-MeTHF to Hcy, catalysed by MS is

labile to oxidation, rendering the enzyme inactive. CBS, on the other hand contains haem group in which the oxidised ferric moiety is more favourable to Hcy binding. Thus an oxidative environment stimulates the transsulphuration pathway in order to provide the antioxidant, glutathione.

If oxidative stress is increased in both AD and the elderly in general, MS may be impaired by enhanced oxidation of cob(I)alamin, resulting in a functional B12 deficiency. MS reactivation requires S-AdMet to donate a methyl group to cob(II)alamin to reform methylcobalamin. Thus MS inactivation results in reduced levels of S-AdMet through decreased production and increased utilization. Although oxidative stress would stimulate CBS activity the effect is compromised through loss of the positive allosteric effect of S-AdMet, in a futile attempt to conserve Hcy within the Met cycle to regenerate S-AdMet.

Hcy would therefore accumulate, and be exported from the cells, thus potentiating the generation of further free radicals.

This model, proposed by McCaddon *et al.*[181], accounted for the earlier finding of increased levels of inactive B12 analogues in AD[172]. It also explains the absence of haematological abnormalities in these patients; the activity of methylmalonyl-CoA mutase requires B12 but does not involve the formation of the reactive intermediate cob(I)alamin generated by MS. Oxidative stress therefore selectively impairs MS and spares methylmalonyl-CoA mutase.

Further implications of this hypothesis involve the neuronal metabolism of B12. Neurones can internalise several different forms of the vitamin including methylcobalamin and adenosylcobalamin. On entering the cell, each is converted to glutathionylcobalamin (GS-B12), which appears to be the preferred substrate for the

reductase enzymes that convert the vitamin into its metabolically active forms[184]. GS-B12 requires glutathione for its synthesis, however neurones lack cystathionase. Neurones therefore rely on their neighbouring glial cells to produce Cys, the precursor of glutathione in the transsulphuration pathway. This may be an additional explanation for a functional B12 deficiency in the presence of normal serum B12 levels in AD, as a result of cerebral oxidative stress under which conditions glutathione levels would be depleted for AD, and furthermore may explain poor response to B12 treatment in intervention studies.

Although McCaddon *et al.* described a plausible model relating Hcy and oxidative stress in an escalating feed-forward cascade[181], there is very little published information about the related aminothiols in AD. In Paper IV, total plasma Hcy, Cys, and glutathione was determined in patients with probable Alzheimer's disease and compared to age matched controls. The results confirmed earlier reports of reduced folate and increased Hcy levels in AD, with total B12, and red cell folate levels showing no difference, indicating that the results were not a result of a dietary vitamin deficiency. AD patients also showed increased levels of Cys, but, in contrast to the expected results glutathione was not significant. Yet, within the AD group plasma glutathione levels were a highly significant and independent predictor of cognitive scores. Lower glutathione levels were associated with greater cognitive impairment.

Increased Cys levels occurring in hyperhomocysteinaemia implies that transsulphuration is intact but remethylation is in some way impaired. MS inactivation could explain reduced plasma folate levels, as folate is trapped in the 5Me-THF form which cannot undergo polyglutamation, a prerequisite for cellular retention. As a

consequence folate is excreted in the urine resulting in folate depletion from which the red cells are spared[4;185].

These results fit with the model suggested above and also with the results reported by Mosharov *et al.*[186], who showed that flux of Hcy through the transsulphuration pathway is increased in response to oxidative stress in order to maintain synthesis of the antioxidant glutathione. The reason that levels of glutathione did not differ from controls is unclear, but the correlation with disease severity may indicate that in a prolonged struggle to overcome the effects of oxidative stress, glutathione is eventually overcome.

Paper V reports further findings from the study described in paper IV. In McCaddon *et al.*'s model of a functional B12 deficiency in AD[181], oxidative stress leads to impaired MS activity, but methylmalonyl-CoA mutase is spared as it does not involve the reactive B12 intermediate, cob(I)alamin. If this is the case then AD patients are unlikely to show the haematological symptoms associated with B12 deficiency[187]. Although raised MMA levels were found in one group of hospitalised AD patients compared to controls living at home, comparison with hospitalised controls explained the finding as a coincident mild dietary deficiency[164]. These results were reflected in the comparable prevalence of anaemia and macrocytosis in hospitalised individuals, which differed significantly when compared to the healthy elderly group.

In the investigation of biochemical markers, serum B12, methylmalonic acid, serum folate, and plasma Hcy, the latter appears to be the most consistent marker of tissue deficiencies in nutrient cofactors and cognitive performance in the elderly[151].

Paper V confirms the theory that AD is not associated with an increased incidence of macrocytic anaemia. Furthermore, a failure to develop these symptoms with increased

duration of AD argues against a longitudinal model in which a functional deficiency, with increased levels of metabolites, precedes the structural and morphological changes such as macrocytosis and anaemia. In this model one might explain the neurological features of AD as the primary presentation in the early stages of a classical B12 deficiency.

These results support both previous theories of a functional B12 deficiency in AD. Cerebral oxidative stress may lead to an increased degree of B12 oxidation, additionally impaired delivery to the central nervous system (CNS), would explain impaired MS activity, with spared methylmalonyl-CoA mutase activity. In fact these two theories could be linked by oxidation of B12 resulting in increased levels of B12 analogues, and a shift in binding from TC to haptocorrin, leading to reduced tissue delivery.

4.3 A model for the development of AD associated with increased Hcy

So far this thesis has described how increased Hcy, functional B12 deficiencies, and oxidative stress may be linked with AD, but it remains to be explained how these biochemical markers are associated with the hallmark features of the disease, notably amyloid plaques and neurofibrillary tangles. Figure 14 summarises the observed metabolic changes in Hcy metabolism in AD and shows the effects on brain function, which will be explained within this section.

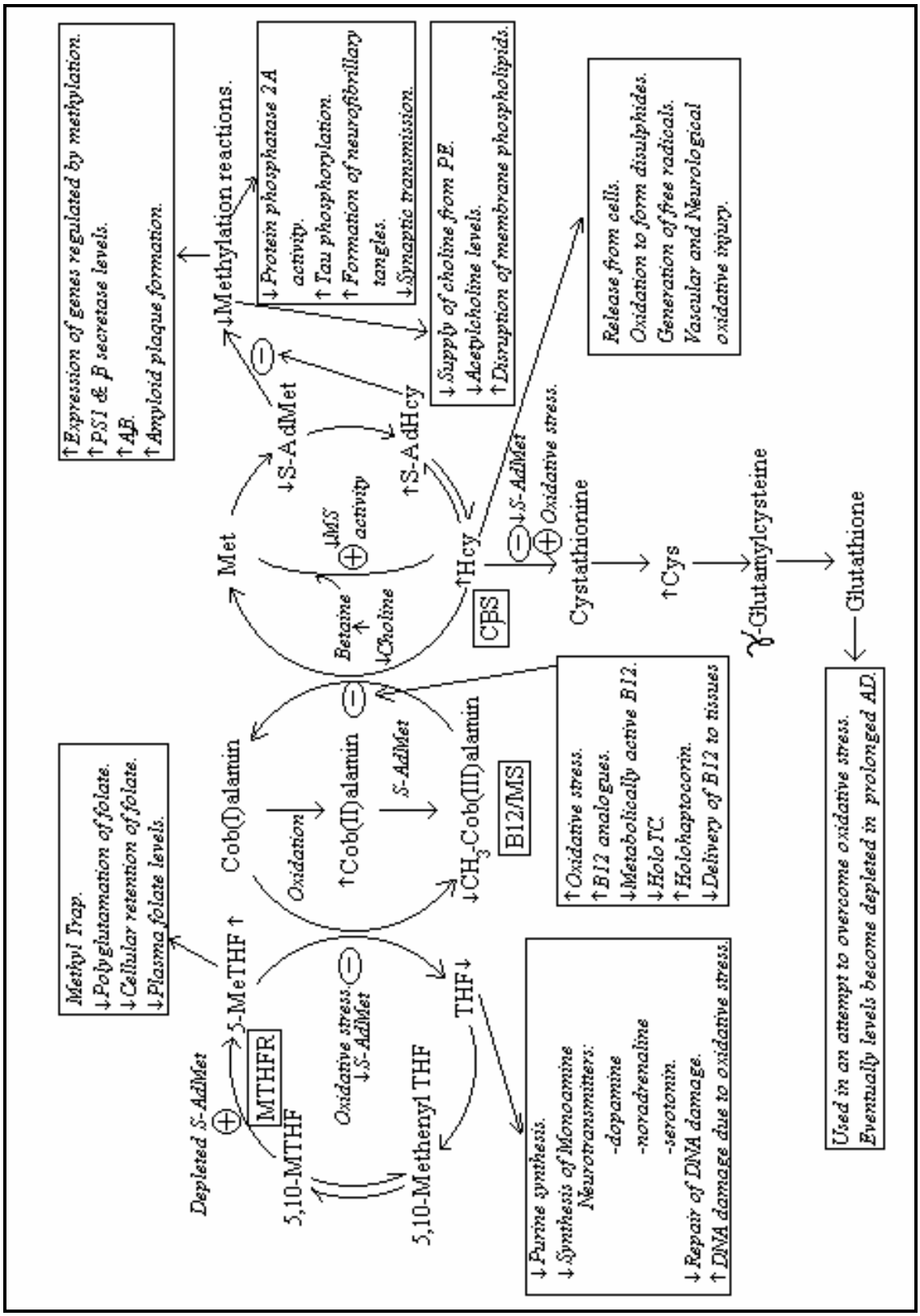

Figure 14 Pathway summarising the metabolic observations in AD and the consequences on brain function.

Before progressing down this path is it worth noting that most studies report levels of Hcy, B12 and carrier proteins in the plasma or serum and there is little point trying to explain how these findings relate to the pathological patterns observed in the AD brain if plasma levels do not correlate with levels in the CSF. Agnati *et al.*[170] proved that such a correlation was valid. They injected Hcy into the veins of experimental animals which penetrated the blood-brain barrier within 15 minutes.

Perhaps understandably, until recently any hypothesis on the pathophysiology of AD has focused on the hallmark featuresof the disease[188], and theories have been generated from studies of a small minority of patients in which AD is inherited as an autosomal dominant trait. The majority of early-onset, familial AD is accounted for by mutations in 3 genes associated with the formation of Aβ: APP, PS1 and PS2, with PS1 mutations being most frequent[157]. Late onset AD is associated with the possession of the apoE allele ε4[157].

APP is a transmembrane glycoprotein expressed in a variety of cell types, however its function is still unclear. Synthesised in the endoplasmic reticulum, APP passes through the golgi apparatus and is inserted into the membranes of intracellular vesicles[147]. Differential cleavage of the protein by three forms of secretase enzymes can result in the generation of fragments of differing chain lengths depending on the particular enzymes involved. Amyloid-$\beta_{(1-42)}$, here, for simplicity referred to as Aβ, has limited solubility, and is potentially amyloidigenic depositing in senile plaques and in the walls of cerebral blood vessels. Secreted forms are generated when α-secretases act on the protein as they cleave in the middle of the Aβ region of the precursor protein. B-secretases, also release a secreted form of APP, but they leave behind a membrane

spanning protein that contains the intact Aβ fraction. Further processing by γ-sectretases releases intact Aβ with the potential to form amyloid plaques.

Deposition of Aβ may be triggered by a number of genetic or environmental factors[188]. A mis-sense mutation in the APP gene seen in some families leads to altered metabolism and increased production of Aβ. In Down's syndrome there are three copies of chromosome 21 where the APP gene is located and patients almost invariably develop the pathology of AD before they reach the age of 40 years.

Whether Aβ forms deposits is dependent on its physical state. Freshly formed Aβ possesses a random coil or α-helical structure that elicits a neurotrophic action. Following prolonged incubation *in vitro* changes in the local pH bring about a shift in the conformation into β-pleated sheets, a process stimulated by nucleation, which may be caused by proteins such as apoE, and acetylcholinesterase. This shift into β-sheet conformation results in the formation of fibrils, which are neurotoxic[188].

Whilst possession of the apoE ε4 allele is associated with familial late onset AD, it also appears to be risk factor in sporadic cases. Several studies have shown an increased carriage of the allele in AD patients compared to controls[169;171], and possession of the allele alone may account for approximately 14% of cases[188]. In fact it appears that the other apoE alleles, especially ε2 may protect against the development of AD. *In vitro* studies suggest that apoE2 binds to Aβ more strongly than apoE3, and that apoE3 binds much better than apoE4. ApoE2 therefore may protect against the formation of amyloid plaques by maintaining amyloid protein in a soluble state[188].

PS1 and PS2 are integral membrane proteins found mainly in the nucleus, Golgi apparatus and endoplasmic reticulum with very little if any present in the plasma membrane[188]. Mutations in the presenilin genes do not seem to affect either the

processing or intracellular location of these proteins, but they do seem to alter processing of APP resulting in increased formation of Aβ in the brains of patients with AD. These two proteins, known as presenilins, make up the catalytic subunit of γ-secretase, therefore one might expect that mutations within the genes encoding them could lead to altered activity, and hence altered APP processing. Whilst, in normal brains, the predominant amyloid protein (~90%) is 40 amino acids in length and the isoform with limited solubility (1-42) constitutes about 10%[150], in AD the relative proportions of each are shifted to approximately equal. Furthermore, it is not just the balance that is shifted but the total burden of amyloid in the brains of patients with AD is increased dramatically, with increases in both forms of peptide[189;190].

PS1 activity is also required for processing of Notch, a signalling receptor that is essential for specifying the fate of neurones during neurogenesis and differentiation. Furthermore, PS1 involvement has been implicated in the formation of network connections (synaptogenesis)[191]. This might explain why investigations into the potential use of secretase inhibitors in the treatment of AD resulted in neuronal death, but non-neuronal cells were spared[189].

Increased expression of PS2 in neuronal cells has also been shown to increase their susceptibility to induced cell death, whereas lack of PS2 expression is related to increased formation of neurofibrillary tangles[188]

In the majority of cases AD is not inherited, however presenilins may still be important in the development of disease. As described earlier, hyperhomocysteinaemia caused by whatever means, is associated with impaired remethylation and consequentially reduced levels of S-AdMet. Increased S-AdHcy levels lead to inhibition of virtually all methyltransferase enzymes, with a global decrease in cellular

methylation. The regulation of gene expression is often controlled by methylation of their promoter regions. Hypomethylation may lead to over expression of genes when the cytosine in CpG moieties becomes unmethylated. Such control is important in regulating PS1 activity; S-AdMet administered to cell cultures lead to a down regulation of PS1, and hence a reduction in Aβ production[192]. Down regulation of PS1, in cultured cells treated with S-AdMet was confirmed in a later study, but it was also shown that depriving the culture medium of folate and B12, lead to a decrease in S-AdMet levels and induced expression of both PS1, and a β-secretase (BACE or β site APP-cleaving enzyme) which was reversed when S-AdMet was administered[193]. APP, PS2 and α-secretase expression was unaffected. If α-secretases were regulated in the same manner as BACE and PS1, there would be an unchanged ratio in APP processing.

Many of the above papers report the results of studies conducted on secretase expression and Aβ production in response to hypomethylation *in vitro.* For the effects described, and in fact for many of the other mechanisms relating to hypomethylation described here, to be involved in the brain pathology of AD one would expect to find increased S-AdHcy, reduced levels of S-AdMet and increased 5-MeTHF concentrations in samples taken from AD cases compared to healthy subjects. Mulder *et al.*[194] studied CSF samples in probable AD and found that the levels of each of these metabolites were unchanged compared to controls. They proposed that PS1 activity was regulated by posttranscriptional or posttranslational changes rather than over expression of the gene due to undermethylation of its promotor. It is clear that further studies are required before either theory is ruled out, and these should include cases with confirmed AD, as correct diagnosis may be an explanation for the conflict between *in vitro* and *in vivo* results.

Whilst looking at Hcy and its effect on gene regulation, Kokame *et al.*[195] identified a novel protein which was termed Herp. The term comes from Homocysteine-responsive eR-resident protein. The protein is located in the membrane of the endoplasmic reticulum, and Hcy activates its production through endoplasmic reticulum stress. The endoplasmic reticulum is responsible for protein synthesis, folding, assembly and trafficking. Changes with the cell environment which cause the accumulation of unfolded proteins trigger a stress response, which results in a signalling pathway out of the endoplasmic reticulum, leading to transcriptional and translational regulation in order to prevent the further accumulation of unfolded protein. Herp is one of the proteins that are upregulated under such conditions. Sai *et al.* [196] found that Herp interacts with PS1 and PS2, and that a high expression of Herp was associated with increased generation of amyloid protein.

Thus the formation of amyloid plaques in AD may be explained in relation to impaired remethylation of Hcy. The other key feature in AD is the presence of neurofibrillary tangles consisting primarily of tau, a microtubule associated protein. In healthy axons tau promotes polymerisation and stabilisation of tubulin, into "train track" structures, which guide nutrients and other molecules from the cell body down the length of the axon (see Figure 15). The functional properties of tau are determined by the degree of phosphorylation. For example, tau phosphorylation at the distal end of the axon regulates interaction between the cytoskeleton and the plasma membrane. The integrity of microtubule structure is critical for proper neuronal function and synaptic transmission however in AD and other neurodegenerative diseases, tau becomes highly phosphorylated and may be less effective at binding and stabilising the microtubules, leading to aggregation into paired helical filaments which are redistributed into the

somato-dendritic compartment of the cell in the form of neurofibrillary tangles[147;188]. Thus the microtubule transport system is disrupted and leading to impaired function and degeneration of neuronal cells[197].

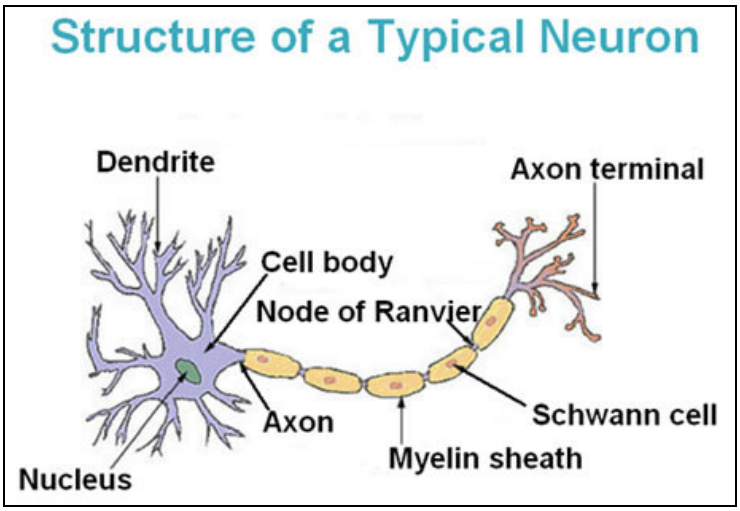

Figure 15 The structure of a typical neurone showing the long axon where microtubules are normally located.
Reproduced from: http://en.wikipedia.org/wiki/Neurone

Phosphorylation of tau is catalysed by two specific kinases, whereas dephosphorylation is mediated by the enzyme protein phosphatase 2A. Tau hyperphosphorylation, therefore, is the result of an imbalance between the activities of these enzymes. Recent evidence suggests that tau hyperphosphorylation is the result of decreased phosphatase activity rather than increased kinase activity[197]. Phosphatase expression seems to decrease with age, but has been found to be significantly reduced in the brains of AD patients relative to controls.

Protein phosphatase 2A, is a multimeric protein complex. Methylation is important in the assembly of this complex as it increases the affinity between the various subunits, which are crucial for directing activity towards tau[197]. As described above, impaired remethylation of Hcy, and accumulation of S-AdHcy, leads to hypomethylation. In this

case the result would be decreased phosphatase activity, leading to hyperphosphorylation of tau, a breakdown in microtubule structure, the formation of neurofibrillary tangles and eventually neuorodegeneration and cell death.

Although the presence of amyloid plaques and neurofibrillary tangles are central features in AD, there are many other biochemical effects leading to cognitive decline and neuronal degeneration. The effects of Hcy can be broadly divided into two groups: vascular and neurotoxic. Vascular effects are increased proliferation of the smooth muscle cells, increased platelet aggregation and increased number of stokes and white matter lesions, much of which can be explained by oxidative stress leading to inflammation. Besides the neurotoxic effects of Hcy on the deposition of Aβ and the formation of neurofibrillary tangles, Hcy may also be involved in neurotoxic events through disruption of neurotransmitter synthesis and function, hyperactivation of N-Methyl-D-Aspartate (NMDA) receptors, and apoptosis[153].

Before much experimental work had been done in this field. McCaddon and Kelly published their "cobalaminergic" hypothesis[183], describing a mechanism whereby B12 deficiency may result in the neurotransmitter and indeed the structural changes of AD. This hypothesis is divided into explanations of a monoaminergic deficiency and a cholinergic deficiency.

Monoaminergic deficiency is explained essentially through an understanding of the "methyl-folate" trap, which is described in more detail in Chapter 1. If remethylation of Hcy to Met is impaired, or S-AdMet levels are depleted in anyway, folate is diverted from purine synthesis, involved in cell growth and division, and diverted into the production of S-AdMet in order to maintain methylation reactions, which are more important for cellular survival. Where B12 is deficient or inactive, such a survival

technique is futile, impaired MS activity results in folate becoming trapped in the 5-MeTHF form. As a result, formyl polyglutamte forms of folate are depleted and these are essential in the synthesis of nucleotides. GTP, in turn is an essential precursor of tetrahydrobiopterin, which is a regulatory factor in the synthesis of the monoamine neurotransmitters dopamine, noradrenaline and serotonin. Additionally disruption to the Met cycle can affect the function of these neurotransmitters, as methylation is important in their deactivation[147].

Depletion of nucleotides, resulting from the folate trap, also affects the cells' ability to repair DNA damage. Under oxidative conditions damaged DNA will accumulate within the cells and when this happens, factors that would normally shift a dividing cell's activities from mitosis to repair can also trigger apotosis[149].

Cholinergic neurones play a key role in memory and learning, thus degeneration of this system contributes to cognitive deficits in AD[147]. Choline is required for the synthesis of the neurotransmitter acetylcholine. Choline is obtained from three main sources: intrasynaptic degradation of acetylcholine, extracellular choline and intraneuronal choline via sequential methylation of phosphatidyethanolamine located in neuronal membranes. Cholinergic deficiency may arise through a lack of B12 due to reduction of both extracellular supplies and intraneuronal synthesis. When MS activity is impaired some tissues exhibit an alternative pathway utilising betaine as methyl donor in place of 5-MeTHF, in order to regenerate Met, and maintain a supply of S-AdMet. Even though the brain does not possess this alternative mechanism for Hcy remethylation[151], extraneuronal supplies of choline will be depleted as other tissues in the body use choline to synthesise betaine in order to overcome the MS block. Intraneuronal choline synthesis may be compromised in that supplies of

phosphatidylcholine, formed by sequential methylation of phosphatidyethanolamine, will be reduced through lack of S-AdMet and inhibition of methylation reactions due to increased levels of S-AdHcy[147;183].

Cellular destruction may then be explained by neurones resorting to membrane phospholipids as a supply of choline. Membrane disruption could result in increased permeability, with loss of proteins and enzymes, or an increased influx of secondary messengers. Both theories may account for the selective destruction of central cholinergic neurones[183]. Membrane fluidity may also be influenced by methylation of phospholipids important in the function of various membrane receptors, influencing signal transmission across the cell membrane[147]. An asymmetry exists in the distribution of phospholipids within the membrane, with phosphatidylethanolamine largely facing the cytoplasm and phosphatidycholine located on the outer cell surface, facing the extracellular space. As phosphatidyethanolamine is sequentially methylated to become phosphatidylcholine it is translocated to the cell's exterior surface in a "phospholipid flip-flop". This rapid rearrangement increases membrane fluidity, which can lead to calcium influx and the release of extracellular secondary messengers, in this way a variety of neurotransmitters work by stimulating methylation when they bind to the cell surface.

