The health economic benefits of improved diagnosis of vitamin B12 deficiency in primary care patients, associated with a new diagnostic testing algorithm

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The health economic benefits of improved diagnosis of vitamin B12 deficiency in primary care patients, associated with a new diagnostic testing algorithm

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Declaration

I declare that the data presented in this thesis has been composed solely by myself and that to the best of my knowledge has not previously been submitted for any other degree or diploma except where due reference has been made in the text. I understand that plagiarism and/or the use of unacknowledged third-party data is dealt with seriously as misconduct by Manchester Metropolitan University.

Signed: 

Dated: 24th June 2024

Print Name: Jayne Patricia Parkes

List of abbreviations

Ado-Cbl	Adenosyl Cobalamin
AfC	Agenda for change
ALTM	All Methods Laboratory Trimmed Mean
ANOVA	Analysis of Variance
AUC	Area under the curve
B12	Vitamin B12
BCPS	Black Country Pathology Services network
cB12	Combined B12
Cbl	Cobalamin
CBS	Cystathionine Beta Synthase
CCG	Clinical Commissioning Group
CEA	Cost-effectiveness analysis
CE-IVD	*Conformité Européenne* – in vitro diagnostic device
CI	Confidence Interval
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CLSI	Clinical and Laboratory Standards Institute
CMIA	Chemiluminescent Microparticle Immunoassay
CoA	Coenzyme A
CPB	Competitive Protein Binding
CPS	Counts per second
CRM	Certified reference material
CV	Coefficient of Variation
DA	Diagnostic accuracy
DHFR	Dihydrofolate Reductase

DNA	Deoxyribose Nucleic Acid
dTMP	Deoxythymidine Monophosphate
dUMP	Deoxyuridine Monophosphate
EDTA	Ethylenediaminetetraacetic Acid
EFLM	European federation of clinical chemistry and laboratory medicine
eGFR	Estimated Glomerular Filtration Rate
EPU	Extended Information Processing Unit
EQ-5D	EuroQol questionnaire
EQA	External Quality Assurance
ESI	Electrospray Ionisation
FBC	Full Blood Count
FBCM	Food bound cobalamin malabsorption
FBP	Folate Binding Protein
fL	Femtolitre
FN	False negative
FP	False positive
GC-MS	Gas Chromatography Mass Spectrometry
GI	Gastrointestinal
GIRFT	Getting it right first time
GLD	Glutamate dehydrogenase
GP	General Practitioner
GPC	Gastric Parietal Cell
Hb	Haemoglobin
HC	Haptocorrin
Hct	Haematocrit

Hcy	Homocysteine
HH2	Histamine H2
HO-Cbl	Hydroxocobalamin
Holo-TC	Holotranscobalamin (Active B12)
HPLC	High Performance Liquid Chromatography
HRA	NHS Health Research Authority
HRT	Hormone replacement therapy
ICB	Integrated Care Board
IC	Incremental cost
IE	Incremental effect
ICER	Incremental Cost-Effectiveness Ratio
ICT	Integrated Chip Technology
IDMS	Isotope Dilution Mass Spectrometry
IF	Intrinsic Factor
IgG	Immunoglobulin Gamma
IM	Intramuscular
IQC	Internal Quality Control
IQR	Inter Quartile Range
IS	Internal standard
ISE	Ion Selective Electrode
KSD	Kolmogorov-Smirnov D statistic
LC	Liquid chromatography
LCMS/MS	Liquid Chromatography Tandem Mass Spectrometry
LIMS	Laboratory Information Management System
LoB	Level of Blank

LoD	Level of Detection
LoQ	Level of Quantification
MCM	Methylmalonyl-CoA Mutase
MCV	Mean Cell Volume
Me-Cbl	Methylcobalamin
MLTM	Method Laboratory Trimmed Mean
MMA	Methylmalonic Acid
MRP1	Multidrug Resistance Protein 1
MS	Methionine Synthase
5-MTHF	5-Methyltetrahydrofolate
MTHFR	Methylene Tetrahydrofolate Reductase
MU	Measurement of Uncertainty
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NEQAS	National External Quality Assurance Scheme
NHANES	National Health and Nutrition Examination Survey
NICE	National Institute for Health and Care Excellence
NMB	Net monetary benefit
N_2O	Nitrous Oxide
NPV	Negative predictive value
NRBC	Nucleated Red Blood Cells
OCP	Oral contraceptive pill
PA	Pernicious Anaemia
PPI	Proton Pump Inhibitor
PPV	Positive predictive value