Hcy, or a derivative, may play a role in the direct stimulation of neuronal receptors. Glutamate is the major neurotransmitter in the CNS involved in some degree in all CNS functions from perception to cognition[147]. Postsynaptic membranes contain NMDA glutamate receptors that have unique modulatory features mediating synaptic plasticity, necessary for learning and long-term memory potentiation[147;163]. Glycine acts as co-agonist for the receptor, and although its binding does not trigger the receptor, it is

required before glutamate can act on the receptor. Hcy can act as both a partial agonist at the glutamate binding site, and a partial antagonist at the glycine co-agonist site[170]. Under normal conditions, Hcy levels are not significant enough for it to have a noticeable effect on the receptor, and it may actually be neuroprotective through its antagonistic action. In pathological conditions, however, the excitotoxic (agonist) affects result from over stimulation of the receptor leading to excessive intracellular calcium, free radical generation, collapse of the mitochondrial membrane potential, release of cellular proteases and eventually neuronal cell death. In fact homocysteic acid, an oxidised metabolite of Hcy is more likely to be the cause of the disruption to the neuronal network as it is 250 times more effective than Hcy itself[147;163].

Hcy oxidation, and the generation of reactive oxygen species may also have an indirect effect on NMDA receptors through modulators of neuronal activity. Oxidative stress has also been implicated as a factor in the severely reduced levels of hydrogen sulphide seen in the brains of AD patients[198]. In the brain, hydrogen sulphide is produced by CBS, and acts as neuromodulator selectively enhancing NMDA receptor mediated responses. Dwyer *et al.*[198] proposed that reduced CBS activity resulted from Hcy oxidation, an increase in redox-active iron in AD neurones, mobilisation of ferritin stores of iron and decreased levels of haem, vital for CBS activity, through increased activity of the haem degrading enzyme haem oxygenase 1. Activity of haem oxygenase 1 is stimulated by oxidative stress, and may lead to a localised haem deficiency in the brain of AD patients, resulting in the loss of CBS redox responsiveness. This provides another explanation of how hyperhomocysteinaemia can be both the cause of consequence of oxidative stress.

Supporting evidence that Hcy or a derivative may play a role in excitotoxicty through NMDA receptors comes from the finding that, in healthy individuals, high Hcy levels predicted poor scores of constructional praxis (i.e. the ability to copy shapes). The association was stronger than for other tests of cognition including learning and memory. NMDA receptor mediated long-term potentiation is believed to be important in visuospatial processing[166]. For Hcy to exert a maximal effect on spatial copying skills, it seems that neurones may be more sensitive to NMDA receptor mediated damage than to the generalised vascular or oxidative damage that Hcy may cause.

There is evidence supporting several mechanisms whereby hyperhomocysteinaemia is involved in the development of dementia. It is still not clear whether raised Hcy is the primary cause of these events or a marker of altered metabolism. In fact it seems likely that disruption of the Met cycle may be caused by any one of a number of factors, each of which potentiates the others in a cascading series of events leading to global cellular hypomethylation and eventually to neuronal death.

Of course not all these mechanisms may play a part in each individual with AD, and B12 deficiency in whatever form may not be the primary cause of AD in all cases. It is likely that subgroups of patients exist with differing causes leading to a common pathological condition. Nevertheless an understanding of some of the potential mechanisms involved, and the possible consequences of interference, will assist in the development of suitable treatments with an eventual aim of disease prevention.

4.4 Treatment Strategies

As described above the differential diagnosis of dementia is a difficult task, however a number of causes are fully reversible. In addition some pharmacological treatments

may be beneficial in one type of dementia, whereas their possible side effects mean that they should not be used indiscriminately. It is important that a correct diagnosis is made as early as possible. Yet the long time frames involved in the development of AD means that many of the treatments aimed at alleviating the symptoms and pathological findings connected with the disease may simply be doing just that and not targeting the real cause. By the time the symptoms and pathology appear it may be too late to prevent, or reverse the disease and the best that may be possible is to delay progression, as much of the damage may be irreversible.

Treatment may be targeted at preventing the development of Aβ, or neurofibrillary tangles, but whether either of these is a primary cause of the disease or merely a consequence is still uncertain. Other treatment strategies may involve repair of DNA damage, or targeting the prevention of a hypomethylation state, or oxidative state.

An understanding of the mechanisms involved in the formation of Aβ plaques lead some to suggest that inhibitors of secretase enzymes might have potential in the treatment of AD. However *in vitro* studies showed that inhibition of β- or γ-secretase activity induced neuronal cell death, whilst sparing non-neuronal cells[189]. Incubation with the soluble form of amyloid (1-40) prevented the toxicity, suggesting a protective, physiological role for this peptide. Furthermore, loss of PS1 activity may result in severe defects in the formation of neuronal networks[191]. In fact, the formation of amyloid plaques results from an imbalance between the activities of the different forms of secretase enzyme and a more suitable form of treatment might be to re-establish a correct balance. Specific inhibitors designed to reduce the production of Aβ by 30-40% may be beneficial in the treatment of the early phases of the disease, where cognitive impairment is apparent[150]. Mild intervention by administration of S-AdMet has been

shown to reduce Aβ production through down regulation of PS1 and BACE rather than complete loss of gene expression[192;193].

Some non-steroidal anti-inflammatory drugs (such as ibuprofen) have been associated with protection from AD, and it was thought that the effect was due to a reduction in neurotoxic inflammatory responses through inhibition of cyclooxygenase-1, however experiments in animal models imply that they may modulate the processing of γ-secretases, without lowering overall amyloid production or affecting neuronal differentiation[150].

Far fewer inhibitors of β-secretases, than for γ-secretases have been found to date but the fact that the crystal structure of a β-secretase with an inhibitor in place has been resolved suggests that effective inhibitors will soon be designed through the use of molecular modelling techniques. Such inhibitors may be more beneficial therapeutically as knock out mice for the β-secretase gene no not show an adverse phenotype[150].

An alternative approach to lowering the burden of Aβ protein in AD is to vaccinate patients against the protein, however early trials showed that a small number of patients (6%) developed an inflammatory response in the CNS, causing the trials to be abandoned. Nevertheless at subsequent follow-up, those who had developed antibodies towards Aβ seemed to show a slowing down of their cognitive and behavioural decline, such that human trials of passively administered antibodies are to be conducted[150].

Pharmaceutical treatment of the dementia in AD is currently restricted to 2 types of drug. Cholinergic deficiency may be addressed through the use of acetylcholinesterase inhibitors, and more recently a NMDA receptor antagonist, memantine has been approved to tackle excitotoxicity[147;183]. However these agents only seem able to

temporarily relieve some of the symptoms and do not address the underlying pathology of the disease, or substantially slow its progress[150].

As oxidative stress appears to be a major player in the development of AD, whether causative or part of an escalating feed-forward effect, it seems sensible that treatments be aimed at reducing potential production of free radicals. A healthy balanced diet, with plenty of antioxidants should reduce the risk of developing AD, along with several other diseases associated with aging, but any lifestyle change may need to be introduced several years before as it is unclear just how long a latency period may be involved. The long time spans involved in this chronic disease also make it difficult to conduct thorough prospective studies.

Antioxidants available in food and supplements include vitamin E (tocopherol), vitamin C (ascorbate) and carotenes. *In vitro* studies show suppressed levels of inflammation and oxidative stress, affects on catecholamine synthesis, and a reduction in lipid peroxidation respectively[158]. Antioxidants may also prevent the development of vascular events associated with dementia; in fact several treatments and dietary interventions aimed at lowering cardiovascular risk may help to prevent AD through vascular mechanisms.

Another way of tackling oxidation is the administration of chelating agents in order to remove trace metals such as zinc, copper, and aluminium which may all play a role in the generation of free radicals[150;182].

Reduction in oxidative stress and the restoration of neurotransmitter and gene function may be possible through dietary or pharmaceutical intervention however it unlikely that the structural abnormalities associated with AD can be reversed. Therefore, primary prevention, rather than cure seems to be key to alleviating the

burden on society caused by the increasing numbers of patients suffering from AD. Screening of the elderly particularly those at risk of developing dietary deficiencies of B12 and folate should enable identification of those where intervention might be beneficial before any signs of cognitive decline are noted. Such screening requires that suitable plasma markers are measured and evidence supports the use of Hcy and holo-TC.

If vitamin deficiency and / or a raised Hcy level are found, due to the complex pathology involved, and the possibility that different mechanisms are involved in different subsets of individuals, it seems sensible to supplement with all the nutrients required for Hcy and Met metabolism, including folate, B12 and B6. Supplementation of B vitamins may lower Hcy[78], but whether this is effective in the treatment of AD remains to be proved. Some studies have shown improvements in cases of cognitive impairment, but reversal of dementia is rare[163]. Although trials have shown conflicting reports of the benefits of each of these treatments, case control studies may be trying to treat too late in the disease progression. Well designed primary intervention trials may provide more convincing evidence that these treatment plans will assist in lowering the incidence of AD and cognitive decline, but the chronic nature of the diseases means that the trials require long periods of follow up. Three large trials into the benefits of B12, folate and B6 in slowing down or preventing the development of dementia are currently underway and the first, VITAL (VITamins to Slow ALzheimer's Disease) is due to report in 2006[199]. Dietary supplementation is inexpensive and relatively safe as long as folate is provided with B12, therefore it seems sensible to encourage all those at risk of developing vitamin deficiency, whether functional or dietary, to take all the nutrients required for effective metabolism of Hcy and Met, including B12, folate and B6.

One of the factors complicating trials of vitamin B12 is the different forms of cobalamin that exist. The two metabolically active forms methyl-cobalamin and 5-deoxyadenosylcobalamin, are cofactors in the reactions catalysed, respectively by MS and methylmalonyl-CoA mutase[173]. Oral B12 preparations are obtainable but are rarely used, perhaps due to concerns regarding the unpredictability of absorption, therefore intramuscular administration is currently used in most countries[200]. Two forms of intramuscular preparation are available: cyanocobalamin and hydroxocobalamin. The latter is more widely used as it is retained in the body longer and can be administered at intervals of up to three months[200]. Yet hydroxocobalamin may be easily reduced to the supernucleophilic cob(I)alamin, which is highly reactive towards xenobiotic epoxides formed by mammalian metabolism of dienes such as the industrial chemicals chloroprene and 1,3-butadiene. Glutathione, however, inhibits reduction of hyroxocob(III)alamin by forming GS-B12 which potentially affords greater protection against B12 depletion[201]. In fact, as described above, on entering the cell all currently available pharmaceutical forms of B12 require conversion to GS-B12, which is used much more efficiently in the synthesis of the metabolically active cofactors[184]. The apo-enzyme of MS binds cob(II)alamin, S-AdMet then supplies a methyl group to give the active form, thus reduced GS-B12, binds quicker than methyl-cob(III)alamin.

Under conditions of oxidative stress, where glutathione and S-AdMet are depleted, administered forms of vitamin B12 may not be utilised by neurones. In such cases antioxidant therapy, GS-B12, or B12 given with a glutathione precursor may be preferential forms of treatment.

N-acetylcysteine is a precursor of glutathione and also a potent antioxidant in its own right. In a recent publication McCaddon and Davies presented case studies in which

they had evaluated the co-administration of N-acetylcysteine with B vitamin supplements in three cognitively impaired hyperhomocysteinaemic patients[202]. In each case initial treatment with B12 (injections of hydroxocobalamin) and oral folic acid successfully lowered the patients Hcy levels but cognitive function failed to improve. As the patients were also found to have low glutathione levels, N-acetylcysteine was added to their treatment regimes. In all three cases mood, well being and general alertness were noticeably improved. Two of the patients also showed improvements to cognitive scores within one month; in the third case N-acetylcysteine was commenced late in the course of the disease when the patient already had a very poor score on MMSE (5/30), although he failed to show an improvement in that score, he had started to recognise and communicate with his wife for the first time in many years.

In light of these results COBALZ Ltd., has been issued with a US licence to have suitable preparations produced in the US where trials should soon be underway to determine whether the antioxidant (N-acetylcysteine) together with high-dose B vitamins is superior to standard B-vitamin supplements in slowing the rate of cognitive decline and the accumulation of structural abnormalities in the brains of patients with AD[203]. COBALZ Ltd. is actively seeking partners to conduct similar studies in other countries. Should the results of these trials, and other studies looking at novel therapies prove successful, we may soon have ways to treat AD patients rather than simply alleviate the symptoms of the disorder. Such treatment combined with measures to screen those at risk of developing vitamin deficiencies and the initiation of prophylactic treatment, may soon enable a drastic reduction to the severe burden caused by dementia on the health systems of the Western world.

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Appendix A Included Papers & Posters

Clinical Chemistry 48:11
2017–2022 (2002)

General Clinical Chemistry

Effects of Temperature on Stability of Blood Homocysteine in Collection Tubes Containing 3-Deazaadenosine

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Background: **The accuracy of homocysteine (Hcy) results is currently compromised by the requirement to separate the plasma within 1 h of sample collection. We studied the effect of temperature on the stability of plasma Hcy over a 72-h time course in blood collected into evacuated tubes containing either EDTA alone or both EDTA and 3-deazaadenosine (3DA).**

Methods: **We recruited 100 volunteers, including both diseased and healthy individuals with a range of baseline plasma Hcy values, from two centers. Blood samples were collected into tubes containing EDTA, and EDTA plus 3DA and stored at ambient temperature (20–25 °C) or refrigerated (2–8 °C). Aliquots of blood were centrifuged at various times up to 72 h, the plasma was removed, and Hcy was measured by HPLC.**

Results: **Plasma Hcy measurement covering the sample collection and storage conditions during the whole time course was possible on samples from 59 of those recruited. One-way ANOVA for repeated measures within subjects revealed that only samples that were collected into tubes containing EDTA plus 3DA and stored refrigerated were stable over 72 h ($P = 0.2761$).**

Conclusions: **A combination of 3DA and storage at 2–8 °C will allow collection of samples for plasma Hcy measurement outside of the hospital setting and wider population screening.**

Homocysteine (Hcy),[4] usually present in small amounts in plasma, is formed in mammals solely from methionine *(1)*. McCully and Ragsdale *(2, 3)* highlighted the importance of this pathway when they found that marked increases in plasma Hcy are a common factor in the presence of vascular lesions, which were brought about either by enzyme deficiencies in methionine metabolism or experimentally in rabbits. Since these initial observations were reported, links between hyperhomocysteinemia and a multitude of disorders have been postulated, including cancer *(4)*, neural tubes defects *(5)*, cognitive decline *(6)*, and dementia *(7)*. However, there is still argument about whether increased Hcy is the cause or result of tissue damage *(8)*. Those studies exhibiting a link between Hcy and vascular disease tend to show that relatively small changes in Hcy concentration lead to large increases in relative risk. In a metaanalysis of 27 such studies *(9)*, a 5 μmol/L Hcy increase led to odds ratios for coronary artery disease of 1.6 (95% confidence interval, 1.4–1.7) in men and 1.8 (95% confidence interval, 1.3–1.9) in women. Dietary fortification with folic acid supplements has been shown to decrease Hcy concentrations *(10–12)*, but the results of prospective studies are needed to show whether lowering Hcy is beneficial in reducing the risk of disease *(13–15)*. If confirmed, then the debate is strengthened in favor of screening programs designed to lower the incidence of heart disease. Such moves have been facilitated by the recent emergence of commercial systems allowing the routine clinical laboratory to perform testing previously performed only in research facilities.

The remaining problem is how to stabilize plasma Hcy concentrations in blood before processing, centrifugation, and storage. Erythrocyte Hcy concentrations are ~10-fold

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[4] Nonstandard abbreviations: Hcy, homocysteine; 3DA, 3-deazaadenosine; and SAHH, *S*-adenosylhomocysteine hydrolase.

lower than in plasma; therefore, any increase in plasma Hcy after sample collection is not attributable to leakage from the cells, but from continued metabolism and excretion into the plasma *(16)*. The liver and pancreas are mainly responsible for Hcy removal in vivo *(1)*. Because this removal pathway is absent in vitro, Hcy has been shown to increase by as much as 10% per hour over the first few hours after sample collection *(16, 17)*. The current recommendation is to place samples on ice and centrifuge within 1 h. Plasma Hcy may then be stable for at least 24 h at room temperature and for several months, if not years, when stored frozen *(17)*.

If a 5 μmol/L increase is associated with an 80% increased risk of vascular disease, artifactual increases in plasma Hcy caused by delays in sample processing could easily lead to false-positive results being reported. Although the treatment for hyperhomocysteinemia is simple and noninvasive, proper risk assessment demands accurate data.

Storage temperature *(18–21)*, acid citrate *(18, 22–24)*, sodium fluoride *(25, 26)*, and erythrocyte lysis *(27–29)* have all been considered in attempts to stabilize plasma Hcy. However, several of these methods cause sample dilution, either through the addition of liquid or, particularly with sodium fluoride, through osmotic effects caused by production of hypertonic plasma *(30)*. To date, the only method that has been shown to stabilize Hcy concentrations in whole blood for any longer than a few hours, without readjustment of reference intervals or immediate centrifugation, is the use of 3-deazaadenosine (3DA) *(31)*. At 100 μmol/L in EDTA whole blood, al Khafaji et al. *(31)* reported that 3DA stabilized plasma Hcy for 72 h at room temperature before centrifugation. 3DA prevents Hcy production through competitive inhibition of the enzyme *S*-adenosylhomocysteine hydrolase (SAHH), the final enzyme in Hcy production from methionine.

In pilot studies *(32)*, we investigated the use of 100 μmol/L 3DA in EDTA whole blood before developing a commercially available evacuated blood collection system. However, because 3DA acts through competitive inhibition, its effectiveness is influenced by temperature. To avoid ambiguity when using phrases such as "ambient" temperature, we controlled storage temperatures between 20 and 25 °C; however, the mean Hcy increased from 8.5 μmol/L to 11.5 μmol/L over 72 h. At 2–8 °C, the mean Hcy decreased by a statistically insignificant 0.5 μmol/L.

In light of the pilot study results, a trial batch of evacuated tubes was produced that contained 3DA spray-dried into tubes containing EDTA. Here we report clinical validation of these blood collection tubes, conducted in such a way as to confirm whether a combined effect of low temperature and 3DA was sufficient to provide long-term stability of plasma Hcy in whole blood.

Most studies on sample stability have been conducted on apparently healthy populations. In the clinical validation of our tubes, we were interested in comparing the stability of samples collected from a more diverse population, including patients with vascular disease and elderly patients, in addition to healthy volunteers.

Materials and Methods

Volunteers were recruited in accordance with the current revision of the Helsinki Declaration of 2000 *(33)*. The only exclusion criterion was individuals known to have recently taken drugs that can interfere with the method used for Hcy analysis (captopril, cysteamine, *N*-acetylcysteine, and *N*-2-mercaptopropionyl glycine).

To ensure a range of starting concentrations, two centers were selected such that blood was collected from 50 individuals, patients, and employees at a hospital in the United Kingdom (Birmingham Heartlands Hospital) and from another 50 volunteers, mainly healthy students and employees, at a university campus in the US (University of Maryland at Baltimore). Local approval was obtained from the appropriate ethics committees, and informed consent was given by all participants.

Blood was collected, by venipuncture, into tripotassium EDTA Vacutainer™ Tubes (Becton Dickinson) and DS30 Hcy Blood Collection Tubes (Drew Scientific Ltd., Barrow in Furness, United Kingdom). The DS30 Hcy blood collection tubes contain dipotassium EDTA as anticoagulant and 100 μmol/L 3DA (final concentration) to inhibit Hcy production. Each tube was evacuated to collect 2.5 mL of whole blood. Once collected, each tube was mixed and aliquoted for storage at ambient temperature (20–25 °C) and under refrigeration at 2–8 °C. Samples were taken from each blood tube for baseline Hcy measurements. At 3, 6, 24, and 72 h after blood collection, an aliquot of blood was removed from each tube at each storage temperature and centrifuged for 10 min at 11 000*g*. The plasma was removed and stored at −80 °C until analysis.

Hcy measurement was performed at two sites using the Drew DS30 Hcy Analyzer (Drew Scientific). The Drew analyzer uses reversed-phase HPLC with fluorescence detection to separate ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F)-derivatized thiols in plasma. Reduction of disulfide bonds by tris(2-carboxyethyl)phosphine allows measurement of all forms of homocyst(e)ine. To avoid between-batch variability, each individual's set of samples was measured within a single run. Within-batch imprecision for this method has been shown to be <2% *(34)*.

Changes in Hcy concentration for each storage condition over time were assessed for statistical significance by one-way ANOVA within subjects with repeated measures.

Results

Of the 100 volunteers recruited, 59 yielded a complete set of results for all time points and storage conditions under investigation. Reasons for lost results included poor

bleeds from elderly volunteers and identifiable mistakes in sample analysis. Incomplete datasets were not included.

The 59 volunteers comprised 38 females and 21 males. The median age was 47 years, with a range of 17–91 years. Initial plasma Hcy values ranged from 3.9 to 28.7 μmol/L.

Shown in Fig. 1 are the changes in plasma Hcy concentration over time. One-way ANOVA within subjects with repeated measures showed that only a combination of the DS30 Hcy blood tube and refrigerated storage provided stability of plasma Hcy concentrations in whole blood at all times up to 72 h (no significant difference, P = 0.2761). Hcy changes under the three other storage conditions were all highly significant (P <0.0001).

Using the Tukey ω-procedure *(35)*, we assessed the time taken for Hcy values to change significantly from baseline for each condition. In EDTA alone, Hcy increased significantly before 3 h had elapsed, regardless of whether the tubes were refrigerated or left at ambient temperature. However, in DS30 Hcy blood collection tubes, 3DA used in combination with EDTA provided added stability at ambient temperature (6 h).

DS30 Hcy blood tube samples from 17 individuals yielded enough blood to allow analysis of plasma after 1 week (168 h) at 2–8 °C. However, these volunteers did not produce a full dataset for the other storage conditions. Again, no change in Hcy concentration was observed (P = 0.4690). The initial mean Hcy for this group was 14.0 μmol/L. Even after 1 week at 2–8 °C, the concentration had increased by only 0.3 μmol/L.

Previous stability studies have reported changes in Hcy over time as either percentages or absolute changes against initial values. In the present study, in the absence of inhibitor, whole-blood samples stored at 20–25 °C showed an average Hcy increase of 8% per hour in the first few hours after sample collection, but those individuals whose Hcy was at the lower end of the reference interval [5.2–15.1 μmol/L *(36)*] showed increases as large as 20% per hour. Dividing the study population into 5 μmol/L bands (Table 1), according to baseline Hcy, highlighted a significant difference (P <0.001) in the percentage change in plasma Hcy in the whole-blood samples over 72 h across the different bands. When analyzed according to absolute changes in Hcy, this statistical significance disappeared (P = 0.1460). This shows that the mass of Hcy produced over time varies little between blood samples taken from a large group of individuals, even over a wide range of initial Hcy concentrations. It therefore follows that the percentage change will be greater in those samples with the lowest starting concentrations.

In light of these findings, we believed that data obtained from individuals with higher starting Hcy might mask any changes over time in those samples with initial Hcy in the lower groups. We therefore subdivided the data into two groups (≤10 μmol/L and >10 μmol/L) containing 43 and 16 individuals, respectively, and analyzed them (Table 2). Irrespective of the baseline Hcy, storage of whole blood at 2–8 °C in EDTA or 20–25 °C in the presence or absence of 3DA led to significant changes in plasma Hcy over 72 h (P <0.0001), whereas samples showed stability over 3 days of storage at 2–8 °C in the DS30 Hcy blood tube independent of whether the initial Hcy was less than or greater than 10 μmol/L (P = 0.3682 and 0.3140, respectively).

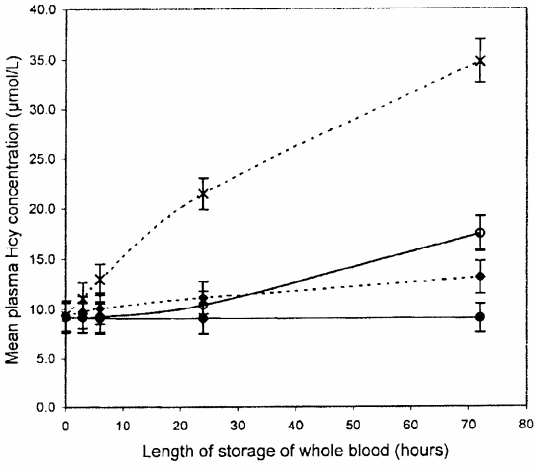

Fig. 1. Effect of whole-blood storage on plasma total Hcy over 72 h in standard EDTA tubes or in DS30 Hcy blood tubes.

Data points are the mean ± 2 SE (*error bars*) for samples from 59 individuals. ●, DS30 Hcy blood tube stored at 2–8 °C; ◆, EDTA tube stored at 2–8 °C; ○, DS30 Hcy blood tube stored at 20–25 °C; ×, EDTA tube stored at 20–25 °C.

Discussion

If blood samples are stored under ambient conditions before centrifugation, Hcy production by erythrocytes leads to an increase in plasma concentrations *(16, 17)*. Previously, 10% per hour has been quoted as the initial rate of Hcy production after venipuncture; a similar average rate was observed in the present study (8% per hour). However, an inverse relationship existed between baseline Hcy and the rate of Hcy production (expressed as a percentage of the initial Hcy). Consequently, because a 5 μmol/L increase in Hcy may be associated with a 80% increased risk of vascular disease *(9)*, strict sampling conditions must be observed to prevent false increases in plasma concentrations.

Storage on ice before centrifugation may stabilize plasma Hcy for up to 6 h *(20)*, although current recommendations suggest that processing should occur within 1 h. As we were interested in providing a solution to sample collection away from a centralized laboratory, we chose to look at refrigerated conditions. However, for samples in which EDTA alone was used, the maximum stability was only 3 h.

If ice is unavailable, acid citrate may also provide

Table 1. Percentage and absolute changes in plasma Hcy concentrations in EDTA whole blood over 72 h of storage at 20–25 °C.

Hcy values, μmol/L	Initial Hcy,[a] μmol/L	Absolute change,[a] μmol/L	Percentage change[a]	n
0 to <5	4.5 (0.1)	23.8 (1.6)	526.9 (36.7)	9
5 to <10	6.8 (0.2)	26.1 (1.2)	397.0 (22.0)	34
10 to <15	12.7 (0.6)	29.8 (4.0)	241.9 (37.2)	7
15 to <20	17.1 (0.8)	20.8 (3.6)	121.2 (20.6)	4
20 to <25	23.0 (0.9)	20.0 (0.9)	87.5 (5.7)	3
25 to <30	27.5 (1.4)	19.8 (2.7)	72.7 (13.6)	2

[a] Results are mean (SE).

stability for up to 6 h at "room temperature". However, at higher temperatures, samples are less stable *(24)*. Ducros et al. *(18)* found that sample stability also depends on the method of analysis. Chromatographic methods showed sample stability for at least 4 h in acid citrate at room temperature, whereas Hcy results increased over the same time course with an immunochemical method (fluorescence polarization immunoassay). Similar findings were reported by Salazar et al. *(23)* and O'Broin et al. *(22)*, who showed a significant increase after 2 h or a 10% increase after 6 h, respectively. Whereas chromatographic methods measure Hcy itself or a Hcy derivative, immunochemical methods are indirect. Hcy is estimated by the amount of its metabolic precursor, *S*-adenosylhomocysteine, formed during the assay by the reverse reaction of the enzyme SAHH. In acid citrate, the low pH may prevent Hcy build up by inhibiting SAHH, but precursor build up is not prevented, thereby giving the impression of sample instability. Where stability is reported, there are conflicting reports about whether acid citrate increases *(22, 24)* or decreases *(23)* baseline Hcy compared with EDTA samples kept on ice. This confusion may be related to corrections for dilution because acid citrate is added as a liquid, requiring hematocrit estimates. Either way, separate reference intervals are required for interpretation.

Similar problems complicate Hcy measurements on capillary whole-blood lysates *(27–29)*. The method appears quite attractive: a simple fingerprick followed by cell lysis, with stability for 2 days at ambient temperature provided by deactivation of the enzymes that produce Hcy. However, even after correction for dilution caused by the lysing agents, results are lower than in plasma because of further dilution by low intracellular Hcy concentrations.

Sodium fluoride samples at 2–3 h after collection have shown Hcy concentrations similar to baseline EDTA values *(25, 26)*. On closer inspection, however, Hcy continued to increase over time. The effect was attributable to an initial concentration drop caused by the formation of hypertonic saline, which led to fluid shifts. This observation was confirmed by Hughes et al. *(30)*, who saw a sodium fluoride concentration-dependent decrease in hematocrit. Fluoride inhibits anaerobic glycolysis and, therefore, ATP production, which is required for methionine conversion to *S*-adenosylmethionine, the first step in Hcy production. However, Hcy production may continue because of cellular reserves of *S*-adenosylmethionine, first postulated by Andersson et al. *(16)*.

Despite these studies, each method has its own weaknesses. After the publication by al Khafaji et al. *(31)*, we investigated the production of evacuated blood tubes containing 3DA. These tubes promised stability for up to 72 h under ambient conditions, without recalculation of Hcy results or changes to currently accepted reference

Table 2. Plasma Hcy concentrations in whole blood over time, subdivided into initial values of ≤10 μmol/L (n = 43) and >10 μmol/L (n = 16), for each storage condition.

Sample	Storage temperature, °C	Initial Hcy, μmol/L	Mean (SE) Hcy concentration, μmol/L, at incubation time of 0 h	3 h	6 h	24 h	72 h	P[a]	ω
EDTA	20–25	≤10	6.3 (0.2)	8.0 (0.3)[b]	10.0 (0.3)[b]	18.9 (0.6)[b]	32.0 (1.0)[b]	<0.0001[c]	1.6
		>10	17.6 (1.4)	19.4 (1.5)	21.1 (1.3)	28.6 (1.4)[b]	42.0 (2.0)[b]	<0.0001[c]	3.8
DS30 Hcy blood tubes	20–25	≤10	6.2 (0.3)	6.2 (0.3)	6.2 (0.2)	7.6 (0.3)[b]	14.9 (0.7)[b]	<0.0001[c]	1.1
		>10	17.3 (1.5)	17.1 (1.4)	17.3 (1.5)	17.9 (1.2)	24.2 (1.6)[b]	<0.0001[c]	2.8
EDTA	2–8	≤10	6.4 (0.2)	6.6 (0.3)	6.9 (0.3)[b]	8.0 (0.3)[b]	9.9 (0.3)[b]	<0.0001[c]	0.3
		>10	17.5 (1.5)	18.1 (1.5)	18.7 (1.4)[b]	19.7 (1.5)[b]	21.6 (1.4)[b]	<0.0001[c]	0.9
DS30 Hcy blood tubes	2–8	≤10	6.2 (0.2)	6.1 (0.2)	6.1 (0.3)	6.1 (0.3)	6.1 (0.2)	0.3682[d]	
		>10	17.2 (1.5)	17.4 (1.4)	17.0 (1.5)	17.1 (1.5)	16.8 (1.4)	0.3410[d]	

[a] *P* values calculated using one-way within-subject ANOVA (repeated measures).
[b] Significant differences from 0 h by Tukey's ω-procedure ($\alpha = 0.05$).
[c] Highly significant.
[d] Not significant.

ranges. However, as observed with acid citrate, stability was temperature-dependent *(32)*. In pilot studies at 20–25 °C, plasma Hcy increased from a mean of 8.5 μmol/L to 11.5 μmol/L over 72 h, an increase of 35%, which conflicted with the 10% increase previously reported *(31)*. Even with a 3DA concentration of 200 μmol/L, Hcy production could not be prevented (D.M. Hill and A.C. Kenney, unpublished results). We therefore considered a combination of SAHH inhibition by use of 3DA and a slowing of Hcy precursor production by chilling to 2–8 °C. Controls were used to ensure that pilot study results were confirmed and to verify that chilling alone was not sufficient for sample stabilization.

Because volunteers were recruited to observe the effects of Hcy stabilization at a range of baseline Hcy values, ANOVA was performed within subjects. Only samples that were collected in DS30 Hcy blood collection tubes and stored refrigerated (2–8 °C) showed stability over 72 h.

The mean plasma Hcy for each storage condition over time is shown in Fig. 1. Under ambient conditions (20–25 °C), the rapid increase in Hcy in samples collected into EDTA alone is clearly visible. Even at 72 h, Hcy production is evident. Therefore, if whole blood is left at room temperature for only a few hours without a preservative to stabilize Hcy, false-positive results may be reported. Chilling samples or storage at ambient temperature in the presence of a SAHH inhibitor (3DA) may both stabilize Hcy to some degree. In fact, 3DA at 20–25 °C stabilizes samples for 6 or even 24 h, depending on the initial Hcy concentration. This offers advantages for samples collected near the site of processing, providing some relief in the requirement to have ice on hand and to deliver the sample quickly to the laboratory. However, if there is a longer delay in transport, refrigerated storage in DS30 Hcy blood collection tubes can offer prolonged stability for 72 h and possibly up to 1 week.

Sample cooling may slow the processes involved in Hcy production. This theory appears to be supported by the data shown in Fig. 1, where a slow but steady increase in plasma Hcy occurs over 72 h in EDTA samples stored at 2–8 °C. In contrast, 3DA inhibits Hcy production in the early period under ambient conditions. However, the block is at the final stage in Hcy production. As the precursors to Hcy accumulate, SAHH inhibition by 3DA is finally overcome. A combination approach appears to be more effective, as chilling will prevent the build up of *S*-adenosylhomocysteine.

Despite the wide range of initial Hcy values observed, the absolute increase in Hcy in unpreserved samples showed very little difference after 72 h of storage. Consequentially, we observed significant differences in the percentage change over time that showed an inverse relationship to baseline Hcy. Similar phenomena have been noted previously over 24 h by Fiskerstrand et al. *(19)* and most recently over 4 h by Duarte et al. *(37)*. It follows that the thiol pool or the rate of Hcy production must be very similar within the collected samples. Andersson et al. *(16)* suggested that Hcy in whole-blood samples may be produced from a preformed pool of *S*-adenosylmethionine. If this is the case, it appears that the concentration of this pool is very similar within blood samples, is independent of Hcy concentration, and therefore, is independent of the efficiency of remethylation in vivo.

We observed significant differences in the degree of error in measurements when samples were stored for prolonged periods without a preservative, according to baseline Hcy concentrations (Table 1). Investigators and clinicians must be aware of this effect when claims are made about stability.

This trial was designed to investigate stability in as wide a range of starting Hcy concentrations as was possible to obtain from an unscreened group, such that stability could be compared in a healthy population vs a group that could be defined as having increased Hcy. When the sample population was divided into two groups, those with higher initial Hcy concentrations were stable over longer periods, e.g., up to 24 h compared with up to 6 h, in the Drew DS30 Hcy collection tubes stored at 20–25 °C. Moreover, any concern over whether significant differences in Hcy had been masked in the samples that were stored at 2–8 °C in DS30 Hcy blood tubes because of the presence of individuals with high baseline Hcy concentrations was unfounded.

We report the first commercially available blood collection tube to allow stability of plasma Hcy in whole blood over the course of 3 days without the need for centrifugation. Although we realize that the requirement for refrigeration is not ideal, several samples may be collected and stored in doctors' offices or in mobile clinical trial facilities before they have to be transported to a centralized laboratory for processing, thereby facilitating wider population screening. If refrigerated conditions are not readily available, the use of DS30 Hcy blood collection tubes may offer sample stability over 6 h, within a hospital environment, which may be particularly useful in cardiovascular clinics. These tubes can be used with a range of methods, providing that the method does not rely on the action of SAHH; consequently, tubes containing 3DA should not be used in some of the immunochemical methods currently available *(38, 39)*.

Ultimately, our aim is to produce a method of whole-blood collection that allows transport at a range of ambient temperatures. In the interim, we have developed a method that allows samples to be collected and stored for up to 72 h before laboratory intervention. We have successfully used this procedure to offer a mail-in Hcy test where local testing was not available.

We thank all those who helped in the course of this study, including Dr. Paul Haggart, who assisted in collection of blood samples at Birmingham Heartlands Hospital; F. Giltrap and R. Hadley, who assisted in sample handling

and analysis; and R.E. Ashby (Cranfield University, UK), who helped with the statistical interpretation of the data collected. This study was funded entirely by Drew Scientific Ltd.

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supplementation with 0·5–5·0 mg folic acid lowered blood homocysteine concentrations by 25%. From our data, the shift in homocysteine dependency from folate to vitamin B12 with folic acid intervention becomes apparent only as the doses of folic-acid supplementation increase.

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Sir—E P Quinlivan and colleagues (Jan 19, p 227)[1] report a relation between total plasma homocysteine (tHcy) and vitamin B12 in folate-supplemented individuals. There are two B12 carrier proteins in serum. Haptocorrin binds most serum B12 but does not deliver the vitamin to metabolically active cells; this function is done by transcobalamin. Only 5–20% of serum B12 is bound to transcobalamin as holotranscobalamin. Current laboratory assays measure total serum B12 and are relatively poor indicators of the ability of serum to deliver the vitamin to tissues.[2]

We measured tHcy, folate, total serum B12, and holotranscobalamin concentrations in 111 elderly individuals (51 patients with dementia and 60 controls) with a mean age of 77 years (SD 9·5) recruited to a continuing study of vitamin B12 status and cognitive function. tHcy was measured by an automated high-performance liquid chromatography system, and folate and total serum B12 by an automated chemiluminescence analyser.

We used generalised linear models to investigate the relation between known tHcy determinants (age, sex, creatinine, smoking history, and serum folate) and B12 status, assessed by total serum B12 or holotranscobalamin concentrations. Diagnosis was included as an additional independent variable. Individuals receiving B vitamin supplements were excluded.

There was a significant and independent relation between tHcy and B12 status assessed by holo-transcobalamin concentrations, but not by total serum B12 (table). Current laboratory assays lack sensitivity to measure biologically available B12. We suggest this might be an additional explanation as to why the effects of B12 on tHcy concentrations are frequently masked by folate status.

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Gene-expression profiling and identification of patients at high risk of breast cancer

Sir—André Ahr and colleagues (Jan 12, p 131)[1] report on the use of cDNA microarray and cluster analysis of gene-expression patterns to prospectively identify a subset of patients with primary breast cancer at high risk of subsequent disease recurrence.

In their initial report,[2] tumours were split into four main groups, namely I–IV, on the basis of cluster analysis.[3] Group III was further separated into class A and class B. Class A was described as containing transcriptionally related samples that were further separated into two subpopulations designated A1 and A2 based on the differences in transcription of several genes. A2 has an exceptionally low expression of oestrogen receptor α and progesterone receptor, *BAD* gene, and insulin-like growth factor binding protein 2, compared with A1.

In the first report, Ahr and colleagues analysed and presented the data on A1 alone, and presented the data on A2 along with the tumours in the other groups, since the A1 subpopulation was characterised by a disproportionately high frequency of lymph-node-positive tumours and distant metastasis at the time of diagnosis compared with the other tumours.

However in the follow-up analysis of these patients,[1] the investigators no longer refer to the molecular subdivision in class A and the differences previously noted between A1 and A2 for lymph-node status and distant metastasis; they simply refer to patients as being class A and non-class A.

Given that the molecular differences between A1 and A2 tumours are small, if the clinical behaviour of these two subpopulations continues to differ it could provide important clues as to the gene or genes that may best predict the risk of disease recurrence and metastasis in breast cancer. Therefore, the presentation of data on the subpopulations A1 and A2 in the follow-up analysis is important.

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Variable	GLM including total serum B12		GLM including holotranscobalamin	
	Estimate (95% CI)	p	Estimate (95% CI)	p
Dementia	−0·86 (−1·48 to −0·23)	0·007	−0·57 (−1·2 to 0·02)	0·06
Sex	−0·10 (−0·82 to 0·62)	0·78	−0·20 (−0·93 to 0·52)	0·57
Smoker	−0·26 (−1·12 to 0·58)	0·54	−0·23 (−1·07 to 0·61)	0·59
Age	−0·01 (−0·09 to 0·08)	0·87	0·01 (−0·07 to 0·08)	0·89
Creatinine (μmol/L)	0·09 (0·07 to 0·12)	<0·0001	0·08 (0·06 to 0·10)	<0·0001
Folate (μg/L)	−0·23 (−0·36 to −0·10)	0·001	−0·15 (−0·28 to −0·03)	0·02
B12 (ng/L)	−0·003 (−0·01 to 0·001)	0·12	..	..
Holotranscobalamin (pmol/L)	..	..	−0·04 (−0·06 to −0·02)	0·001

Generalised linear models (GLMs) of potential tHcy determinants showing estimates and strengths of their respective effects

Sir—Emerging high-throughput molecular analyses have the potential to reveal previously unknown prognostic subclasses of tumours with more homogeneous clinical outcome. By use of DNA array technology, André Ahr and colleagues[1] identify a class of breast-cancer patients with a high risk of metastasis.

These results are clinically important since progress in breast cancer therapy is expected to come as much from the

Response:

Traumatic lumbar puncture at diagnosis of childhood acute lymphoblastic leukemia

We agree with the recommendations of Kebelmann-Betzing et al that initial lumbar puncture should be performed by an experienced clinician and followed immediately by intrathecal therapy. As also stated in our article,[1] we now routinely perform this procedure with patients under short-acting general anesthesia. When the diagnosis of leukemia is uncertain because of the lack of circulating leukemic cells, we postpone lumbar puncture and intrathecal therapy until the diagnosis is established by bone marrow examination.

It is difficult to compare the frequency of traumatic lumbar puncture in the series of Kebelmann-Betzing et al with that of ours because the status of their patients was determined retrospectively on the basis of "preserved" cerebrospinal fluid; in contrast, we used fresh samples of cerebrospinal fluid. The integrity of the erythrocytes and the morphology of the leukocytes are expected to be altered in preserved samples. Since we first implemented steps to reduce this iatrogenic complication, we have substantially reduced the frequency of traumatic lumbar punctures with blast cells from 11% to 5% and that of traumatic lumbar punctures without blast cells from 10% to 7%. It should be noted that we stringently define traumatic lumbar puncture as at least 10 erythrocytes per microliter.

As shown by Total Therapy Study XIII,[2] early intensive intrathecal and systemic therapy is now more successful in treating and preventing central nervous system (CNS) leukemia, even in patients whose leukemic blast cells are iatrogenically introduced into the cerebrospinal fluid by traumatic lumbar puncture. Therefore, cranial irradiation is seldom necessary in contemporary treatment programs. But others have reported neuropsychologic deficits in children who received CNS-directed therapy consisting solely of approximately 20 intrathecal treatments of methotrexate, hydrocortisone, and cytarabine over 3 years.[3] The challenge now is to optimize intrathecal and systemic therapy to maximize efficacy and minimize toxicity. Developing appropriate measures to avoid traumatic lumbar puncture and hence the need of extra intrathecal therapy is but one step toward this goal.

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To the editor:

Transcobalamin polymorphism and homocysteine

Namour et al[1] suggest that codon 259 of the transcobalamin *(TC)* gene is a major determinant of TC polymorphism in Caucasians and that heterozygous individuals have higher plasma homocysteine. Total serum homocysteine (tHcy) is increased in patients with Alzheimer disease (AD) and may be a risk factor for cognitive decline.[2-5] Furthermore, TC saturation declines with age in AD.[6]

We therefore determined nonfasting tHcy, TC phenotype, and codon 259 polymorphism in 144 (93 female, 51 male) healthy elderly volunteers (73) and dementia patients (71) recruited to a study of tHcy and cognition (the COBALZ II project) after ethical committee approval. Phenotypes were identified by polyacrylamide gel electrophoresis (PAGE) of neuraminidase-treated radiolabeled serum samples (Figure 1) and genotypes by solid-phase minisequencing (Figure 2).[7,8] Nonfasting tHcy was assayed with the Drew Scientific DS30 Hcy Analyser (Barrow in Furness, England); Vitamin B_{12} and folate were assayed with the Bayer ACS 180 Automated Chemiluminescence System (Newbury, England).

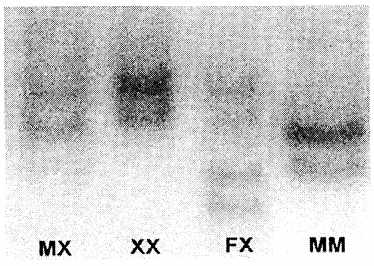

Figure 1. TC phenotypes revealed by PAGE. Serum (25 μL) saturated with 10 μL of radioactive B_{12} (^{57}Co-B_{12}; 200mCi/mg, 1 μCi/mL [7.4 GBq/mg, 37 kBq/mL]; Amersham Pharmacia Biotech "CT2" diluted 1:5 N-saline) was incubated with 25 μL of neuraminidase (0.2 IU/mL) in 25 μL of buffer (0.05 M sodium acetate, 0.1% $CaCl_2$, and 0.95% NaCl, pH 5.5) at 37°C for 30 minutes. Twenty-five μL was replaced with buffer (65% [wt/vol] sucrose) and bromophenol blue as an albumin- and front-marker. PAGE was performed in a 10% polyacrylamide gel (1.5 mm × 7.0 cm × 12.5 cm) with 3.5% concentrating top gel (1.5 cm high) and Tris-glycine electrode buffer at pH 8.3 and electrophoresed at 250 V.

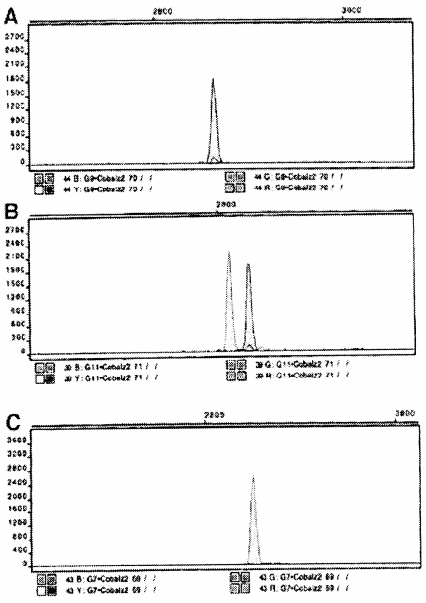

Figure 2. TC genotypes identified by solid-phase minisequencing. (A) TCII-259C homozygotes (PP); (B) TCII-259C/G heterozygotes (PR); (C) TCII-259G homozygotes (RR). Genomic DNA was amplified by PCR using the sense primer biotin-5′-GTGCGAGAGGAGATCTTGAA-3′ and the antisense primer 5′-GTAGGTCT-TGTGGTTCAGAA-3′. Biotinylated products were bound to streptavidine-coated microtiter plates (Wallac, Turku, Finland) and denatured with NaOH. Thermo Sequenase DNA polymerase (Thermo Sequenase Dye Terminator Kit, Amersham, Buckinghamshire, United Kingdom), fluorescent ddNTP, and the antisense detection primer 5′-CTGTTCCCAGTTCTGCCCCA-3′ were added to generate an allele-specific pattern. After the minisequence reaction, the plates were washed and the extended sequence primers were released by incubation with formamide. These were separated and analyzed by capillary electrophoresis and laser-induced fluorescence in an ABI 310 genetic analyzer (Perkin-Elmer, Cambridge, England).

Codon 259 polymorphism and phenotype were highly concordant (Table 1). PAGE reveals a doublet pattern in homozygotes, triplets due to overlapping bands in heterozygotes, and a quadruplet pattern in FX phenotypes (Figure 1). Alternative cleavage of the signal peptide might generate doublets rather than definitive isotypes.[9] Weaker radiolabeling of TC-X in heterozygotes resulted in misclassification of some phenotypes; it perhaps reflects differential transcription.[1] Codon 259 polymorphism and phenotypic distributions paralleled previous studies (PP = 32.6%, PR = 49.3%, and RR = 18.1%), (MM = 34%, MX = 43.7%, XX = 21.5%, and FX = 0.07%).[1,7]

Table 2. Results of a generalized linear model of known tHcy determinants together with diagnosis as an additional independent variable

	Log likelihood	χ^2	*P*
Sex	−317	0.0007	.98
Age	−317	0.05	.83
TC isotype	−317	1.41	.49
B_{12}	−318	2.79	.09
Diagnosis	−319	4.77	.09
Folate	−324	14.89	< .001
Creatinine	−346	58.19	< .0001

Thirty individuals receiving B vitamins or homocysteine-disruptive medication were excluded from further analysis. Vitamin B_{12}, folate, and tHcy did not differ among isotypes (Table 1). The apparent trend for increasing tHcy in the presence of an X variant or codon 259R allele was not significant using the Jonckheere-Terpstra test (asymptotic *P* value for genotype was .16 one-sided and .33 two-sided; *P* value for phenotypes was .08 one-sided and .17 two-sided) or using ANOVA with a Bonferroni correction. Table 2 shows the results of a generalized linear model of known tHcy determinants, together with diagnosis as an additional independent variable.

We therefore confirm that codon 259 is a major determinant of TC polymorphism in populations where M and X phenotypes predominate. The genetic basis underlying F and S phenotypes remains elusive. TC isotype is not a significant risk factor for the development of hyperhomocysteinemia in elderly Caucasians, especially when considered in relation to established risk factors such as creatinine and folate.

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Table 1. Distribution of TC phenotypes and genotypes with their respective median and interquartile ranges for serum B_{12}, folate, and tHcy

	259 Polymorphism			Serum values		
	PP (n = 47)	PR (n = 71)	RR (n = 26)	B_{12} (ng/L)	Folate (μg/L)	tHcy (μM)
Phenotype						
MM (n = 49)	42	7	0	326 (276-354)	10.7 (6.6-14.2)	9.9 (7.8-13.2)
MX (n = 63)	4	58	1	320 (261-389)	8.9 (6.9-12)	11.0 (8.7-13.3)
XX (n = 31)	1	5	25	353 (303-468)	10.4 (9.2-15.1)	11.8 (9.1-16.2)
FX (n = 1)	0	1	0	NA	NA	NA
Serum values						
B_{12} (ng/L)	317 (276-348)	324 (268-391)	416 (311-468)	NA	NA	NA
Folate (μg/L)	9.8 (6.4-14.5)	9.1 (7-12)	10.4 (9.2-18.1)	NA	NA	NA
tHcy (μM)	10.2 (8.2-13.4)	11.0 (8.5-13.2)	11.8 (9.1-16.2)	NA	NA	NA

Interquartile ranges, where applicable, are given in parentheses.

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Response:

Transcobalamin polymorphism, homocysteine, and aging

The DNA sequencing of the transcobalamin *(TC)* gene expressed in various cells identified 5 amino acid substitutions at codons 198, 219, 234, 259, and 376.[1,2] Codon 234 and 259 variability corresponded to the replacement of proline by arginine.[2] By analyzing the DNA and sera from 159 healthy Caucasians from 3 regions of northeastern France (Lorraine, Franche-Comté, and Bourgogne), we found that the codon 259 substitution was a single nucleotide polymorphism with a biallelic distribution, while no substitution was found in the other codons.[3] There is therefore no doubt that codon 259 is a major genetic determinant of the TC polymorphism.

We compared the TC genotype to the isoelectric point of TC isoforms and found a concordance between the amino acid substitution and the isoelectric point. Urea was used to avoid the formation of aggregates in our isoelectric focusing (IEF) analysis of TC. McCaddon et al confirm our findings on the TC codon 259 polymorphism's biallelic distribution. They compared it to a SDS-PAGE phenotyping of TC, which identifies 4 phenotypes, instead of the 2 isoforms observed in IEF. Apparently, no urea was used in their electrophoresis analysis. Urea avoids the formation of TC aggregates and induces denaturation of TC conformation, as shown by the comparison of IEF profiles of TC from HT-29 cells with and without urea.[3,4] In our opinion, the role of alternative cleavage of the signal peptide in generating TC isoforms needs therefore to be confirmed, as only 2 isoforms, not 4, were found in IEF with urea. The TC codon 259 polymorphism affects apo (unsaturated) TC isoforms concentration as shown by IEF analysis of heterozygous HT-29 cells and by sera apo TC assay from homozygous and heterozygous subjects.[3] This is why we thought that this polymorphism could affect the vitamin B_{12} cellular availability and, consequently, the homocysteine concentration.

Surprisingly, the relatively lower concentration of apo TC concentration observed in the Arg/Arg homozygous subjects was not related to an increase of homocysteinemia. Indeed, a significantly higher homocysteine concentration was found in heterozygous subjects than in subjects with either homozygous genotype, while the highest apo TC concentration was observed in subjects with Pro/Pro genotypes. An explanation could be that the binding of TC to the dimeric TC receptor described by Bose et al[5] is associated with a dimerization of TC and that the heterodimer of Pro/Arg isoforms has a lower affinity than Pro/Pro or Arg/Arg homodimers. This hypothesis is currently under investigation. McCaddon et al found no relation between homocysteine concentration and TC codon 259 polymorphism. They performed their study in elderly patients, but no description of the age distribution is given.

Since the publication of our paper, we performed *TC* genotyping and homocysteine analysis in a population of 76 healthy subjects from a preventive medicine center in Lorraine, including 40 elderly subjects. The data summarized in Table 1 confirm the influence of the TC codon 259 polymorphism on homocysteine concentration. But the difference in homocysteine concentration between heterozygous and homozygous subjects was not significant in our elderly subgroup. The influence of age as a determinant of homocysteine concentration may explain the results obtained by McCaddon et al in elderly subjects. Our and McCaddon et al's data suggest therefore that investigations of *TC* genotype in diseases associated with mild hyperhomocysteinemia should take into consideration the age distribution. With regard to the literature, the homocysteinemia increase in the elderly is not fully understood. One should be careful not to transpose to the elderly the role of genetic and nutritional homocysteine determinants characterized in the general population.

Table 1. Plasma homocysteine concentration according to transcobalamin codon 259 genotype in elderly and younger subjects

		Homocysteine		
	Age	Codon 259 TC homozygous	Codon 259 TC heterozygous	*P* (Mann-Whitney)
Entire group, n = 76	61 (33-80)	8.7 (5.5-16.1)	11.0 (5.9-21.6)	.0027
Elderly subgroup, n = 40	69 (60-80)	9.3 (6.2-16.1)	11.0 (5.9-21.6)	.1219
Younger subgroup, n = 36	53 (33-59)	8.4 (5.5-11.5)	10.7 (6.0-18.1)	.0063

Median and extreme values are given in μM. Ranges, where applicable, are in parentheses.

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Alzheimer's Disease and Total Plasma Aminothiols

Andrew McCaddon, Peter Hudson, Diane Hill, Joan Barber, Alwyn Lloyd, Gareth Davies, and Björn Regland

Background: *Plasma homocysteine is elevated in Alzheimer's disease, but little is known regarding levels of related aminothiols in the disease. We therefore determined total plasma homocysteine, cysteine, and glutathione levels in patients and control subjects and investigated their relationship with cognitive scores.*

Methods: *We performed a prospective, case-controlled survey based in two UK Psychogeriatric Assessment Centres. Fifty patients with features compatible with DSM-IV criteria for primary degenerative dementia of Alzheimer type were recruited together with 57 cognitively intact age- and gender-matched control subjects. Mini-Mental State and Alzheimer's Disease Assessment Scale–Cognitive Subsection (ADAS-Cog) scores were determined for patients and control subjects. Aminothiols were assayed with an automated high-performance liquid chromatography (HPLC) system.*

Results: *Patients had significantly elevated total plasma homocysteine ($p < .001$) and cysteine ($p < .01$), but there were no group differences for total plasma glutathione. Glutathione was, however, a highly significant and independent predictor of cognitive scores in patients ($p = .002$); lower plasma levels were associated with more severe cognitive impairment.*

Conclusions: *Total plasma homocysteine and cysteine are elevated in Alzheimer's disease, suggesting intact transsulphuration but defective remethylation of homocysteine in the disease. Total plasma glutathione levels in patients correlate with cognitive scores. Taken together, these observations perhaps reflect the differential effects of Alzheimer's disease–related oxidative stress on the two key pathways of homocysteine metabolism.* Biol Psychiatry 2003;53:254–260 © *2003 Society of Biological Psychiatry*

Key Words: Alzheimer's disease, homocysteine, cysteine, glutathione, folate, vitamin B_{12}

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Introduction

Homocysteine is a sulphydryl-containing amino acid, or "aminothiol," derived from the breakdown of methionine. It is a sensitive, though nonspecific, marker of vitamin B_{12} and folate status. Its plasma levels also increase in vitamin B_6 deficiency, in various defects of enzymes involved in its metabolism, and with aging, impaired renal function, and male gender (Hankey and Eikelboom 1999).

Homocysteine suffers two major metabolic fates: remethylation to methionine, catalyzed by methionine synthase, or irreversible transsulphuration to cystathionine, catalyzed by cystathionine β-synthase (see Figure 1). Methionine, in the activated form of S-adenosyl methionine, is the methyl-donor for essential cellular methylation reactions. Cystathionine is subsequently converted to cysteine, a precursor of glutathione and a source of inorganic sulphate, by cystathionase (Finkelstein and Martin 2000).

Evidence for a relationship between homocysteine and cognition has accumulated since the seminal observation of increased serum levels in patients with primary degenerative dementia (Regland et al 1990). Bell et al (1992) found a significant negative relationship between homocysteine and cognitive function in a small group of depressed elderly subjects. An association between homocysteine and spatial copying skills was observed in healthy elderly men in the Boston Veterans Affairs Normative Aging Study (Riggs et al 1996).

We found significantly elevated homocysteine in serum of patients with clinically diagnosed Alzheimer's disease (AD) and described its relationship with cognitive scores of the Cambridge Examination for Mental Disorders of the Elderly (McCaddon et al 1998). A similar relationship was reported in 186 patients with AD, vascular dementia, and minor cognitive impairment and subjective symptoms (Lehmann et al 1999).

Clarke et al (1998) also observed elevated plasma homocysteine in histopathologically confirmed AD and found that, after 3 years, patients with the highest initial levels had greater radiological evidence of disease progression.

An increased plasma homocysteine level is a strong independent risk factor for the development of dementia

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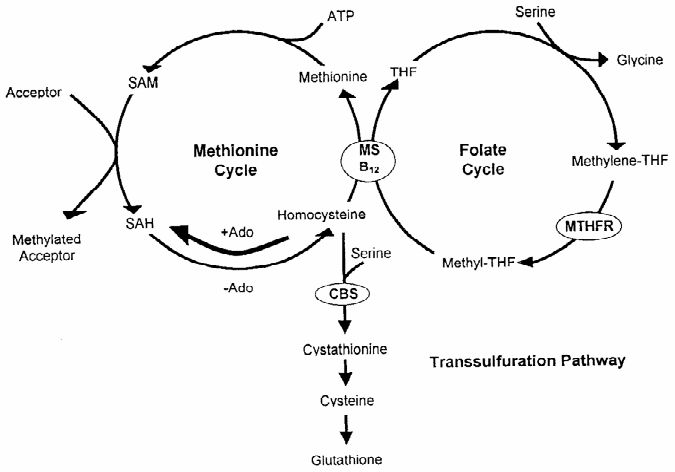

Figure 1. Homocysteine metabolism. In the methionine cycle, B_{12}-dependent methionine synthase (MS) catalyses the transfer of a methyl group from methyl-tetrahydrofolate (methyl-THF) to homocysteine, producing THF and methionine. Activation of methionine by adenosine triphosphate (ATP) generates S-adenosylmethionine (SAM), the universal methyl-group donor. Methylation of an acceptor by SAM yields S-adenosylhomocysteine (SAH). The regeneration of homocysteine by SAH hydrolase completes this cycle. This reaction is driven in the forward direction by the efficient removal of homocysteine and adenosine (Ado). In the folate cycle, free THF accepts single carbon units from serine to form methylene-THF and glycine. Methyl-THF is formed by the reduction of methylene-THF by methylene tetrahydrofolate reductase (MTHFR). In the transsulphuration pathway, cystathionine β-synthase (CBS) catalyzes the synthesis of cystathionine from homocysteine. The pathway continues with the synthesis of cysteine by cystathionase. Cysteine is a rate-limiting precursor for glutathione synthesis.

and AD (Seshadri et al 2002). Furthermore, we recently reported that total homocysteine is an independent predictor of cognitive decline in healthy elderly subjects (McCaddon et al 2001). The mechanisms by which it exerts this effect are unknown, but possibilities include a direct toxicity, either on glutamate neurotransmitter receptors or cerebrovascular endothelium, and an indirect inhibitory effect on various transmethylation reactions via its conversion to S-adenosyl homocysteine. Elevated homocysteine might also contribute to amyloid accumulation, including the formation of senile plaques and amyloid angiopathy, via its effect on Herp—an endoplasmic reticulum membrane protein that interacts with presenilins (Sai et al 2002).

Little is known regarding the plasma levels of related aminothiols in AD. Evaluating plasma levels of homocysteine-related metabolites might assist in elucidating the biochemical locus of hyperhomocysteinemia in AD. We therefore determined total plasma homocysteine, cysteine, and glutathione in elderly patients with AD and healthy age- and gender-matched control subjects and investigated their relationship with cognitive scores.

Methods and Materials

Patients

Patients were recruited from Psychogeriatric Assessment Services (Wrexham Maelor and Ayrshire Central Hospitals) with features compatible with DSM-IV criteria for primary degenerative dementia of Alzheimer type (American Psychiatric Association 1994). Control subjects were healthy, cognitively intact age- and gender-matched elderly volunteers from a group general practice in a comparable semirural area of predominantly lower socioeconomic class. Subjects using homocysteine-disruptive medication, including vitamin B_{12}, folic acid, and hormone replacement therapy were excluded. Mini Mental State Examination (MMSE) and ADAS-Cog scores were recorded for patients and control subjects (Folstein et al 1975; Rosen et al 1984). The latter instrument addresses several cognitive domains and is well validated. It is sensitive to cognitive changes over time and so provides a useful baseline for future studies.

Prior education was determined ("none, primary, intermediate, secondary, and further"), because this relates to cognitive decline in normal aging (Jacqmin-Gadda et al 1997). Smoking status ("current, ex, and never"), previous history of vascular disease, including myocardial infarction, cerebral and peripheral vascular disease, and hypertensive history were also documented, these

being associated with modest elevation of homocysteine (Nygard et al 1995). For the purpose of statistical analysis, prior education and smoking were treated as ordinal variables and hypertensive and past medical history of vascular disease as categorical variables. Blood pressure was also determined at recruitment, as were height and weight for body mass index (BMI). Ethical approval was granted by the North Wales Health Authority Research Ethics Committee, and informed consent was obtained.

Laboratory Methods

Non-fasting blood samples were taken for full blood count, red cell folate (RCF), creatinine, B_{12}, folate, and aminothiol assays. Blood for the aminothiol assays was collected into tubes containing potassium-ethylenediamine tetraacetic acid (Safety Monovette, Sarstedt Ltd, Leicester, UK). The tube was not placed in ice, but separation was performed at 15°C within 1 hour of venepuncture and plasma removed and stored frozen at −20°C until aminothiol analysis (Andersson et al 1992).

An automated cell counter was used to measure hemoglobin (Hb) concentration and mean corpuscular volume (MCV) (Coulter Gen-S, Beckman Coulter, High Wycombe, Bucks, UK). An automated biochemistry analyzer was used to analyse creatinine (Synchron LX-20 analyser, Beckman Coulter, High Wycombe, Bucks, UK), and an automated chemiluminescence analyzer was used to measure folate, vitamin B_{12}, and red cell folate (RCF) (ACS:180 SE, Bayer plc, Newbury, Berks UK) using the manufacturers' recommended protocols. Aminothiols were assayed with an automated high-performance liquid chromatography (HPLC) system (DS30 Hey Analyser, Drew Scientific Group plc, Barrow-in-Furness, Cumbria, UK). For each aminothiol, all forms including protein-bound, non–protein-bound, free forms both oxidized (dimer and mixed disulphides) and reduced were measured. Aminothiol values therefore always refer to "total" plasma levels.

Statistical Methods

Comparisons between groups (Wilcoxon–Mann–Whitney test), 95% confidence intervals (95% CI) for differences between medians (Hodges–Lehmann estimates), and measures of association (Spearman rank-order correlation coefficient) were performed using exact, nonparametric methods (StatXact 4 for Windows, Cytel Software Corporation, Cambridge, MA).

Conventional techniques were used for regression analysis and generalized linear modelling (Statistica for Windows v5.5, StatSoft, Inc., Tulsa, OK). Ridge regression, an extension to conventional regression analysis, was used to correct for possible correlations among the independent variables, and a robust regression method (least absolute deviation regression) was used to investigate possible outlier effects (Pynnönen and Salmi 1994). For the purpose of generalized linear modelling, continuous variables were specified as the dependent variables, a linear link function was used and mixtures of categorical, ordinal, and continuous variables specified as the independent variables. The significance of the model parameters were assessed using a χ^2 test. Median results are presented with interquartile ranges, and simple regression coefficients are presented with their 95% CI.

Results

There were 50 AD patients (17 male and 33 female) and 57 control subjects (23 male and 34 female). The median age of both groups was 79 years (75–83 for AD and 72–85 for control subjects). Alzheimer's disease patients had a median duration of disease of 24 (13–36) months and a median age of onset of 77 (74–82) years.

There was no difference in BMI, prior education, smoking status, or past medical history of vascular disease between patients and control subjects. Patients had lower median systolic blood pressure than control subjects (patients: 130 [120–140] mm Hg; control subjects: 145 [130–160] mm Hg; 95% CI for difference: 0 to 20, p = .01) and lower median diastolic blood pressure (patients: 80 [70–82] mm Hg; control subjects: 85 (79–95) mm Hg, 95% CI for difference: 5 to 10, p = .0005).

Plasma samples were stored for a median of 12 months prior to aminothiol assay (range 1–23 months). Regression analysis was used to assess the effect of storage time on these assays. Within the aggregated data (patients plus control subjects), storage time had no effect upon homocysteine or glutathione levels. There was a small decrease in cysteine levels with storage time: cysteine = 142.3 − 2.33 × storage time in months (p = .0002, 95% CI: −3.52 to −1.14). The addition of diagnosis as a categorical independent variable demonstrated no difference in the effect of storage between patients and control subjects.

Alzheimer's disease patients had significantly decreased serum folate and significantly increased plasma homocysteine and cysteine. Hemoglobin, platelets, MCV, creatinine, B_{12}, RCF, and glutathione did not differ between groups (Table 1).

Among the patients, a decrease in MMSE score was associated with a decrease in plasma glutathione: MMSE = 8.27 + 3.56 × glutathione (p = .002, 95% CI: 0.29 to 6.82). Similarly, an increase in ADAS-Cog score was associated with a decrease in plasma glutathione: ADAS-Cog = 54.9 − 8.39 × glutathione (p = .002, 95% CI: −13.6 to −3.22) (Figure 2). To protect against the possibility that these relationships were artefacts caused by a correlation between plasma glutathione and homocysteine, itself known to affect cognitive function (Lehmann et al 1999; McCaddon et al 1998), a ridge regression analysis was performed, with plasma homocysteine and cysteine as additional independent variables. This analysis confirmed that plasma glutathione was the only aminothiol to be an independent statistically significant predictor of MMSE and ADAS-Cog scores. In addition, to exclude the possibility that the relationship was an effect of several "outliers" (see Figure 2), the data was analyzed using a robust regression method: MMSE = 7.20 + 4.00 × glutathione (p = .029, 95% CI: 0.48 to 7.52); ADAS-Cog

Table 1. Summary of Results

	Control Subjects	Patients	*p* Value (95% CI of Difference between Medians)
BMI	24.1 (22.1–28.4)	24.6 (22.5–27.4)	
MMSE	28 (27–29)	18 (14–21)	*p* <.0001 (8–12)
ADAS-Cog	9 (7–12)	32 (24–39)	*p* <.0001 (19–26)
Hb, g/dL	13.4 (12.5–14.4)	12.9 (12.0–14.2)	
Platelets, × 10^9/L	218 (184–277)	216 (182–273)	
MCV, fL	90.4 (87.8–94.0)	90.4 (88.4–93.6)	
Creatinine, μmol/L	84 (72–102)	87 (72–115)	
B_{12}, ng/L	324 (276–445)	334 (268–421)	
Folate, μg/L	10.5 (8.1–14.5)	9.0 (6.3–11.6)	*p* = .018 (.4–3.8)
RCF, μg/L	330 (272–425)	282 (243–399)	
Homocysteine, (μmol/L)	9.6 (8.2–12.5)	12.6 (9.6–15.8)	*p* = .0006 (1.2–4.3)
Cysteine, (μmol/L)	107.2 (94.7–129.4)	120.1 (102.0–142.4)	*p* = .007 (3.6–23.7)
Glutathione, (μmol/L)	2.5 (2.2–2.9)	2.7 (2.2–3.3)	

CI, confidence interval; BMI, body mass index; MMSE, Mini Mental State Examination; ADAS-Cog, Alzheimer's Disease Assessment Scale–Cognitive Subsection; Hb, hemoglobin; MCV, mean corpuscular volume; RCF, red cell folate.

= 49.27 − 7.22 × glutathione (p = .0104, 95% CI: −12.55 to −1.89).

There was no correlation between disease duration and levels of total plasma aminothiols. Among the control subjects, there was no relationship between MMSE and aminothiols. There was an increase in ADAS-Cog with decreasing plasma glutathione: ADAS-Cog = 29.6 − 0.61 × glutathione (p = .04, 95% CI: −1.21 to −0.002), but this relationship was abolished with the addition of homocysteine and cysteine in a ridge regression model.

Other possible confounding factors that might influence the relationship between plasma glutathione and cognitive score are age, smoking and hypertensive status, and years of education. A generalized linear modelling technique was used to assess the effect of these factors plus plasma glutathione on ADAS-Cog and MMSE scores in patients. The results confirmed that glutathione is an independent predictor of cognitive performance in this study (Table 2).

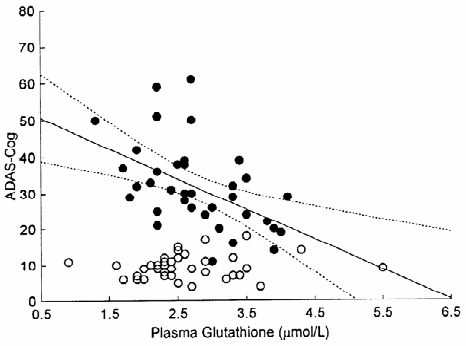

Figure 2. Scatter diagram showing the relationship between the Alzheimer's Disease Assessment Scale–Cognitive Subsection (ADAS-Cog) score and plasma glutathione in patients (filled circles) and control subjects (open circles). The regression line indicates the increase in ADAS-Cog score in patients with decreasing plasma glutathione: ADAS-Cog = 54.9 − 8.39 × plasma glutathione (p = .002, 95% confidence interval for regression parameter: −13.6 to −3.22).

Discussion

Plasma homocysteine was increased and serum folate reduced in AD, confirming earlier reports (Clarke et al 1998; Lehmann et al 1999; McCaddon et al 1998). Plasma cysteine was also increased in AD, but glutathione was unchanged; however, within the AD group plasma total glutathione was a highly significant and independent predictor of cognitive scores; lower levels were associated with greater cognitive impairment.

A limitation of our study is that AD was clinically diagnosed; patients lacked any confirmatory tissue diagnosis. Furthermore, ApoE4 status was not available for the study population, and past history of alcohol use was not obtained.

Body Mass Index did not differ between patients and control subjects. It is unlikely then that group differences for folate and aminothiols arise simply as a consequence of impaired nutritional status in dementia. This is consistent with earlier studies (McCaddon et al 1998; Renvall et al 1989) and suggests that differences in aminothiol metabolism arise by some other mechanism.

All aminothiols were assayed with an automated HPLC system (DS30 Hcy Analyser, Drew Scientific Group). This system is currently commercially available only for homocysteine assays; however, a development version made available by the supplier is capable of measuring other plasma thiols in addition to homocysteine (Ranga-

Table 2. Results of a Generalized Linear Model of Possible Determinants of Cognitive Scores in Patients

Scoring System	Independent Variable	Log-Likelihood	χ^2 (1 df)	p Value
ADAS-Cog	Age	-72.33	.35	.55
	Education	-76.55	8.78	.003
	Smoking	-72.42	.52	.47
	Glutathione	-75.15	5.98	.014
	Hypertension	-76.83	9.34	.002
MMSE	Age	-56.98	.0001	.99
	Education	-58.26	2.56	.11
	Smoking	-57.33	.69	.40
	Glutathione	-60.57	7.18	.007
	Hypertension	-58.59	3.22	.07

ADAS-Cog, Alzheimer's Disease Assessment Scale–Cognitive Subsection; MMSE, Mini Mental State Examination.

nath et al 2001). These novel observations provide a useful initial insight into altered homocysteine metabolism in AD. Values obtained for total plasma glutathione and cysteine are somewhat lower than those cited by Ranganath et al; however, our sample is from an older population, and it is possible that these metabolite levels decline with age. The Drew Scientific assay results compare well with other thorough studies of plasma thiol status (Kleinman and Richie 2000).

Increased plasma cysteine in patients implies intact transsulphuration of homocysteine; aberrant remethylation must therefore account for AD-associated hyperhomocysteinemia. Homocysteine flux through these two pathways is regulated by the inherent kinetic properties of the relevant enzymes, alterations in their tissue distribution, and the differential effects of cellular redox state on methionine synthase and cystathionine β-synthase (Finkelstein 2001). Methionine synthase is vulnerable to oxidation, and maintenance of its activity requires periodic reductive methylation by methionine synthase reductase. In contrast, cystathionine β-synthase is a heme protein, and its activity increases in an oxidative environment (Taoka et al 1998). Increased transsulphuration probably represents a physiological response to oxidative stress, because a major function of this pathway is the synthesis of the intracellular antioxidant glutathione (Mosharov et al 2000).

Glutathione is composed of glutamate, glycine, and cysteine; the latter is a rate-limiting precursor. Glutathione is a nucleophilic scavenger and enzyme-catalyzed antioxidant that allows the detoxification of free radicals and oxygen-reactive species involved in diseases such as atherosclerosis and rheumatoid arthritis; consequently its plasma concentration decreases in such diseases (Uhlig and Wendel 1992).

Although it is impossible to determine cause and effect from a cross-sectional study, the above facts suggest a plausible biological explanation for our findings.

There is now abundant evidence implicating oxidative stress in AD and, regardless of whether it is a primary or secondary event, it is an important neurodegenerative element (Smith et al 2000). Oxidative damage is common in the aging brain, but more severe in AD. Redox-active iron is increased in AD brains; it catalyzes hydroxyl radical formation from hydrogen peroxide (Smith et al 1997). Hydroxyl radicals react readily with membrane lipids, generating lipid peroxides and peroxyl radicals. These kindle a chain reaction of lipid peroxidation that propagates through surrounding membranes like a spreading forest fire. This process of partial combustion has been elegantly described as a "simmering biological fire of oxy-radical-based pathology" (Cohen 1994).

Excess cerebral oxidative stress in AD might progressively deplete body glutathione stores. This perhaps explains the observed association between plasma levels of glutathione and disease severity; our findings presumably reflect physiological changes in sulphur amino acid metabolism in response to AD-related oxidative stress (McCaddon et al 2002). This hypothesis requires further investigation, however; it is not clear to what extent plasma aminothiol levels reflect alterations within brain tissue.

In this model, the relationship between AD and folate status presumably represents excess folate catabolism. Methionine synthase inactivation results in folate being trapped in the methyltetrahydrofolate form. It is unavailable for polyglutamation, a necessary prerequisite for cellular retention, and is released from cells (McGing et al 1978). It is subsequently excreted into the urine, eventually resulting in folate depletion (Stabler et al 1991). Haematopoietic tissue will be spared in this process, explaining the normal red cell folate values and absent anemia in our patients. Herbert and Colman (1979) speculated over two decades ago that such a selective or "cryptic" folate deficiency might exist in brain tissue in certain situations.

In summary, total plasma homocysteine and cysteine are elevated in AD. This implies intact transsulphuration but aberrant remethylation of homocysteine in patients. Plasma glutathione levels correlate with cognitive scores in the disease. Taken together, we suggest that these observations reflect the differential effects of AD-related oxidative stress on the two key enzymes of homocysteine metabolism.

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Absence of macrocytic anaemia in Alzheimer's disease

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Summary There is an association between Alzheimer's disease (AD) and low serum levels of vitamin B_{12} and folic acid. Patients also have elevated serum levels of homocysteine and disease progression might therefore be associated with the development of a macrocytic anaemia. We investigated the relationship between disease duration, homocysteine and haematological indices in patients with clinically diagnosed AD and healthy elderly controls. Haemoglobin and platelet counts fell only slightly with increasing dementia duration, but there were no other changes in haematological indices. In particular, macrocytosis and red cell distribution width were unrelated to disease duration and no patients were anaemic. Our results support previous observations that the neurological and haematological features of B_{12} and folate deficiency are often unrelated in these patients.

Keywords Vitamin B_{12}, folic acid, Alzheimer's disease, homocysteine, anaemia

Introduction

Vitamin B_{12} is essential for two mammalian metabolic reactions – the conversion of methylmalonate to succinate, and of homocysteine to methionine; the latter reaction is also folate-dependent. Serum levels of methylmalonic acid rise in B_{12} deficiency, whereas homocysteine levels rise in both folate and B_{12} deficiency. The advent of assays for these metabolites has facilitated the detection of 'subtle' vitamin B_{12} and folate deficiencies and broadened their clinical spectra (Carmel, 1996).

Haematological features of B_{12} and folate deficiencies include anaemia, macrocytosis, thrombocytopenia, neutropenia, and neutrophil hypersegmentation. In severe deficiency there is also poikilocytosis with some fragmentation. In more subtle deficiencies although macrocytosis (indicated by an increased MCV) may be absent, there is a progressive widening of the red cell distribution width (RDW) reflecting increasing anisocytosis.

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Herbert and Herzlich described a 'longitudinal model' for the development of B_{12} and folate deficiency based partly on observations of other nutrient-deficient anaemias (Herbert, 1987). They suggested that deficiency develops via sequential stages: negative vitamin balance, decreased body stores, functional (biochemical) deficiency with raised metabolites, body store depletion, and finally structural and morphological changes such as macrocytosis and anaemia.

Vitamin B_{12}-deficient patients may have inconspicuous haematological changes, often presenting primarily with neurological features (Lindenbaum *et al.*, 1988). It has been suggested that such patients are in the early stages of deficiency, and that macrocytic anaemia is not yet evident (Lindenbaum *et al.*, 1988).

We recently described elevated serum homocysteine in patients with Alzheimer's disease (AD) (McCaddon *et al.*, 1998). Other studies have confirmed this observation and suggested that elevated homocysteine is a risk factor for cognitive decline and dementia (Clarke *et al.*, 1998; Lehmann Gottfries & Regland, 1999; McCaddon *et al.*, 2001; Seshadri *et al.*, 2002).

Elevated serum homocysteine in these patients might represent the early stage of a 'classical' B-vitamin deficiency and precede haematological change. We therefore

investigated the relationship between homocysteine, haematological indices, and dementia duration in patients with clinically diagnosed AD. We hypothesized that increasing duration of dementia should be associated with the development of haematological abnormalities.

Materials and methods

Patients

Patients were recruited from the Wrexham Maelor Psychogeriatric Assessment Service with features compatible with DSM-IV criteria for primary degenerative dementia of Alzheimer-type (American Psychiatric Association, 1994). Controls were healthy cognitively intact age- and sex-matched elderly volunteers from a group General Practice in a comparable semirural area of predominantly lower socioeconomic class. Patients or controls receiving vitamin B_{12} or folate supplements, or taking medication known to influence homocysteine, were excluded.

Cognitive scores [Mini-Mental State Examination (MMSE) and the cognitive component of the Alzheimer Disease Assessment Scale (ADAS-Cog)] were recorded for cases and controls (Folstein Folstein & McHugh, 1975; Rosen Mohs & Davis, 1984).

Body mass index (BMI) was determined from height and weight measured at assessment. Duration of dementia in months was determined from records wherever possible, or alternatively from next of kin or carers. Ethical approval was granted, and informed consent obtained.

Laboratory methods

Nonfasting blood samples were taken for full blood count, red cell folate (RCF), creatinine, B_{12}, folate, and homocysteine assays. Separation and freezing were performed within 1 h of venepuncture.

An automated cell counter was used to measure haemoglobin (Hb) concentration, and MCV (Coulter Gen-S; Beckman Coulter, High Wycombe, Bucks, UK). The RDW was mathematically derived from the frequency over red cell volume histogram on the same instrument. A broad curve, resulting from cells with wide range of cell volume, will yield a raised RDW. An automated biochemistry analyser was used to analyse creatinine (Synchron LX-20 analyser; Beckman Coulter) and an automated chemiluminescence analyser was used to measure folate, vitamin B_{12} and RCF (ACS:180 SE, Bayer plc, Newbury, Berks, UK) using the manufacturers' recommended protocols. Homocysteine was assayed with an automated high-performance liquid chromatography (HPLC) system (DS30 Hcy Analyser, Drew Scientific Group plc, Barrow in Furness, Cumbria, UK). All forms of homocysteine were assayed including protein-bound, nonprotein-bound, free forms both oxidized (dimer and mixed disulphides) and reduced. Values presented therefore always refer to 'total' plasma levels.

Statistics

Comparisons between groups were performed using the Mann–Whitney *U*-test (Statistica for Windows v5.5, StatSoft, Inc., Tulsa, OK, USA). Median values are presented with interquartile ranges. A generalized linear model, using a linear link function, was used to assess relationships between variables. Regression parameters are presented with their 95% confidence intervals.

Results

Forty-four patients and 55 control subjects were recruited to the study. There were no significant differences between the two groups regarding age, sex, BMI and creatinine. Patients scored significantly worse on scores of cognitive function ($P < 0.0001$) (Table 1).

There were no significant differences for Hb, WBC, platelets, MCV, RDW, B_{12}, or RCF between groups. Patients had significantly higher serum homocysteine ($P = 0.0008$) and lower serum folate ($P = 0.02$) than controls (Table 2).

Males had higher levels of homocysteine than females [12.5 (9.5–14.5) μM and 10.2 (7.9–12.7) μM, $P = 0.02$].

A generalized linear model was used to determine the relationship between haematological variables and disease duration in the patient group. Gender was included as an additional variable. Increasing duration of dementia was

Table 1. Age, sex, BMI, creatinine and cognitive scores for patients and controls

	Age	Sex (M/F)	BMI	Creatinine	MMSE	ADAS-Cog
Controls ($n = 55$)	79 (72–86)	22/33	24.1 (22.1–28.4)	84 (72–102)	28 (27–29)	9 (7–12)
Patients ($n = 44$)	79 (75–84)	14/30	24.5 (21.8–27.6)	85 (72–115)	17 (13–21)	32 (24–40)

BMI, body mass index; MMSE, Mini-Mental State Examination; ADAS-Cog, cognitive component of the Alzheimer Disease Assessment Scale.

Table 2. Haematological parameters, B_{12} and folate status, and homocysteine values for patients and controls

	Controls	Patients	Significance (*P*-value)
Hb (g/dl)	13.4 (12.5–14.4)	12.8 (12–14.0)	
WBC ($\times 10^9$/l)	6.9 (6.1 8.0)	6.9 (6.0–8.2)	
Platelets ($\times 10^9$/l)	219 (184–277)	219 (181–271)	
MCV (fl)	90.6 (87.8–94.0)	90.0 (87.7–93.5)	
RDW	13.4 (12.9–14.0)	13.6 (12.9–14.4)	
B_{12} (ng/l)	324 (291–445)	328 (268–421)	
Folate (μg/l)	11.1 (8.2–14.5)	9.1 (6.4–11.7)	<0.02
RCF (μg/l)	332 (272–429)	282 (243–399)	
Homocysteine (μM)	9.8 (8.5–12.5)	12.2 (9.4–15.7)	<0.0008

Hb, haemoglobin; RDW, red cell distribution width; RCF, red cell folate.

associated with a slight decline in haemoglobin [Hb = 13.67–0.023 (−0.003 to −0.04) × duration + 0.20 (−0.2 to 0.61) × gender; $P = 0.02$], and platelet count [platelets = 246.6–0.96 (−1.79 to −0.13) × duration − 5.3 (−22.2 to 11.7) × gender; $P = 0.03$].

There was no relationship between disease duration and WBC, MCV, or RDW.

Homocysteine, but not B vitamins, declined with increasing dementia duration (homocysteine = 16.4–0.085 (−0.17 to −0.002) × duration + 1.95 (0.25–3.65) × gender; $P = 0.048$).

Discussion

This study confirms earlier observations of increased plasma homocysteine and reduced serum folate levels in patients with clinically diagnosed AD (Clarke *et al.*, 1998; McCaddon *et al.*, 1998.) Hb and platelet counts fell only slightly with increasing dementia duration, but there were no other changes in haematological indices; macrocytosis and RDW in particular were not related to disease duration, and no patients were anaemic. Surprisingly, homocysteine declined with time in the AD patient group rather than rising.

It is difficult to obtain a 'true' measure of disease duration because of the insidious onset of AD. Also, although medical records were consulted wherever possible, we were often solely reliant upon the recall of next of kin or carers for this information. Prospective studies might resolve some of these difficulties.

In B_{12} or folate deficiency the number of nuclear lobes in neutrophils is typically increased; cells with five or more lobes are evidence of an underlying megaloblastic process. This is generally regarded as the earliest morphological change in megaloblastic anaemia and is the last to disappear after treatment. This was not evaluated in the present study, but we have previously found no relationship between neutrophil segmentation and homocysteine levels in AD (McCaddon *et al.*, 1998).

The AD patients therefore exhibit a functional B_{12}/folate deficiency, but in the absence of 'classical' haematological indicators. Low serum folate, but normal red cell folate, in AD suggests that these patients are in negative folate balance, i.e. more folate has been catabolized than absorbed (Herbert & Colman, 1979).

Lindenbaum *et al.* (1988) suggested that neuropsychiatric features of B vitamin deficiency might precede haematological change. Our study suggests that this is not the case in patients with AD.

It is likely then that the association between B vitamin deficiency and AD reflects an entirely different pathogenic mechanism from that associated with malnutrition- or malabsorption-related syndromes such as pernicious anaemia. We suggest that a 'selective' cerebral B_{12}/folate deficiency probably exists in AD, with relative sparing of haematopoietic tissue.

One possibility is that this arises from impaired delivery of these vitamins to the central nervous system. AD patients have significantly lower levels of serum holotranscobalamin (Refsum & Smith, 2003), and a transcobalamin codon 259 genetic polymorphism influences holo-transcobalamin concentration in AD cerebrospinal fluid (Zetterberg *et al.*, 2003). Levels of cerebrospinal fluid B_{12} (but not folate) are lower in patients with AD (Nijst *et al.*, 1990).

An alternative hypothesis proposes a selective impairment of cerebral B_{12} metabolism in AD (McCaddon *et al.*, 2002). AD-related cerebral oxidative stress will augment the oxidation of an intermediate form of vitamin B_{12} generated in the methionine synthase reaction, thereby impairing homocysteine metabolism. Furthermore, oxidative stress will compromise the intraneuronal reduction of vitamin B_{12} to its metabolically active state (McCaddon *et al.*, 2002). Folate might also undergo irreversible

oxidation (Fuchs *et al.*, 2001). If such stress were confined to brain tissue haematopoietic methionine synthase activity would be relatively unaffected, accounting for the absence of anaemia and macrocytosis.

In the latter model, the relationship between AD and folate status probably reflects excess folate catabolism. Methionine synthase inactivation results in folate being trapped in the methyltetrahydrofolate form. It is unavailable for polyglutamation, a necessary prerequisite for cellular retention, and is released from cells (McGing *et al.*, 1978). It is subsequently excreted in the urine, eventually resulting in folate depletion (Stabler *et al.*, 1991). Haematopoietic tissue will be spared in this process, explaining the normal red cell folate values and absent anaemia. Herbert and Colman (1979) speculated over two decades ago that such a selective or 'cryptic' folate deficiency might exist in brain tissue in certain situations.

In conclusion, metabolic evidence for B_{12} and folate deficiencies in AD is neither associated with nor precedes the development of a macrocytic anaemia. Practitioners should be aware that, in such patients, metabolic abnormalities arise in the absence of these traditional haematological markers. Homocysteine has recently been identified as an independent predictor of cognitive decline (McCaddon *et al.*, 2001), and dementia patients with elevated plasma levels improve clinically following vitamin substitution (Nilsson Gustafson & Hultberg, 2001). It therefore seems prudent to actively seek this abnormality at the initial presentation of any dementing illness. Current evidence based guidelines for the management of dementia should be altered to include, at least, an assay of vitamin B_{12} and folate, irrespective of whether there is evidence of macrocytic anaemia (Eccles *et al.*, 1998). This novel concept of a 'functional vitamin B_{12} deficiency' also has potential implications for treatment options (McCaddon *et al.*, 2002). Further research is now underway to evaluate the efficacy of alternative forms of B_{12}, such as glutathionylcobalamin, in treating such patients.

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- W073 -
A PLASMA HOMOCYSTEINE REFERENCE RANGE STUDY CONDUCTED USING THE DREW SCIENTIFIC DS30 HCY ASSAY.

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There is now considerable interest in the possibility that elevated plasma homocysteine levels may be a precursor of vascular disease. We have developed a fluorescence-based HPLC assay that is simple to use and is intended for routine measurement and screening. As part of the validation of the assay we have determined the normal range for plasma homocysteine in a panel of adult volunteers. Blood was collected into anticoagulant tubes and the plasma separated by centrifugation within 30 minutes. Plasma was frozen at -20°C until analyzed. The assay involves derivatization of thiols with a fluorescent tag followed by reverse phase chromatography with fluorescence detection. Patient volunteers were selected to exclude those individuals with a history of vascular or renal disease. Also, patients who had taken methotrexate, cyclosporine, captopril or anticonvulsants were excluded. Currently prescribed medication was recorded. The 143 volunteers comprised 56 males and 87 females in the age range 18 - 60 yrs (mean = 37yrs). The 95% reference was calculated to be 5.2 - 15.1 µmol/L with 90% confidence limits of 4.9 - 5.7 and 13.9 - 18.4 µmol/L respectively. The range obtained with our method is similar to the majority of published ranges using a variety of measurement methods.

- W074 -
INDIRECT NEUROTRAUMA CAUSED BY PULMONARY BLAST INJURY

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It is suspected that among important mechanisms of indirect neurotrauma (INT) development is oxidative stress, i.e. oxygen reactive metabolites in connection with NO. The experiments were performed on anesthetized rabbits exposed to pressure wave, focused on the right middle thoracic region in the lung area (LD_{30}). In brain tissue (cortex, brainstem) we assayed malondialdehyde (MDA) and intesivity of superoxide anion radical (O_2-)generation. Antioxidant protection was estimated by the SOD and GSH-Px. Experimental animals were devided into three groups : control(C), animals exposed to pressure wave (B) and animals which immediately after the injury have received N-nitro-L-arginine methyl ester (L-NAME) in order to inhibit constitutive and inducible nitric-oxide synthase (B+L-NAME). The obtained results proved the development of oxidative stress in brain. The concentration of MDA and O_2- were increased in the group of traumatized animals compared to the control one. The therapy by L-NAME was more efficient in decrease of lipid MDA stimulated by blust injury. An enhancement of antioxidant enzymes was detected in brain structures of traumatized rabbits. The activity of SOD was higher in L-NAME group , while those of GSH-Px was increased in traumatized rabbits without therapy.

- W075 -
OXIDATIVE STRESS AND LUNG BLAST INJURY

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Pulmonary blast injury is the consequence of pressure wave generated during the explosion. Target organs are those containing air i.e. lungs. It is suspected that oxidative stress, stimulated by direct morphological tissue damage and hipoxy is an important factor in pulmonary blast injury (PBI). The hypothesis was that NO as free radical contribute to the harmful effect of oxidative stress. Experiments were performed on anesthetized rabbits exposed to pressure wave, focused on the right middle thoracic region in lung area (LD_{30}). Of the markers of oxidative stress we assayed MDA and the intensity of superoxide anion (O_2-) generation. Antioxidant protection was estimated by the activity of SOD and GSH-Px. Animals were devided into three groups : control (C), exposed to pressure wave (B) and animals which immediately after the injury received N-nitro-L-arginine methyl ester (L-NAME) to inhibit constitutive and inducible nitric oxide synthase (B+L-NAME). Results showed that MDA was increased in the group B compared to the control, while enhancement in group C was not statistically significant. Increment of O_2- in lungs after PBI was not significant. In bronchial and alveolar area antioxidant enzymes were increased both in group B and group under the therapy (B + L-NAME).

- W076 -
LABORATORY EVALUATION OF THYROID FUNCTIONS IN HYPERESTROGENISM

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Estrogen-induced increase in thyroxin binding globulin (TBG) might interfere with the evaluation of thyroid functions. Aim of the present investigations was to determine the most reliable thyroid parameters in hyperestrogenism. Sera of healthy women (36 controls, 44 taking oral contraceptives, 154 pregnant) were investigated for TSH, TBG, total-T4 (TT4), total-T3 (TT3), T3-uptake (T3U), thyroxin binding capacity (TBC), free-T3 (fT3) and free-T4 (fT4). Estrogen-dependent differences were found between the three patient groups in TBG, TT3, TT4, T3U, TBC, fT4, and T4/TT3 ratio. Between pregnant and contraceptive treated women, differences were found in fT3, fT4-index, fT3-index, TT4/TT3 ratio and fT4 levels. The ratio of TT4/TBC was also different in control, versus contraceptive treated women. The correlation of thyroid parameters with the type of the contraceptive drug (Marvelon, Anteovin, Tri-Regol, Ovidon, Rigevidon, etc.) was also investigated. It has been found that TSH and the free hormone indices (T4*T3U, T4*TBC, T3*T3U) as well as the fT4 values, measured with various two-step assays, were in the normal range in all three patient groups. Other hormone values (e.g. fT4 values measured with an one-step assay) were out of this range. Since the incidence of high TT3 was higher than that of high TT4, the results argue for the necessity of fT3 (or fT3-index) measurements in hyperestrogenism.

Poster display: Wednesday, 9 June, 10:00-17:00 – Author's attendance: 13:15-14:15

91
Serum bile acids: the mystery of the missing blank

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A nationwide bile acid service for obstetric cholestasis was introduced at University Hospital Birmingham in 1994. The current method (Nycomed Enzabile Kit, Bio-Stat Diagnostics) employs 3α-hydroxysteroid dehydrogenase (3α-HSDH), which oxidizes 3α-hydroxy bile acids with release of NADH and formation of formazan, which is monitored at 540 nm on a Cobas Fara. A separate blank assay, omitting 3α-HSDH, is also run. This method is slow, requiring manual cuvette changing and manual data handling. The aim of the study was to transfer the method to a Cobas Mira S to overcome these disadvantages. Using the test parameters provided by Bio-Stat, samples were run on the Mira using an integral blank. This involved pre-incubation of sample and reagent for 100 s, followed by blank readings, prior to addition of the 3α-HSDH reagent. Results from the Mira were higher than with the current Fara method. Using a reference limit of <14 μmol/L, 7 out of 46 results (15.2%) would have been misclassified as abnormal. When a completely separate blank assay was run, this fell to 2 out of 88 results (22%). Only by using a separate sample blank can erroneously high results due to non-3α-HSDH-mediated formazan production be avoided.

92
Measurement of boron by graphite furnace atomic absorption spectroscopy and its relevance in boron neutron capture therapy

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(All experimental work was carried out at the Regional Laboratory for Toxicology, Birmingham City Hospitals NHS Trust.) A method for the measurement of boron by graphite furnace atomic absorption spectroscopy has been developed using a zirconium-nickel nitrate modifier to pre-treat the graphite tube before injection of the sample. The method was based on the procedure reported by Varian (*Instruments at Work*, May 1997, AA-125) and can be used to determine boron in aqueous soluton, serum and whole blood. Calibration curves for boron have been established from 1 to 100 mg/L of boron. The sensitivity (i.e. characteristic mass) was recorded at 354 pg, and the tube lifetime was about 100 atomization cycles at 2800°C.

Boronophenylalanine (BPA) is a boronated compound currently being used in boron neutron capture therapy (BNCT). BPA-spiked blood samples of different concentrations were prepared to send to the University of Birmingham as internal Quality Control material, from where they would also be sent to various other BNCT facilities around the world to be measured by the local technique. A comparison study for the analysis of boron in different centres was piloted. Within- and between-batch precision were measured at 5 mg/L of boron (BPA in citrated blood), n=5, CV=3.9% and CV=11.0%, respectively.

93
Screening for hyperhomocysteinaemia: comparison of a commercial assay with a laboratory assay

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Hyperhomocysteinaemia is an established risk factor for vascular disease. Widespread screening might now be facilitated by the availability of commercially available assays since many laboratories no longer have the resources to contemplate in-house assay development. The aim of this study was to compare an in-house laboratory HPLC method (reverse-phase, electrochemical detection) with a commercial assay (Drew Scientific Ltd; DS30 Hcy, reverse-phase, fluorescence detection). A fasting specimen of EDTA plasma was separated and frozen within 1 h of venesection from each of 93 patients. Total homocysteine (tHcy) was measured using both methods. The results were compared by linear regression (unweighted and Passing and Bablok) and by difference plots (Bland and Altman). There was a close correlation between the assays with r^2=0.90 (95% CI 0.86-0.94). The commercial assay produced a small positive bias for fasting tHcy concentrations of 2.95 μmol/L (95% CI 2.50-3.40). There was a close agreement between the assays with 95% of values lying between 7.23 μmol/L above (95% CI 6.47 to 8.00) and 1.33 μmol/L below (95% CI -2.09 to -0.56) the corresponding value for the electrochemical method. The commercial assay provides an easy-to-use alternative to the in-house method.

94
Detection of clonal T-cell receptor β rearrangements in paraffin-embedded tissue by RT-PCR

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Knowledge of T-cell clonality can help considerably in cases where diagnostic uncertainty still exists after conventional histology and immunocytochemistry.

Clonal T-cell populations can be detected by consideration of both the rearranged T-cell receptor (TCR) β and

methods. In conclusion, PPD-1 method for HDL-C assay with phthalic acid exhibits no acceptable performance to approach 1998 NCEP goal.
Passing Bablok regression data (in comparison with UC method)

		Slope	Intercept	r
	II/UC	1.45	-13.19	9.726
High trigliseride sera (n=22)	PPD-1/UC	2.0	-44.5**	0.314
	PPD-2/UC	0.66	11.33**	0.599
	II/UC	0.97	8.162**	0.816
High bilirubin sera (n=28)	PPD-1/UC	0.66*	10.76**	0.751
	PPD-2/UC	0.876	5.10**	0.732
	II/UC	1.07	4.85**	0.955
High bilirubin and trigliseride (n=46)	PPD-1/UC	0.664	10.769	0.751
	PPD-2/UC	0.91	7.708**	0.876

* Slopes are significantly different from 1.00
** Intercepts are significantly different from 0.00

139

Guidelines on preanalytical criteria for the measurement of pyridinoline and deoxypyridinoline as bone resorption markers in urine. H. W. Vesper[1], L. M. Demers[2], R. Eastell[3], P. Garnero[4], M. Kleerekoper[5], S. P. Robins[6], A. K. Srivastava[7], R. G. Warnick[8], N. B. Watts[9], *[1]CDC: Atlanta, GA; [2]Milton S. Hershey Med. Center: Hershey, PA; [3]Northern General Hosp.: Sheffield, United Kingdom; [4]INSERM Unite 403 & Synarc: Lyon, France; [5]Wayne State Univ.: Detroit, MI; [6]The Rowett Research Inst.: Aberdeen, United Kingdom; [7]J.L. Pettis VAMC: Loma Linda, CA; [8]Pacific BioMetrics Res. Foundation: Seattle, WA; [9]Emory Univ.: Atlanta, GA.*

Biochemical bone markers are well established tools in the management of metabolic bone diseases like osteoporosis. Preanalytical factors may contribute significantly towards the observed intra- and inter-individual variability as well as to interlaboratory differences. This variability has a profound effect on the reproducibility, comparability and interpretation of results. Preanalytical criteria include patient status prior to sample collection, the sample collection and treatment as well as other non-bone related factors affecting bone marker levels.
To assess the effects of preanalytical factors and to improve the measurement of biochemical bone markers, the Centers for Disease Control and Prevention (CDC) initiated a standardization effort on the measurement of the bone resorption markers pyridinoline (PYD) and deoxypyridinoline (DPD). As one part of this standardization effort, CDC established an international working group to identify and characterize preanalytical factors affecting PYD and DPD measurement and values in urine. The goal of this working group is to define guidelines on preanalytical factors, which help minimizing preanalytical variability. A thorough literature search on PYD and DPD was performed. Literature selection was limited to studies on human subjects and urine as specimen, except for citations dealing with stability issues of PYD and DPD. In addition, literature on creatinine measurement in urine was collected. The obtained citations were reviewed by the working group members. The following preanalytical issues likely to affect PYD and DPD levels in urine have been identified and characterized:

Diurnal changes,
Lifestyle including diet and exercise,
Menstrual changes, pregnancy and lactation,
Specimen storage and treatment,
Seasonal variation,
Age, gender and race,
Certain diseases and medications
Creatinine measurement.

Based on the available data, recommendations about subject status, time-point of sample collection and sample treatment are formulated. Issues like multiple measurements versus single measurement and population-based variability that are important for the assessment of reference ranges are discussed. Areas with data insufficient to detect any impact on PYD and DPD levels have been identified. The recommendations are intended to be used for sample collection in the assessment of metabolic bone diseases as well as for establishing reference ranges.

140

Effect of temperature on the stabilization of blood homocysteine concentration by 3-deazaadenosine. D. M. Hill, A. C. Kenney, *Drew Scientific Ltd.: Barrow-in-Furness, United Kingdom.*

The recent emergence of commercial assays makes analysis of plasma total homocysteine (tHcy) much more accessible to the routine clinical laboratory. The problem that remains is how to stabilize plasma tHcy prior to centrifugation of a blood sample, since red blood cells continue to produce and excrete Hcy into the plasma. The way in which this instability is currently overcome is to put the samples on ice and centrifuge within one hour of collection, however this method is often inconvenient and does not lend itself readily to large-scale clinical studies.
A recent report (Al-Khafaji *et al.* Ann Clin Biochem 1998; 35:780-782) suggested that 3-deazaadenosine (3DA) could be used in EDTA blood collection tubes to inhibit the enzyme S-adenosylhomocysteine hydrolase (SAHH). This enzyme is involved in the final step of Hcy production from methionine. 3DA competes against the enzyme's substrate, S-adenosylhomocysteine, for the active site of the enzyme thus slowing down the production of Hcy.
It is possible that both the activity of SAHH and the degree of its inhibition by 3DA might be altered by temperature. The purpose of this study was to determine the effectiveness of 3DA in stabilising tHcy in blood samples that have been stored at ambient temperatures of 20-25°C, and to investigate whether cooling the samples would extend stability prior to separation of the plasma.
Although the concentration of 3DA used in this study (100 µmol/L), matched that of the previously mentioned report, tHcy concentrations rose significantly over 72 hours in whole blood stored at 20-25°C (Table 1). Plasma tHcy was measured using HPLC with fluorescence detection. When samples were stored at 2-8°C in the presence of 3DA, plasma tHcy remained stable over a period of one week. Cooling the samples to 2-8°C in the absence of 3DA was not sufficient to stabilize plasma tHcy.

Table 1. Effect of temperature and 3-deazaadenosine on plasma Hcy (µmol/L) in whole blood.

		Time of Incubation					
Sample	Temp	0		3 days		1 week	
EDTA	2-8°C	8.8	(0.8)	16.7	(1.8)	22.5	(1.7)*
EDTA/3DA	2-8°C	8.5	(0.9)	8.0	(0.8)	9.1	(2.4)ns
EDTA/3DA	20-25°C	8.5	(0.7)	11.5	(2.3)	19.3	(4.3)*

Results are mean (SD). *P<0.05, Friedman ANOVA, n=4
ns=not significant, 3DA=3-deazaadenosine

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Short-term storage of serum at room temperature in gel separator tubes: effect on common chemistry tests determined with Hitachi 917. J. N. Murthy, M. R. George, N. N. Rehak, *National Institute of Health: Bethesda, MD.*

Direct sampling from gel separator tubes usually means that the leftover serum is not aliquoted into separate tubes but is stored in contact with the gel material. This may affect the serum analytes. We investigated the effect of short-term contact of serum with the gel in Vacutainer Plus SST tubes (Becton Dickinson #7983) on the results of Hitachi 917 common chemistry methods. Random patient specimens (n=35) were analyzed promptly upon receipt by the laboratory (fresh samples). The specimens were then stored in tightly stoppered SST tubes at room temperature (»25°C) for 8 h (stored samples). The stored samples were reanalyzed and the results were compared with those obtained for fresh samples. The effect of storage on each serum analyte, calculated as the difference DIF=stored-fresh (mean, SD and paired t-test)
Five analytes (Alt, Ast, CO_2 Cl and Trig) showed statistically significant ($p<0.05$) difference. The values obtained (mean, mean difference, SD and significance (t value) are as follows: Alt: 70.2, -1.34, 8.00, <0.05; Ast: 46.8, 0.77, 6.00, <0.01; CO_2, 23.4, -1.45, 2.0, <0.001; chloride 104.8, 0.61, 4.0, 0.001; Trig: 159.5, -0.36, 10, <0.05. Although these analytes showed statistically significance mean difference, the SDs were within the allowable ±2 SD values (no effect if DIF£ fresh ±2 SD) which reflects the day-to-day imprecision (3 levels) of the appropriate chemistry method for acceptable performance of the instrument/method.
Thus, based on our results, we believe that majority of common analytes can be measured with Hitachi methods using serum specimens stored at room temperature for up to 8 h in tightly stoppered Vacutainer Plus SST tubes.

142

Two cases of immunoassay interference with clinical consequences. G. F. Johnson, R. D. Feld, J. M. Beranek, P. L. Roberts, *University of Iowa Hosp.: Iowa City, IA.*

Two site sandwich immunoassays are subject to positive interference by human antibodies directed against the animal immunoglobulins used in these assay systems. Modern commercial immunoassay reagents usually contain excess immunoglobulins from the animal source to block any human anti-animal immunoglobulin that might be present.
We present two patients in which the blocking agents in the immunoassay were inadequate to prevent interference from endogenous human anti-animal immunoglobulins and which resulted in negative clinical consequences. Patient 1 was being investigated for an elevated serum TSH in the presence of normal free thyroxine. The differential diagnosis included a TSH producing pituitary adenoma as well as genetic thyroid hormone resistance. Unnecessary radiological tests and laboratory tests were performed. Patient 2 had a testicular mass and an elevated serum HCG. The testicle was removed and the lesion found to be cystic rather than malignant but the serum HCG remained elevated after testicular excision.
Antibody interference was evaluated in these two patients by reassay on another immunoassay instrument and by addition of mouse and bovine sera to the patient samples

EFFECT OF TEMPERATURE ON THE STABILIZATION OF BLOOD HOMOCYSTEINE CONCENTRATION BY 3-DEAZAADENOSINE

137

Hill D.M., Kenney A.C.

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The measurement of plasma tHcy is becoming much more accessible to the routine clinical laboratory through the emergence of commercial assays. The problem that remains is how to prevent Hcy production by red blood cells prior to centrifugation of a blood sample. This instability is currently overcome putting the samples on ice and centrifuging within one hour of collection, however this method is often inconvenient and does not lend itself readily to large-scale clinical studies.
Al-Khafaji et al. (Ann Clin Biochem 1998; 35: 780) suggested that 3-deazaadenosine (3DA) could be used in EDTA blood collection tubes to inhibit the enzyme S-adenosylhomocysteine hydrolase (SAHH) which is involved in the final step of Hcy production from methionine. It is possible that both the activity of SAHH and the degree of its inhibition by 3DA might be altered by temperature.
A pilot study was performed to evaluate the effectiveness of 3DA in stabilising tHcy in blood samples that have been stored at ambient temperatures of 20-25°C, and to investigate whether cooling the samples would extend stability prior to separation of the plasma.
Although the concentration of 3DA used in this study (100µmol/L), matched that of the previously mentioned report, tHcy concentrations rose significantly over 72 hours in whole blood stored at 20-25°C (Table 1). Plasma tHcy was measured using HPLC with fluorescence detection. When samples were stored at 2-8°C in the presence of 3DA, plasma tHcy remained stable over a period of one week. Cooling the samples to 2-8°C in the absence of 3DA was not sufficient to stabilize plasma tHcy.

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EDTA/3DA	20-25°C	8.5	(0.7)	11.5	(2.3)	19.3	(4.3)*

*Results are mean (SD). *P<0.05, ns=not significant. Friedman ANOVA. n=4.*

RIBOFLAVIN CONCENTRATIONS IN HUMAN PLASMA AND ERYTHROCYTES AT BASELINE AND AFTER RIBOFLAVIN SUPPLEMENTATION

138

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BACKGROUND: Riboflavin is a determinant of plasma total homocysteine and serves as a cofactor for enzymes involved in the metabolism of several vitamins. The activation coefficient of erythrocyte glutathione reductase has traditionally been used to assess riboflavin status. Determination of riboflavin concentrations has been used less frequently.
AIMS: To evaluate the response of riboflavin, and its coenzyme forms, FMN and FAD, to riboflavin supplementation.
METHODS: Riboflavin, FMN and FAD were determined in 118 elderly adults. 46 subjects took part in a 12-week placebo controlled riboflavin (1.6 mg/d) intervention. Riboflavin, FMN and FAD were analysed by CE-LIF.
RESULTS: Baseline plasma concentrations were 10.4 nmol/L (4.3 – 31.9) (median, 5 - 95 pct) for riboflavin, 6.6 nmol/L (3.7 – 13.0) for FMN and 73.4 nmol/L (54.3 – 101.3) for FAD. There were only traces of riboflavin in the erythrocytes, while FMN and FAD concentrations were 44.5 nmol/L (24.2 –82.7) and 468.7 nmol/L (333.9 – 630.0), respectively. In the supplementation group, plasma riboflavin increased 83 % (28 – 139) (mean, 95% CI), FMN increased 27 % (11 – 43), and plasma FAD did not change. Erythrocyte FMN increased 87 % (55 – 118), and FAD increased 14 % (8 – 20).
SUMMARY/CONCLUSIONS: We determined riboflavin, FMN and FAD concentrations in elderly adults at baseline and after low dose riboflavin supplementation. In plasma, riboflavin was the most responsive parameter, followed by FMN, but there was no change in FAD. In erythrocytes, there were only traces of riboflavin, and both coenzyme forms increased.

system (X) as judged by linear regression: Y = 1.05X - 0.29, r = 0.99, Syx = 1.71, range = 0.0 - 50.0 ng/mL. The improved CTNI method increases the robustness of the Dimension® system while maintaining safe, rapid and accurate cardiac test performance.

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Evaluation of the Drew Scientific 3DA sample collection tube for homocysteine analysis. L. J. Johnson[1], A. M. Neale[1], D. H. Harmening[1], D. M. Hill[2], A. C. Kinney[2], *[1]Dept. Medical & Research Technology, Univ. MD School of Medicine: Baltimore, MD; [2]Drew Scientific, Inc.: Providence, RI*

Much attention has been focused on the thiol-containing amino acid homocysteine (Hcy) because of the existing correlation between increased levels and the presence of cardiovascular disease. Normal fasting plasma levels range from 5–15 umol/L. Artifactual increases occur in whole blood due to continued metabolism. The rate of increase in unprocessed specimens collected in EDTA is approximately 1 umol/L per hour at room temperature. It is often inconvenient, if not impossible, to place samples on ice and follow with timely centrifugation and freezing of the plasma. This study compared the effects of delayed processing between samples collected in EDTA and 3 deazaadenosine (3DA) at room temperature (RT) and 4°C.

EDTA and 3DA samples were collected from 36 healthy volunteers; after processing a baseline sample from both tubes, samples were maintained at RT and 4°C, with aliquots processed at 3, 6, 24, and 72 hours after collection. Plasma aliquots were frozen at –72°C until analysis was performed using the Drew D530 Hcy analyzer. All aliquots from each subject were analyzed on the same run. Data were analyzed and expressed as a multiple of the baseline value. The mean change was determined for both sample types, at both temperatures and at each time point. Results were as follows:

Time Point	3DA – RT+	EDTA – RT+	p-value	3DA – 4°C+	EDTA – 4°C+	p-value
3 hour	1.00	1.30	<0.05*	0.99	1.02	<0.05*
6 hour	1.02	1.65	<0.001*	0.98	1.08	<0.001*
24 hour	1.29	3.22	<0.001*	0.99	1.25	<0.001*
72 hour	2.54	5.47	<0.001*	0.99	1.60	<0.001*

+data shown as *mean multiple from baseline value*; *significant

Data demonstrate a significant difference between the 3DA and EDTA tubes at RT and 4°C for all aliquots processed at each time point. Though EDTA is the most commonly used additive, 3DA offers greater stability of Hcy in whole blood both at RT and 4°C. The enhanced stability is greatly needed, especially for large epidemiological studies involving different collection sites which may not have access to ice to preserve sample integrity until processing, as well as clinical sites where processing is often delayed if testing is performed off-site.

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On the interaction of cardiac troponin I (cTNI) and heparin. A possible solution. T. L. Wagner, H. M. Schessler, L. A. Liotta, A. R. Day, *Immunomatrix, Inc.: Gaithersburg, MD*

It is generally accepted that heparin binds to cardiac troponin I (cTNI) via a charge-charge interaction, forming complexes which may change antibody-epitope interactions in some assays. To reverse heparin toxicity in the clinical setting, protamine is used. We theorized that the addition of protamine to plasma samples might inhibit cTNI/heparin binding thereby increasing the sensitivity for a given assay system. With this goal in mind, we evaluated the OPUS and Vitros cTNI assays in the presence and absence of protamine. A series of cTNI calibrators were prepared in heparinized plasma (SeraCare). The native cTNI was purchased from Hytest (Finland) and diluted as per the manufacturer's instructions. The concentration range of cTNI used was 0 – 100ng/ml. The assays were performed at Suburban Hospital (Bethesda MD, Vitros), Washington Hospital Center (Washington DC, Vitros) and at Immunomatrix (OPUS). The results we obtained are in Table 1.

Gravimetric		Heparin		Heparin with protamine		
ng/ml	OPUS	Suburban	WHC	OPUS	Suburban	WHC
0	<0.500	0.001	0.000	<0.500	0.285	0.440
0.5	<0.500	0.215	0.163	4.13	1.840	2.330
2	<0.500	0.562	0.482	11.90	3.600	4.250
5	<0.500	1.57	1.29	22.30	8.77	10.10
10	1.00	3.29	2.78	50.70	16.60	19.70
50	8.57	16.30	14.30	>150	100	226
100	16.90	35.00	31.60	>150	206	477

For the OPUS, inclusion of protamine (2mg/ml) increases the cTNI values significantly relative to those determined in the absence of protamine. The over-estimation of the true gravimetric level may be related to:

1. The calibrators for OPUS are not in a plasma matrix
2. The manufacturer indicates that heparinized plasma samples should not be employed in their system, presumably because of a significant negative bias.

The data obtained for Vitros (2 sites) are in reasonable agreement. Similarly, protamine appears to "complex" heparin, which is translated into higher cTNI values across the calibrator range. The data presented herein is very preliminary, nonetheless we feel it may be of interest to those investigators involved in the difficult and complex task of designing cTNI assays.

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Ischemia-reperfusion induced novel phosphorylation of TnI: Implications for serum diagnostics. R. Labugger[1], L. Organ[2], I. Neverova[2], J. E. Van Eyk[2], *[1]Queen's University: Kingston, ON, Canada; [2]Queen's University: Kingston, ON, Canada*

Identification of the exact TnI post-translational modifications that occur in the ischemic heart is vital for optimization of serum TnI diagnostics. Moreover, there is a potential to correlate TnI modifications with the extent of ischemic injury. TnI undergoes extensive modification, including phosphorylation as well as selective and progressive proteolysis during ischemia. Most of the TnI products result from ischemic-induced modifications that occur in myocardium prior to their release into serum. The full spectrum of these products has yet to be identified. Using immunological analysis and mass spectrometry, we have determined that there is at least one novel phosphorylation site resulting from ischemia. Results: The anti-TnI monoclonal antibody (mAb) 2I-14 (Spectral Diagnostics Inc.), which has specificity for both rat and human TnI, was used to study TnI from both ischemic rat hearts and diseased human myocardium (transplant rejection). mAb 2I-14 was epitope mapped to TnI amino acid residues 104-136, a region not previously known to be phosphorylated either in vivo or in vitro. Phosphorylated TnI (or its phosphorylated degradation products) present in ischemic (stunned) rat hearts (15 min ischemia followed by 45 min reperfusion) and human diseased myocardium were not detectable with mAb 2I-14 indicating that TnI phosphorylation had occurred at the novel site. To identify this phosphorylation site, TnI and its degradation products were purified from the left ventricle of rat ischemic hearts (n=16), and from the human myocardium using ion exchange chromatography followed by reversed phase high performance liquid chromatography. Intact TnI (95% pure) and several lower molecular weight degradation products (22, 16, and 15 kDa, 75% pure) were analyzed by mass spectrometry, revealing that TnI was phosphorylated at residues Thr119, Thr123 and/or Thr129 during ischemia-reperfusion injury. Conclusion: A novel phosphorylation of TnI occurs during ischemia, which may not only play a role in cardiac dysfunction but may also alter antibody recognition of TnI in serum diagnostics.

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Retrospective database review for use of cardiac troponin I for detection of myocardial infarction. V. Johari, G. K. Davis, K. Hoybook, E. Weber-Shrikant, M. M. Murakami, F. S. Apple, *Hennepin County Medical Center: Minneapolis, MN*

A consensus document from the international cardiology community was recently published regarding the redefinition of myocardial infarction (MI), predicated on the use of cardiac troponin (cTnI or cTnT). Since June 1996 our medical center discontinued CKMB testing, replacing it with cTnI as the primary biochemical marker, along with total CK, for the detection of MI. The aim of this study was to determine the impact of the new redefinition of MI based on lower concentration decision cutpoints on patient diagnosis classifi-

145-Poster

INFLUENCE OF CENTRIFUGATION TEMPERATURE ON THE TOTAL PLASMA HOMOCYSTEINE CONCENTRATION

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Background and aim: The total plasma homocysteine (tHcy) concentration falsely increases when whole blood is not centrifuged immediately after drawing, as at room temperature homocysteine will leak from blood cells to plasma. It is not known whether the temperature of centrifugation influences the tHcy concentration. Therefore, we performed a study in which we compared the tHcy concentration in samples centrifuged at +4 C and at +20 C.

Methods: This study was performed in 40 females (aged 43–68 years) of whom we took two fasting venous EDTA blood samples that were centrifuged immediately for 15 min at 2000g; one at +4 C and the other at ambient temperature (+20 C). After centrifugation the plasma was separated from the blood cells immediately and the plasma samples were stored at 20 C until the tHcy measurement.

Results: The mean tHcy concentration for the samples centrifuged at +4 C was 9.18 ($\pm$3.7) µmol/L, and at +20 C it was 9.25 ($\pm$4.1) µmol/L (p = 0.3, paired Student t-test). The Pearson correlation coefficient confirmed a perfect agreement in the tHcy concentrations at both centrifugation temperatures (r = 0.98, p<0.0001).

Conclusion: This study indicates that using non-refrigerated centrifuges does not lead to misclassification of subjects according to their tHcy concentration, if whole blood can be centrifuged immediately after drawing.

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HOMOCYSTEINE EXPORT FROM ERYTHROCYTES IN STORED WHOLE BLOOD

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Background and aims: In a recent study we observed that samples collected from individuals covering a wide range of baseline plasma homocysteine (Hcy) concentrations showed very similar absolute rises over a 72 h period. Here we report further investigation into the initial rates of Hcy production comparing two subgroups of the population based upon baseline concentration.

Methods: EDTA blood samples were collected from 59 individuals at two centres. Vascular patients were recruited from a UK hospital, and a US university campus provided samples from mainly healthy volunteers. An aliquot of blood was centrifuged immediately and the plasma removed. After 3 h storage at 20–25 C, a second aliquot of whole blood was processed. The average rate of each individual's Hcy production per hour was compared in those with baseline Hcy concentrations above and below 10 µmol/L.

Results: The rate of Hcy production from erythrocytes was shown to be independent of baseline Hcy concentration over the first 3 h post sample collection (p = 0.3890, Mann–Whitney test). Median rates were 0.6 µmol/L/h for those whose baseline value was less than 10 µmol/L (n = 43), and 0.7 µmol/L/h for those with baseline values greater than 10µmol/L (n = 16).

Conclusions: The rate of Hcy production from eryth rocytes *in vitro* appears to be independent of the *in vivo* mechanisms that lead to moderate hyperhomocysteinaemia.

EFFECT OF 5-METHYLTETRAHYDROFOLATE AND FOLIC ACID TREATMENT ON PLASMA HOMOCYSTEINE IN HEALTHY SUBJECTS WITH OR WITHOUT THE 677C->T POLYMORPHISM OF MTHFR

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Folic acid has been used to lower homocysteine (Hcy) in many studies and might be influenced by the 677C->T mutation for 5,10-methyleneTHF reductase (MTHFR), which is associated with increased levels of Hcy, especially together with poor folate status. This study tested the effect of nutritional doses of MTHF compared with folic acid on Hcy levels in healthy subjects, with and without the 677C->T polymorphism. 20 wild type and 20 homozygous subjects were investigated. Within each genotype group, half received 400 µg/day of [6 S,R] MTHF (equivalent to 200 µg of the natural isomer), the other half 400 µg/day of folic acid, both orally. tHcy and folate were determined before, after 7 weeks of treatment and 24 weeks after stopping treatment.

After 7 weeks treatment with MTHF, tHcy levels fell from 11.6±1.5 to 8.7±1.8, ($p<0.0001$) and from 16.9±6.8 to 11.6±4.4 µmol/L, mean ± SD ($p<0.0005$) in wild type and homozygous subjects respectively, proving biological availability of MTHF irrespective of the 677C->T genotype.

After folic acid for 7 weeks, values were 12.6±3.3 to 9.2±2.7 ($p<0.0001$) and 15.6±4.9 to 9.1±2.4 ($p<0.0005$) in wild type and homozygous subjects respectively.

Surprisingly 6 months after stopping treatment, tHcy levels remained significantly lower than pre-treatment levels (12.1±2.5 vs. 16.9±6.8, $p<0.02$) in homozygous subjects treated with MTHF, but showed no significant difference from pre-treatment levels in the other groups.

The prolonged effect of MTHF, but not folic acid, only in subjects homozygous for 677C->T represents a further phenotypic effect of this genotype, probably related to folate disposition.

TRANSCOBALAMIN ISOTYPE AND SERUM HOMOCYSTEINE IN THE ELDERLY

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BACKGROUND: Transcobalamin (TC) is a 43kDa transport protein essential for the assimilation, tissue distribution and utilization of cobalamin. Polyacrylamide gel electrophoresis (PAGE) reveals four common phenotypic variants, M, X, S and F, with European population allelic frequencies of 0.59, 0.38, 0.02, and 0.01 respectively. TC phenotype is a determinant of serum cobalamin in healthy Caucasians, but it is not known whether it influences homocysteine (tHcy).

AIMS: To determine whether TC isotype is a determinant of non-fasting tHcy in an elderly population.Methods: 122 (48M, 74F) non-vitamin supplemented healthy volunteers (64) and dementia patients (58) from a UK study of cobalamin metabolism and Alzheimer's Disease (COBALZ II) were phenotyped (PAGE of neuraminidase-treated radiolabelled samples) and genotyped (codon 259 Arg/Pro polymorphism) for TC isotype. tHcy was assayed with the Drew Scientific DS30 Analyser. Results were analysed using a generalized linear model with diagnosis as an additional independent variable.

RESULTS: TC isotype mirrored known population prevalences (38MM (31%), 63MX (51%), 21XX(17%)), but was not a determinant of tHcy:

	Log-like	Chi-square	p-value
Sex	-317	0.0007	0.98
Age	-317	0.05	0.83
TC isotype	-317	1.41	0.49
Cobalamin	-318	2.79	0.09
Diagnosis	-319	4.77	0.09
Folate	-324	14.89	<0.001
Creatinine	-346	58.19	<0.0001

CONCLUSIONS: TC isotype is not a significant risk factor for the development of hyperhomocysteinaemia in an elderly population.

SERUM HOMOCYSTEINE LEVELS, CEREBROVASCULAR RISK FACTORS AND WHITE MATTER HYPOATTENUATION ON CRANIAL CT SCANS IN PATIENTS WITH POST-MORTEM CONFIRMED ALZHEIMER'S DISEASE

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BACKGROUND AND PURPOSE: White matter hypoattenuation (WMH) is recognised as an important risk factor for cognitive decline and dementia. We investigated the association of various cerebrovascular risk factors with WMH on X-ray computed tomography (CT) scans in patients with Alzheimer's disease (AD) and controls.
METHODS: We included those patients with clinical AD (n=157, of whom 104 were post mortem confirmed: AD-PM), other types of dementia (ODS, n=38) and controls (n=279) from the Oxford Project To Investigate Memory and Ageing (age 51-99 years) from whom a CT scan, serum total homocysteine (tHcy) and blood pressure data had been obtained at first episode.
RESULTS: Logistic regression for the whole cohort showed that the presence of WMH was associated with age, dementia severity, cerebral infarcts and systolic blood pressure. Analyses were repeated for only those participants with WMH and were stratified for diagnosis. For controls, only old age was a predictor, while for AD-PM, high tHcy and old age were predictive for Total WMH (severity x extent). In AD-PM, hypointensity of deep white matter, but not of periventricular white matter, was independently associated with tHcy. Gender, diabetes, smoking, presence of the apoE e4 allele, systolic blood pressure, dementia severity, or general brain atrophy were not associated with WMH in subjects with AD.
CONCLUSIONS: tHcy is unlikely to be a risk factor for the presence of WMH, but it is strongly associated with the severity and extent of WMH in subjects with AD.
SUPPORT: MRC, BMS, EU Biomed.

HYPERHOMOCYSTEINEMIA AND OXIDATIVE STRESS – A PATHOGENIC MECHANISM COMMON TO ALZHEIMER'S AND VASCULAR DISEASE?

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BACKGROUND: Elevated total serum homocysteine (tHcy) is associated with Alzheimer's disease (AD) and vascular disease. The mechanism underlying these associations is unclear.
AIMS: To investigate the relationship between tHcy and past history of vascular disease including hypertension, myocardial infarction, peripheral and cerebral vascular disease in patients with AD.
METHODS: The study comprised 65 patients from UK Psychogeriatric Assessment Services with features compatible with DSM-IV criteria for AD and 70 healthy cognitively intact age-sex matched volunteers from a General Practice in a comparable semi-rural area of predominantly lower socio-economic class. Vitamin supplemented subjects were excluded. Non-fasting tHcy was assayed with the Drew Scientific DS30 Analyser using reduction and fluorescence derivatisation followed by reverse phase chromatography. Body mass index, smoking status, haematological parameters, B12, folate, RCF and creatinine were determined. Generalized linear models for binomial distributions with logit link functions were used to investigate the effects of these variables on the development of both AD and vascular disease.
RESULTS: Although tHcy is a determinant of the presence of vascular disease (Log-likelihood -72.2, Chi-Square 10.24, p=0.001), both are independently associated with a diagnosis of AD ("Vascular" = Log-likelihood -52.1, Chi- Square 6.44, p=0.01; "Homocysteine" = Log-likelihood -53.5, Chi- Square 9.28, p=0.002).
CONCLUSIONS: Hyperhomocysteinaemia might reflect a pathogenic mechanism common to the development of AD and vascular disease. Vascular endothelium and brain are particularly sensitive to oxidative-stress. We propose that the influence of ambient redox status on key enzymes of homocysteine metabolism underpins the observed relationships between homocysteine, AD and vascular disease.

Appendix B Product Method Sheet for the Drew Scientific DS30 Hcy Homocysteine Assay Kit

DS30 Hcy 15 Test Assay Kit

Part Number (014-280)

INTRODUCTION

Intended Use

The Drew Scientific DS30 Hcy Homocysteine Assay Kit is intended for in vitro quantitative measurement of total homocysteine (tHcy) in plasma for the detection of hyperhomocysteinemia.
PLEASE NOTE: A number of pharmaceutical compounds have been reported to alter plasma homocysteine levels including methotrexate, tamoxifen, cyclosporine, anticonvulsants and antiepileptics. Also patients who have undergone transplantation surgery or who are suffering from renal disease may have elevated levels of total plasma homocysteine.

Summary and Explanation

Homocysteine (Hcy) is a thiol-containing amino acid derived from methionine by demethylation. The plasma concentration of Hcy is dependent on the two metabolic pathways by which homocysteine can be degraded. These involve re-methylation to methionine, by the enzyme methylene tetrahydrofolate reductase (MTHFR), and demethylation (or transulphuration) to cystathionine, by the enzyme cystathionine synthase (CS). Folic acid, and the vitamins B12 and B6 are important cofactors in these steps [1, 2].
Plasma concentrations of Hcy are normally quite low, as much of the metabolism of Hcy occurs within the intracellular matrix, however when metabolism is impaired, such as in enzyme or nutrient deficiencies, Hcy accumulates and is excreted into the plasma [3,4].
Within plasma some 70-80% of Hcy is protein bound with a variable amount present as Hcy dimers or mixed disulphides [5]. Measurement of tHcy includes all these forms [6].
A number of studies have been published that show a positive association between increased plasma homocysteine and various forms of arterial disease. A meta-analysis [7] of 27 of these studies, relating plasma Hcy to arteriosclerotic vascular disease, suggested that a 5mmol/L increase in plasma Hcy leads to an odds ratio for coronary artery disease risk in men of 1.6 (95%CI, 1.4 to 1.7) and an odds ratio for coronary artery disease risk in women of 1.8 (95%CI, 1.3 to 1.9). The same study also showed a positive association between elevated plasma Hcy and cerebrovascular disease and peripheral arterial disease.

Principle of the Procedure

Following addition of Internal Standard, the disulphide bonds in the calibrant / sample are reduced using the Reducing Agent. Protein is precipitated from solution and the thiol groups in the supernatant are then derivatized with a fluorescent thiol-specific dye. The fluorescent derivative mixture is then separated using the DS30 Hcy analyser which automatically calculates the homocysteine concentration. Alternatively, using a suitable HPLC system, with a reverse-phase column and mobile phase as described below, the derivatives can be separated and detected by their fluorescence (λ_{ex} = 385nm, λ_{em} = 515nm). Quantitative evaluation of the homocysteine concentration is achieved by comparison with a two-point calibration.

TEST COMPONENTS

DS30 Hcy Homocysteine Assay Kit

Reagent	Name	Description
A	Calibrator I	} 2 x 0.5mL, lyophilised calibrator set containing cysteine, homocysteine,
B	Calibrator II	cysteinyl-glycine and glutathione.
C	Internal Standard	1 x 0.5mL, 2-mercaptoethylamine.
D	Reducing Agent	1 x 1mL, Lyophilised, tris(2-carboxyethyl) phosphine hydrochloride.
E	TCA	1 x 1g, trichloroacetic acid. Corrosive.
F	EDTA Reagent	1 x 10mL, ethylenediaminetetraacetic acid.
G	Fluorophore	1 x 1.75mL, Lyophilised SBD-F.
H	Diluent 1	1 x 2mL, 1M sodium hydroxide. Corrosive.
I	Diluent 2	1 x 1.4mL, sodium borate buffer solution, pH 9.0.
J	Stop Reagent	1 x 0.5mL, 5M hydrochloric acid. Corrosive.

Additional Required Items

1. Pipettes to dispense 10µL, 20µL, 100µL, 200µL, 0.5mL, and 0.35mL.
2. Positive displacement pipettes to dispense 10µL and 200µL.
3. 1mL sample vials, to fit micro centrifuge.
4. 0.75mL amber glass vials.
5. Vortex Mixer.
6. Heating block to take 0.75mL amber glass vials.
7. Micro-Centrifuge.
8. Timer.
9. Drew Scientific DS30 Hcy Analyser or HPLC system (see Appendix)

PROCEDURE

The DS30 Hcy Homocysteine Assay Kit is designed for batches of 2 calibrants and up to 13 samples.

Specimen Collection

Blood should be collected into specimen tubes containing EDTA or lithium heparin as anticoagulant and the plasma separated from the red blood cells within one hour of collection, as synthesis and excretion of homocysteine will continue in the cells after sampling. The specimen should be kept cooled (<8°C) but not frozen, until separation.
Prior to analysis plasma should be stored frozen (-20°C), although plasma concentrations of homocysteine have been shown to be stable for up to four days at room temperature [9].

Quality Control

It is recommended that two levels of quality control material be used with each batch of samples. Level 1 should have a value in the normal range, and Level 2 should be above the threshold. Suggested control levels would be 10µmol/L and 20µmol/L respectively (Drew Scientific Part No. 014-185).
Two controls should be run at the beginning of the batch, following the calibrants. If one or more of the results obtained are outside of the manufacturers quoted range then check the equipment has been set correctly. Where no quoted range is available, ensure that obtained values lie within +/- 10% of the assigned value. After determining the source of error, calibration and quality control should be repeated. (It is important that the calibrants and quality control samples are prepared at the same time as the patient samples using the same kit).

Reconstitution of Calibrants (Reagents A & B)

1. Open vials carefully. Lyophilized reagents are bottled under reduced pressure.
2. Add 500µL of deionised water to each of Reagents A & B.
3. Let stand for 10 minutes, then agitate slowly to dissolve.

Reconstitution of Reducing Agent (Reagent D)

1. Open vial carefully. Lyophilized reagents are bottled under reduced pressure.
2. Add 1mL of deionised water to Reagent D.
3. Let stand for 10 minutes, then agitate slowly to dissolve.

Sample Preparation

Note: In order to obtain the best possible precision, it is essential that all timings are strictly observed and that positive displacement pipettes are used for dispensing samples and for the addition of Internal Standard (Reagent C).

1. Turn on the heating block, to allow enough time for it to reach 60°C ± 2°C before step 10.
2. For every calibrator (Reagents A & B), control and patient specimen, pipette 200µL into an appropriate labelled test tube (1mL plastic tube suitable for centrifugation).
3. Add 10µL of Reagent C and vortex mix.
4. Add 20µL of Reagent D, vortex mix and leave to stand at room temperature for 10 minutes. Meanwhile prepare the precipitation reagent by pouring the contents of Reagent F into Reagent E. Mix to ensure contents are dissolved.
5. After the samples have been incubating at room temperature for 10 minutes add 200µL of the precipitation reagent (Reagent E / Reagent F mixture).
6. Mix thoroughly on a vortex mixer, before centrifuging the samples for 2 minutes at 13,000 rpm.
7. Remove 100µL of each supernatant into numbered amber glass vials.
8. Prepare the fluorophore (Reagent G) as follows:
   a) Open vial carefully. Lyophilized reagents are bottled under reduced pressure.
   b) Add 350µL of Diluent 1 (Reagent H) swirl to mix.
   c) When all of the fluorophore has dissolved add all of Diluent 2 (Reagent I).
9. When the fluorophore is prepared immediately add 100µL to each of the samples.
10. Mix thoroughly on a vortex mixer. Place the samples in a heating block and incubate at 60°C ± 2°C for 50 minutes.
11. At the end of the incubation add 10µL of Reagent J and mix on a vortex mixer.
12. The samples are then ready for analysis.
13. Samples that cannot be analysed immediately must be covered until they are needed.

Analysis

1. Switch on the DS30 Hcy Analyser. Check that the reagent is connected and that the waste line runs to a collection container.
2. Place the samples into the DS30 Hcy Analyser carousel taking care to ensure that the low and high calibrants are placed in wells 1 and 2 respectively.
3. Close the Autosampler lid and start the analysis by pressing the Run Batch icon. Refer to the DS30 Hcy User Manual for detailed operator instructions.

NB The use of an HPLC system to analyse the samples is described in the Appendix.

SAFETY PRECAUTIONS

The following reagents are corrosive.

1. Reagent E contains trichloroacetic acid.
2. Reagent H contains a strong base.
3. Reagent J contains a strong acid.

Avoid skin contact or ingestion.

In case of skin or eye contact, wash thoroughly with water and seek medical attention.

In case of ingestion, wash out mouth thoroughly, give plenty of water to drink and seek medical attention.

INTERPRETATION OF RESULTS

Reference Ranges

It is important that each laboratory determine suitable reference ranges for the population it is testing. Homocysteine levels in plasma are known to vary with age, sex and genotype.
However the reference ranges cited in the majority of publications seem to agree with that suggested in reference 9 (Normal Range: £15.9µmol/L)
A study involving healthy volunteers was conducted to validate the literature consensus. A total of 146 samples were analyzed. None of the volunteers admitted to any previous or current cardiovascular or renal disease.
Excluded Results: One sample gave a value of 20.3µmol/L. On investigation, it was discovered that the individual concerned was receiving lamotrigine therapy, a drug known to inhibit dihydrofolate reductase and possibly, therefore, the cause of the slightly elevated homocysteine result. Two other results were excluded on the basis that they were far outliers from the dataset (>3 IQRs).
The volunteer group of 143 results from which the reference interval was calculated included 56 males and 87 females in the age range 18 – 60 yrs (mean age = 37 yrs). One of the volunteers was of Asian origin, whilst the remainder were Caucasian.
A 95% reference interval of 5.2 – 15.1µmol/L was determined.

Limitations

Although there is now substantial evidence that raised plasma homocysteine levels are associated with risk of cardiovascular disease, it has not been demonstrated unequivocally that lowering levels in patients reduces their risk of subsequent cardiovascular disease.

PERFORMANCE CHARACTERISTICS

Linearity

To determine linearity six concentration levels of homocysteine were prepared. Three replicates of each concentration were analyzed.
The results of this study showed that the DS30 Hcy Homocysteine Assay Kit is linear in the range 3.5 – 100µmol/L. The limit of sensitivity being demonstrated as 3.5µmol/L.

Imprecision

Within Batch Imprecision

Within batch imprecision was determined using commercial QC material, and reconstituted lyophilized human plasma spiked with an aqueous solution of homocysteine. Each sample was analyzed five times within a single batch of samples.

	QC I	QC II	Plasma A	Plasma B	Plasma ~15µmol/L	Plasma ~30µmol/L
1	7.4	18.1	7.8	7.1	14.4	27.2
2	7.1	18.8	7.6	6.9	14.3	28.8
3	7.0	18.5	7.4	6.7	14.2	29.0
4	6.7	18.5	7.3	6.8	14.4	29.7
5	6.9	20.7	7.3	6.7	14.5	28.7
Mean	7.0	18.9	7.5	6.8	14.4	28.7
SD	0.25	1.02	0.22	0.17	0.11	0.89
CV	3.6	5.4	3.0	2.6	0.8	3.1
N	5	5	5	5	5	5

Appendix B

Between Batch Imprecision

Between batch imprecision was determined by measuring two commercial QC materials and two plasma samples in duplicate in seven or eight different test batches as shown below.

	Conc. (µmol/L)			
Batch	QC I	QC II	Plasma 1	Plasma 2
1	7.7	18.7	6.6	7.4
	7.9	20.6	6.9	7.6
2	7.0	17.5	6.5	7.0
	7.3	17.2	6.6	7.0
3	7.7	17.6	6.6	7.1
	7.2	17.9	6.7	7.5
4	6.7	15.8	6.3	7.3
	7.0	16.7	6.6	7.5
5	7.4	18.6	7.1	7.8
	7.9	18.4	6.6	7.3
6	7.9	18.3	6.9	7.5
	7.8	18.0	6.7	7.2
7	7.5	18.1	6.7	7.9
	7.8	18.1	6.8	8.0
8	7.5	17.9	6.8	
	7.9	18.4	6.8	
Mean	7.5	18.0	6.7	7.4
SD	0.37	1.02	0.18	0.31
CV	4.9	5.7	2.6	4.1
N	16	16	16	14

Correlation

The DS30 Hcy Homocysteine Assay Kit was compared to the method of Ubbink *et al.* [10].
Using 102 patient samples the following correlation was achieved (Figure 1).

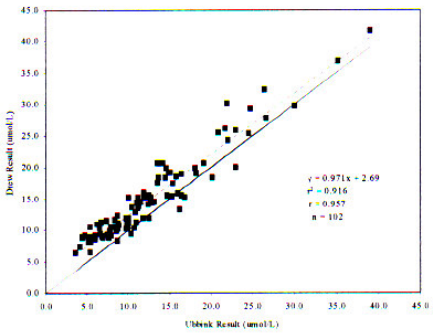

Figure 1 Correlation with the Ubbink method

The DS30 Hcy Homocysteine kit was also compared to the Beckman System 6300 Amino Acid analyser using 25 samples with the following result (Figure 2).

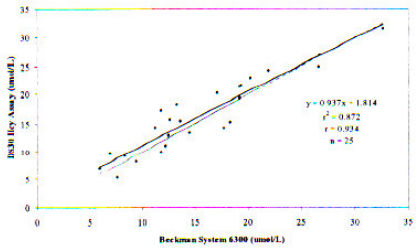

Figure 2 Correlation with Amino Acid Analyser.

Interferences

The fluorophore used is highly specific for thiol groups and therefore patients should refrain from the use of drugs containing this grouping prior to a blood sampling.
Three of these compounds were investigated for their interference in the method. Captopril was found to produce a very late, broad peak which may interfere with the sample following that in which it was present, and cysteamine will cause falsely low Hcy results.
Penicillamine at therapeutic levels did not interfere with the results.
A number of pharmaceutical compounds have been reported to alter plasma homocysteine levels including methotrexate, tamoxifen, cyclosporine, anticonvulsants and antiepileptics.
Patients who have undergone transplantation surgery or who are suffering from renal disease may have elevated levels of total plasma homocysteine.

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APPENDIX

HPLC System Specification

1. Column: C18-DB column (3.3cm x 4.6mm, 3µm particles)
2. Mobile Phase: 50mmol/L KCl, 10mmol/L Heptane Sulphonic Acid, 13mmol/L HCl, 2% (v/v) Methanol.
3. Pump: To maintain a flow rate of 1mL/min.
4. Injector: Capable of injecting 10µL of prepared sample.
5. Column Heater: To hold HPLC column at a constant temperature of 36°C.
6. Fluorescence Detector: Capable of excitation at 385nm and emission at 515nm.
7. Recorder/Integrator: Capable of monitoring and integrating the output of the fluorescence detector.

Preparation of Mobile Phase

1. Dissolve 1.864g of KCl and 1.011g of heptane sulphonic acid in 400mL of deionised water.
2. Add 10mL of methanol and 638µL of 10M HCl. Mix thoroughly and then make up to 500mL in a volumetric flask.

Preparation of HPLC System

1. Switch on the fluorimeter and set the excitation to 385nm and the emission to 515nm.
2. Switch on the column heater and allow to reach a steady 36°C before injecting any samples.
3. Flush the HPLC system through with the mobile phase, insert the column and then leave the column to wash in mobile phase at 0.5mL per minute, for 30 minutes.

Chromotography and Integration

1. Set the mobile phase flow rate to 1mL per minute
2. Inject 10µL of each of the prepared samples and allow elution to continue for 7 minutes before injection of the next sample.
3. Using a suitable integration system, identify each of the peaks as shown in the trace below (Figure 1.)
4. Determine the peak height of the Internal Standard and the Hcy peaks on each sample.

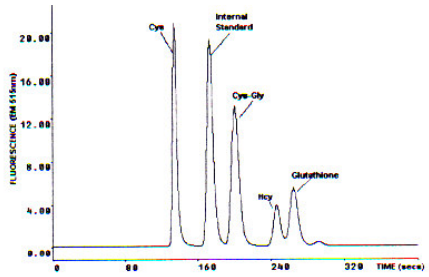

Figure 3 Chromatogram of a calibrant solution.

Calculations

1. Calculate the Hcy/Internal Standard peak height ratios for each sample (RATIO).
2. Plot the ratios obtained for the two calibrants against the concentrations as given on the reagents vials. (Reagents A & B).
3. Calculate the line of best fit in the form y = mx + c, where:
   y = RATIO
   m = the gradient
   x = Hcy concentration
   c = the intercept
4. Determine the Hcy concentration in the controls and specimens using the following formula.

Hcy Concentration = (RATIO − c) ÷ m

Drew Scientific Limited
Sowerby Woods Industrial Estate
Park Road
Barrow-in-Furness
Cumbria LA14 4QR
UNITED KINGDOM

014 IL 280 Rev. R&D A 11/00

Appendix B

Appendix C 510(k) Notification Approval - DS30 Hcy Blood Collection Tubes

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
2098 Gaither Road
Rockville MD 20850

SEP 1 8 2001

Dr. Andrew Kenny
Research & Development Director
Drew Scientific Ltd
Sowerby Woods
Industrial Estate
Barrow-in-Furness
Cumbria, LA14 4QR UK

Re: k012152
Trade/Device Name: DS30 Hcy Blood Collection Tubes
Regulation Number: 21 CFR 862.1675
Regulation Name: Blood Specimen Collection Device
Regulatory Class: II
Product Code: GIM
Dated: July 9, 2001
Received: July 11, 2001

Dear Dr. Kenney:

We have reviewed your Section 510(k) notification of intent to market the device referenced above and we have determined the device is substantially equivalent to devices marketed in interstate commerce prior to May 28, 1976, the enactment date of the Medical Device Amendments, or to devices that have been reclassified in accordance with the provisions of the Federal Food, Drug, and Cosmetic Act (Act). You may, therefore, market the device, subject to the general controls provisions of the Act. The general controls provisions of the Act include requirements for annual registration, listing of devices, good manufacturing practice, labeling, and prohibitions against misbranding and adulteration.

If your device is classified (see above) into either class II (Special Controls) or class III (Premarket Approval), it may be subject to such additional controls. Existing major regulations affecting your device can be found in the Code of Federal Regulations, Title 21, Parts 800 to 895. A substantially equivalent determination assumes compliance with the Good Manufacturing Practice for Medical Devices: General (GMP) regulation (21 CFR Part 820) and that, through periodic GMP inspections, the Food and Drug Administration (FDA) will verify such assumptions. Failure to comply with the GMP regulation may result in regulatory action. In addition, FDA may publish further announcements concerning your device in the Federal Register. Please note: this response to your premarket notification submission does not affect any obligation you might have under sections 531 through 542 of the Act for devices under the Electronic Product Radiation Control provisions, or other Federal laws or regulations.

This letter will allow you to begin marketing your device as described in your 510(k) premarket notification. The FDA finding of substantial equivalence of your device to a legally marketed predicate device results in a classification for your device and thus, permits your device to proceed to the market.

If you desire specific advice for your device on our labeling regulation (21 CFR Part 801 and additionally 809.10 for in vitro diagnostic devices), please contact the Office of Compliance at (301) 594-4588. Additionally, for questions on the promotion and advertising of your device, please contact the Office of Compliance at (301) 594-4639. Also, please note the regulation entitled, "Misbranding by reference to premarket notification" (21CFR 807.97). Other general information on your responsibilities under the Act may be obtained from the Division of Small Manufacturers International and Consumer Assistance at its toll-free number (800) 638-2041 or (301) 443-6597 or at its internet address "http://www.fda.gov/cdrh/dsma/dsmamain.html".

Sincerely yours,

Steven Gutman

Steven I. Gutman, M.D., M.B.A.
Director
Division of Clinical Laboratory Devices
Office of Device Evaluation
Center for Devices and
Radiological Health

Enclosure

K012152

10.0 Indication for use

Plastic, evacuated blood collection tube containing EDTA (anti-coagulant) and 3-deazaadenosine (enzyme inhibitor) for the collection and stabilization of 2.5 mL whole blood prior to removal of plasma for total homocysteine measurement by HPLC for the detection of hyperhomocysteinæmia.

✓ prescription use

Kesia Alexander for Jean Cooper
(Division Sign-Off)
Division of Clinical Laboratory Devices
510(k) Number K012152

Appendix D Product Method Sheet for the Axis Homocysteine EIA Assay Kit

Axis®
Homocysteine EIA
Package Insert

Axis®
Homocystein EIA
Packungsbeilage

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2 Summary and Explanation of the Test 1
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1 INTENDED USE

The Axis® Homocysteine Enzyme Immunoassay is intended for the quantitative determination of total L-homocysteine in human serum or plasma.

The device can assist in the diagnosis and treatment of patients suspected of having hyperhomocysteinemia and homocystinuria.

2 SUMMARY AND EXPLANATION OF THE TEST

Homocysteine is a thiol-containing amino acid produced by the intracellular demethylation of methionine. Homocysteine is metabolised to either cysteine or to methionine. In the vitamin B_6 dependent trans-sulphuration pathway homocysteine is irreversibly catabolised to cysteine. A major part of homocysteine is remethylated to methionine, mainly by the folate and cobalamin-dependent enzyme methionine synthase. Homocysteine accumulates in the cell and is exported to the circulation when these reactions are impaired.[1-4] Homocysteine circulates in plasma mostly in its oxidised forms (i.e, homocystine and cysteine-Hcy disulfide) bound to plasma proteins. Smaller amounts of reduced homocysteine and disulfide homocysteine (Hcy-SS-Hcy) are also present. Total homocysteine, (tHcy), represents the sum of free and protein bound homocysteine.

Severely elevated concentrations of tHcy are found in subjects with **homocystinuria**, a rare genetic disorder of the enzymes involved in the metabolism of homocysteine. Patients with homocystinuria exhibit mental retardation, early arteriosclerosis and arterial and venous thromboembolism.[1, 5]
Other less severe genetic defects which lead to moderately elevated levels of homocysteine are also found.[6, 7, 8]

Epidemiological studies have investigated the relationship between homocysteine levels in blood and **cardiovascular disease (CVD)**. A meta-analysis of 27 epidemiological studies, including more than 4000 patients, estimated that a 5 µmol/L increase in tHcy was associated with an odds ratio for coronary artery disease (CAD) of 1.6 (95% confidence interval (CI), 1.4 to 1.7) for men and 1.8 (95% CI, 1.3 to 1.9) for women. The odds ratio for cerebrovascular disease was 1.5 (95% CI, 1.3 to 1.9) or the same increase in risk as for 0.5 mmol/L increase in cholesterol.[9] Peripheral arterial disease also showed a strong association.

Patients with **chronic renal disease** experiences an excess morbidity and mortality due to arteriosclerotic CVD. Elevated concentration of tHcy is a frequent observed finding in the blood of these patients. Although they may lack some of the vitamins involved in the metabolism of homocysteine, the increased levels of tHcy are mainly due to impaired removal of homocysteine from the blood by the kidney.[10, 11]

Drugs that interfere with the homocysteine metabolism, e.g. nitric oxide, methotrexate, isoniacid, penicillamine, anti-convulsants and various antiepileptic drugs, may give elevated levels of total homocysteine.[12]

3 ASSAY PRINCIPLE

Axis® Homocysteine is an enzyme immunoassay for the determination of total homocysteine in blood.[13] Protein bound homocysteine is reduced to free homocysteine and enzymatically converted to S-adenosyl-L-homocysteine (SAH) in a separate procedure prior to the immunoassay.[14] The enzyme is specific for the L-form of homocysteine, which is the only form present in the blood.

Reduction
Homocysteine, mixed disulphide and protein bound forms of homocysteine in the sample are reduced to free homocysteine by use of dithiothreitol (DTT).

Prot-SS-Hcy
*R1-SS-Hcy —DTT→ Hcy
Hcy-SS-Hcy

*R1 is any thiol-residue.

Enzymatic conversion
Homocysteine in the test sample is converted to S-adenosyl-L-homocysteine by the use of SAH hydrolase and excess adenosine (Ad).

$$Hcy + Ad \xrightarrow{\text{SAH-hydrolase}} SAH + H_2O$$

The following solid-phase enzyme immunoassay is based on competition between SAH in the sample and immobilised SAH bound to the walls of the microtitre plate for binding sites on a monoclonal anti-SAH antibody. After removal of anti-SAH antibody not bound to the plate, a secondary rabbit anti-mouse antibody labelled with the enzyme horse radish peroxidase (HRP) is added. The peroxidase activity is measured spectrophotometrically after addition of substrate, and the absorbance is inversely related to the concentration of total homocysteine in the sample.

4 REAGENTS

Axis® Homocysteine EIA				AXH00001
Reagents	**Colour code**	**Content**	**Format**	**Volumes**
Reagent A Assay buffer	Brown	Phosphate buffer, 0.09% NaN_3	Ready to use.	54 mL
Reagent B Adenosine/DTT	White	Adenosine / dithiothreitol, citric acid.	Ready to use.	3.5 mL
Reagent C SAH-Hydrolase	White	Bovine S-adenosyl-L-homocysteine hydrolase, tris-buffer w/glycerol, methylparabene.	Ready to use.	3.5 mL
Reagent D Enzyme inhibitor	Orange	0.15% Merthiolate, phosphate buffer.	Ready to use. Dark bottle.	55 mL
Reagent E Adenosine deaminase	Red	Adenosine deaminase, phosphate buffer, BSA, 0.09% NaN_3, phenol-red dye.	Ready to use. Red coloured.	55 mL
Reagent F a-SAH antibody	Green	Monoclonal mouse-anti-S-adenosyl-L-homocysteine-antibody, BSA, 0.01% Merthiolate.	Ready to use.	25 mL
Reagent G Enzyme conjugate	Blue	Rabbit anti-mouse-antibody enzyme conjugate, BSA, horse radish peroxidase, blue dye.	Ready to use. Blue coloured.	15 mL

Reagents	Colour code	Content	Format	Volumes
Reagent H Substrate solution	Violet	TMB+ (Tetramethylbenzidine).	Ready to use. Dark bottle.	15 mL
Reagent S Stop solution	Yellow	0.8 M Sulphuric acid.	Ready to use.	20 mL
Wash buffer	Black	Phosphate buffer, 0.01% Merthiolate, Tween 20, BSA.	Concentrated. Dilute × 10 before use.	60 mL
Calibrators	White	S-adenosyl-L-homocysteine. 2 - 4 - 8 - 15 - 30 - 50 µmol/L in Assay buffer.	Ready to use.	6 x 1.5 mL
Microtitre strips		Coated with BSA S-adenosyl-L-homocysteine.	Ready to use.	12 x 8 wells

Axis® Homocysteine EIA Control kit			**AXH00101**
Controls	Diluted serum samples of human origin. Phosphate buffer, 0.09% NaN_3. Low, medium and high levels.	Ready to use.	3 x 1.5 mL

Axis® Homocysteine EIA Wash Buffer			**AXH00102**
Wash buffer	Phosphate buffer, 0.01% Merthiolate, Tween 20, BSA.	Concentrated, Dilute × 10 before use.	1000 mL

5 *PREPARATION AND STORAGE OF ASSAY COMPONENTS*

- All system reagents should be stored refrigerated (2 - 8 °C). Reagents are stable until expiration date when stored and handled as directed. Opened reagent kit is stable for 12 weeks when stored at 2 - 8 °C and handled as directed in this package insert.
- The sample pre-treatment solution (SPS) has to be made by mixing Reagent A, B and C (see instructions under chapter 9; Procedure). SPS solution is stable for one hour and has to be freshly made for each run.
- The wash buffer solution should be diluted 1:10 with distilled water before use. The prepared wash buffer is stable for 4 weeks when stored at room temperature (18 - 25 °C).
- Reagent D and H are stored in dark bottles to avoid exposure to light.
- It is important that the microtitre strips are kept dry, i.e. in the sealed bag with drying capsules, and stored refrigerated. Equilibration for a minimum of two hours is required to reach room temperature (18 - 25 °C). Leave the strips in the bag during equilibration.
- Only the necessary number of microtitre strips should be kept in the frame during the run. Unused strips should be kept in the sealed bag with drying capsules.
- Avoid exposure of the kit to temperatures exceeding 37 °C as this may denature the enzymes.

6 *MATERIALS NEEDED BUT NOT PROVIDED IN THE ASSAY-KIT*

- Homocysteine control (see chapter 11 for more information).
- Plastic or glass tubes for pre-treatment of samples.
- Pipettes / multipipettes 25 µL, 100 µL, 200 µL and 500 µL or 8 channel multipipette for 100 µL and 200 µL.

- Volumetric flask 50 mL and 600 mL.
- Incubator, 37 °C.
- Washer and reader for microtitre plates.

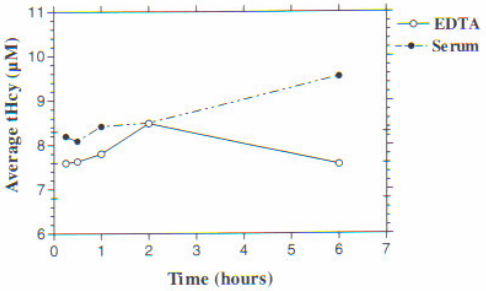
7 *SPECIMEN COLLECTION AND PREPARATION*

- As synthesis of homocysteine will take place in red blood cells after sampling, it is very important to centrifuge and separate plasma and serum from the blood cells as soon as possible.
- EDTA-plasma from anti-coagulated whole blood is recommended in the scientific literature.[15, 16] EDTA-plasma and/or serum may be used with the Axis® Homocysteine EIA assay. Serum samples should be allowed to clot for no more than 30 minutes before centrifugation and separation of serum. Serum samples should be kept on ice prior to separation. EDTA samples has to be centrifuged or put on ice immediately after drawing.

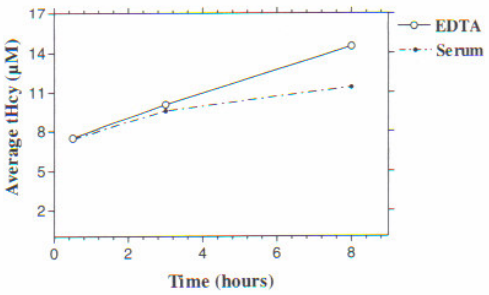
Plasma samples may be stored on ice for up to 6 hours prior to centrifugation and separation.

The following two figures show the effect of storage temperature on plasma (EDTA) and serum samples before centrifugation.

Concentration of tHcy in Serum and EDTA plasma when blood samples are stored on ice before centrifugation



Concentration of tHcy in Serum and EDTA plasma when blood samples are stored at room temperature before centrifugation



- Food consumption can affect circulating homocysteine levels. Protein rich meals give higher total homocysteine values and should be avoided late in the day before sampling.[15, 17]
- Standardised sampling procedures are crucial due to the above mentioned influencing factors.
- Complete mixing of thawed samples is required before use.
- Plasma or serum samples may be stored for 12 weeks at 2 - 8 °C, for up to 3 weeks at room temperature (18 - 25 °C) and have been shown to be stable for at least 8 months if frozen at minus 20 °C.
- Haemoglobin, bilirubin or triglycerides do not interfere with the assay.

8 *LIMITATIONS*

Technical limitations of the procedure

- If an automatic pipetting station is used, thorough washing of the tubing after addition of the blue coloured solution G (enzyme conjugate) may be needed - preferably with diluted acid followed by water. Any remaining solution in the tubing will interfere with the next assay step; i.e. addition of solution H (substrate solution).

- The washing procedure is critical for obtaining good precision. If manual washing is required, use 4 times 350 µL instead of 3 times 400 µL. After washing, empty the wells on paper towels.
- Avoid exposure of the kit to temperatures exceeding 37 °C as this may denature the enzymes.
- Specimens from patients who are on drug therapy involving S-adenosyl-methionine may show falsely elevated levels of homocysteine.
- Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibody (HAMA). HAMA, present in serum or plasma specimens, may interfere in immunoassays which utilise mouse monoclonal antibodies. These specimens should not be assayed with the Axis® Homocysteine EIA assay.

Clinical limitations related to metabolic interference

- Specimens from patients taking methotrexate, carbamazepine, phenytoin, nitrous oxide, anti-convulsants or 6-azauridine triacetate, may have elevated levels of homocysteine due to metabolic interference with the homocysteine metabolism.

9 *PROCEDURE*

Make sure all solutions and microtitre strips are equilibrated to room temperature before use. Leaving the kit at room temperature over night is recommended. We recommend to run the calibrators in duplicate and to perform a new calibration-curve for each run to avoid run-to-run variations using coated microtitre-plates.

Sample pre-treatment procedure:

1. Sample Pre-treatment Solution, **SPS**
   Has to be made within 1 hour prior to the start of the assay. Volume needed per 10 samples: (No dead volume calculated)

   4.5 mL **Reagent A** ***(brown)***
   0.25 mL **Reagent B** ***(white)***
   0.25 mL **Reagent C** ***(white)***

   Mix.

2. Dilute calibrators and samples/controls in plastic or glass tubes as follows:
   25 µL calibrator/sample/control
   + 500 µL **SPS**
   Mix well.
   Incubate 30 minutes at 37 °C.
   Cap the tubes or cover with parafilm during incubation.

   Proceed with step 3 before the samples have cooled.

3. Add 500 µL **Reagent D** ***(orange).***
   Mix well.
   Incubate 15 minutes at 18-25 °C.

4. Add 500 µL **Reagent E** ***(red).***
   Mix well.
   Incubate 5 minutes at 18-25 °C.

Microtitre plate procedure

5. Pipette 25 µL diluted calibrator / sample / control from *step 4* into the wells of the SAH-coated microtitre strips.

6. Add 200 µL **Reagent F** ***(green)*** to each well. Incubate 30 min at 18-25 °C. Use the enclosed lid during all incubations.

7. Wash with diluted **Wash buffer,** 3 × 400 µL (See chapter 8; Limitations)

8. Add 100 µL **Reagent G** ***(blue)*** to each well. Incubate 20 min at 18-25 °C.

9. Wash with diluted **Wash buffer**, 3 × 400 µL.

10. Add 100 µL **Reagent H** ***(violet)*** to each well. Incubate 10 min at 18-25 °C.

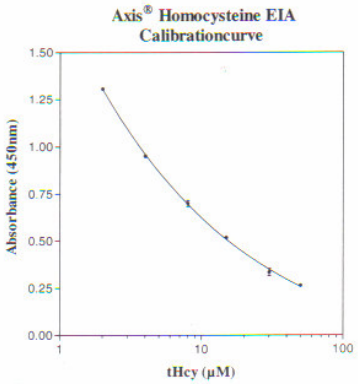
11. Add 100 µL **Reagent S** ***(yellow)*** to each well.

12. Shake and read at 450 nm within 15 minutes. Automatic plate shaker is preferred to ensure proper mixing.

10 INTERPRETATION OF RESULTS

- Results should be interpreted considering all other test results and the clinical status of the patient.
- We recommend that a four parameter logistic curve fit is used for preparing the calibration curve and calculation of unknown samples.

Below is an example of a calibration curve using the four-parameter logistic curve fit.



Example only, not to be used to determine values.

11 QUALITY CONTROLS

We recommend each laboratory to use a homocysteine control with known value.
Axis provides a set of low, medium and high controls. (Ordering number: AXH00101).
The controls contain L-homocysteine in processed human serum (non-reactive for HIV-1/2 Antibody, HCV Antibody, Hepatitis B Core Antibody, HTLV-1 Antibody, HIV-1 Antigen and Hepatitis B Surface Antigen) in the following concentrations:

Control level	Mean value Hcy µmol/L	Range Hcy µmol/L
Low Control	7.0	5.6 - 8.4
Medium Control	12.5	10.0 - 15.0
High Control	25.0	20.0 - 30.0

12 REFERENCE VALUES

The reference range should be determined by each laboratory to confirm with the characteristics of the population being tested. As a point of reference, the following data may be used until the laboratory has analysed a sufficient number of samples to determine its own reference range.
The total homocysteine concentration in plasma or serum of healthy individuals vary with age, gender, geographical area and genetic factors. Scientific literature reports reference values for adult male and females between 5 and 15 µmol/L. They also report men to have higher values than women, and post menopausal women to have higher homocysteine values than pre menopausal women.[18, 19]
Homocysteine values will normally increase with age, giving a reference range among elderly (> 60 years) of 5 - 20 µmol/L.[20, 21]

Samples from 382 males and females (100 Scandinavians; 54 males aged 30 - 60, 46 females

aged 29 - 70. 185 Hispanics; predominantly males aged 20 - 65. 97 Americans; 54 males aged 16 - 74, 43 females aged 15 - 79), apparently healthy, without information on current medications, disease states or known risk conditions for elevated homocysteine, were tested using the Axis® Homocysteine EIA. Median value of the homocysteine concentration among Scandinavians was 8.4 µmol/L, among Hispanics/Arizona 8.9 µmol/L and among American/Cleveland 9.3 µmol/L.
Homocysteine reference range was established based on 95% confidence limit as 5 - 15 µmol/L for the Scandinavian population, 3.6 - 15.0 µmol/L for the American/Cleveland population and 2.9 - 16.0 µmol/L for the Hispanic/Arizona population.

13 MEASURING RANGE

The calibrator range is from 2.0 to 50.0 µmol/L.

14 PERFORMANCE DATA

Assay Precision

Precision was tested running 3 levels of controls for 8 days, 4 replicates per run of each level.
Precision data are summarised in the table below.

	Average Hcy concentration	Intra assay precision	Total precision
Low	6.1 µmol/L	7.3%	9.3%
Med.	10.3 µmol/L	6.8%	8.1%
High	21.4 µmol/L	5.2%	7.1%

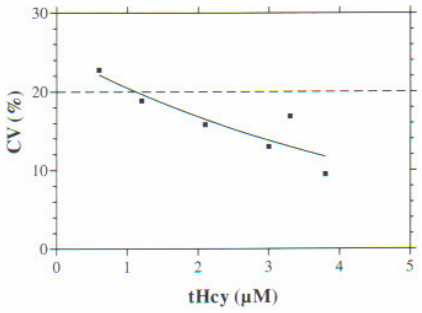
Limit of quantification

The limit of quantification was tested using diluted SAH-calibrators, ranging from 4.0 µmol/L to 0.5 µmol/L.
The quantification limit of the Axis® Homocysteine EIA is 1.0 µmol/L with a CV < 20%.

Limit of quantification

Hcy µmol/L	Average result µM	SD µmol/L	CV %
4.0	3.8	0.4	9.5
3.0	3.3	0.6	17
2.5	3.0	0.4	13
2.0	2.1	0.3	16
1.0	1.2	0.2	19
0.5	0.61	0.14	23

Limit of Quantification
CV as a function of the tHcy concentration



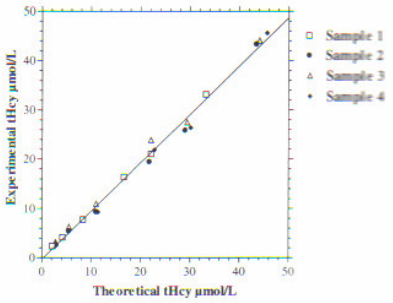
Linearity of diluted plasma samples

If the homocysteine concentration of a sample exceeds the range of the calibration curve, the sample should be diluted with Reagent A; assay buffer, and reanalysed.

The linearity of the Axis® Homocysteine EIA was evaluated by diluting four high patient samples with varying amounts of Reagent A as diluent.

The results below show that the average recovery in % for each dilution step is within 90-110% of the calculated theoretical value.

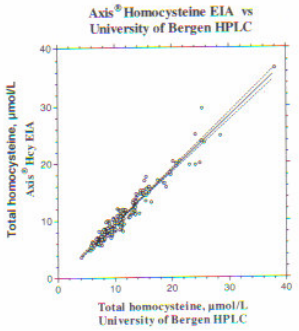
Linearity for Axis® Homocysteine EIA
Dilution recovery of 4 samples



Slope = 0.98
Intercept = - 0.35 µmol/L
Correlation coefficient (r^2)= 0.99

Method Comparison

The Axis® Homocysteine EIA was compared to the University of Bergen HPLC method.[22]
A comparison of 164 patient samples ranging from 3 - 37 µmol/L homocysteine rendered the following:



Slope = 0.94, Intercept = - 0.09 µmol/L.
Correlation coefficient (r^2)= 0.94.

Interfering Substances

Bilirubin, haemoglobin, lipids, red blood cells, protein and sodium fluoride were spiked into plasma samples and tested for interference by Axis® Homocysteine EIA.
The following interference's were found:

Interfering Substance	Interfering Substance Concentration	% Interference
Bilirubin	0.5 g/L	- 4.2
Haemoglobin	10.0 g/L	- 4.6
Triglycerides	2 g/L	6.0
	10 g/L	1.5
Red blood cells	1.0 % v/v	- 9.0
	5.0 % v/v	- 4.9
Protein	80 g/L	1.8
Na-Fluoride	10.0 g/L	- 3.8

Cross reactivity

Solutions of Adenosine, S-adenosyl-L-methionine (SAM), Cystathionine, L-Cysteine, Gluthathione and Thiolactone were spiked into plasma samples in concentrations more than 20-fold the physiological concentrations. The following cross-reactivity was found when tested with Axis® Homocysteine EIA:

Compound	Concentration mmol/L	% Cross Reactivity
Adenosine	5.0	0
S-adenosyl-L-methionine	0.5	16.3
Cystathionine	0.5	0.3
L-Cysteine	100	0
Gluthathione	100	0.01
Thiolactone	0.5	0.3

15 PRECAUTIONS

- The Axis® Homocysteine EIA assay is for *in vitro* diagnostic use.
- The Axis® Homocysteine EIA assay only measures L-homocysteine and not D-homocysteine.
- Reagent F contains mouse antibodies. Reagent G contains rabbit antibodies.
- Reagent D contains 0.15% merthiolate (≤ 0.074% mercury). Please handle and dispose of properly.
- Controls contain sera originating from human blood samples. The source materials have been tested and found to be negative for HIV-1, HIV-2 and Hepatitis B and C. However, the handling of blood derivatives should be done according to recommended procedures for handling infectious material. HAS publication no. (CDC) 84-8395 or local/national guidelines

on laboratory safety procedures should be referred.

- Some of the reagents contain < 0.1% sodium azide, NaN_3, as preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.
- 0.01% merthiolate is used as preservative in some reagents. Each kit contains less than 0.028% mercury. Please handle and dispose appropriately.
- Reagents with different lot numbers must not be interchanged.
- Do not use the kit after the expiration date on the outer box.

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