Q	Quadrupole
QALY	Quality Adjusted Life Years
QoL	Quality of life
RCC	Red Cell Count
RLU	Relative Light Unit
RNA	Ribose Nucleic Acid
ROC	Receiver operating characteristic
Rs	Resolution
RT	Retention time
RV	Reaction Vessel
RWT	The Royal Wolverhampton NHS Trust
SAH	S-Adenosyl Homocysteine
SAM	S-Adenosyl Methionine
SCD	Subacute Combined Degeneration
SD	Standard Deviation
Se	Sensitivity
SLS	Sodium Lauryl Sulphate
Sp	Specificity
TB12	Total B12
TC	Transcobalamin
TCN2	Transcobalamin gene
T-Hcy	Total Homocysteine
THF	Tetrahydrofolate
TN	True negative
TP	True positive

TS	Thymidylate Synthase
TSH	Thyroid Stimulating Hormone
TTG-IgA	Tissue Transglutaminase IgA (coeliac screen)
U+E	Urea and Electrolytes
UKAS	United Kingdom Accreditation Service
WBC	White Cell Count
WHO	World Health Organisation
WTP	Willingness to pay

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Abstract

Introduction: Vitamin B12 is an essential micronutrient. Deficiency is common but can be difficult to diagnose and without early recognition and treatment can lead to significant and irreversible neurological damage.

The aim of this project was to compare a new B12 deficiency diagnostic testing algorithm, where deficiency is diagnosed if the first line test, Holo-transcobalamin (Holo-TC), is <25 pmol/L and if the second line test, methylmalonic acid (MMA) (reflexed for indeterminate Holo-TC results (25-50 pmol/L) is >280 nmol/L and >360 nmol/L for ≤65 years and >65 years, respectively), to an algorithm using Total Vitamin B12 (TB12). A cost-effectiveness analysis (CEA) of the two algorithms was performed. A second CEA was performed to investigate TB12 with MMA because some laboratories do not have access to Holo-TC.

Methods: This was a retrospective study at The Royal Wolverhampton NHS Trust on 1003 patients with suspected B12 deficiency. The Abbott Diagnostics Active B12 kit (CE-IVD) on Abbott Architect System i2000 analyser and the MassChrom® MMA reagent kit for liquid chromatography tandem mass spectrometry (CE-IVD), employing a Shimadzu high performance liquid chromatography system and Applied Biosystems Sciex 6500 Q-Trap tandem mass spectrometer with an Electrospray Ionisation source were implemented and verified against manufacturers pre-defined quality specifications. TB12, Holo-TC, MMA, renal function tests, thyroid stimulating hormone and serum folate were analysed on surplus serum, if not already measured routinely, on the Abbott Architect c16000 and i2000 analysers. Full Blood Count analysis was

performed on Sysmex XN series analysers. Patients that were deficient for TB12 (<187 ng/L) or had results in the indeterminate range (187-300 ng/L) were followed up for one year and additional tests undertaken, and treatment received recorded. The diagnostic accuracies of TB12 and Holo-TC, were calculated using MMA as the proxy 'gold' standard test of deficiency from receiver operator curve (ROC) analysis. CEAs were performed using decision tree analysis (TreeAge Pro software).

Results: Holo-TC (<25 pmol/L) and TB12 (<187 ng/L), had similar diagnostic accuracies for B12 deficiency, using MMA as the proxy 'gold' standard test, 0.78 (95% CI 0.75-0.81) and 0.75 (95% CI 0.72-0.78), respectively. The sensitivity 0.60 (95% CI 0.54-0.66) and specificity 0.99 (95% CI 0.98-0.99) of the new testing algorithm was improved compared to the TB12 algorithm (sensitivity 0.25 (95% CI 0.20-0.30), specificity 0.95 (95% CI 0.93-0.96). When the Holo-TC indeterminate range was modified to the NICE 2024 (NG239), recommendation of 25-70 pmol/L then the algorithm sensitivity further increased to 0.78 (95% CI 0.73-0.83). Haemoglobin, red cell count, mean cell volume and patient symptoms were not reliable indicators of B12 deficiency, when utilised in isolation. The proposed new B12 deficiency algorithm generated both cost and utility benefits to patients with a favourable incremental cost-effectiveness ratio (ICER) of -276.68. Sensitivity analysis, varying the costs or utilities, adjusted the ICER values however most changes still met the cost-effectiveness threshold. A secondary CEA using NG239 Holo-TC indeterminate range of 25-70 pmol/L showed that using a combination of TB12 and MMA was also cost effective.

Conclusion: The new diagnostic testing algorithm utilising the combination of Holo-TC ± MMA assays was cost-effective and provided health benefits for patients. Use of this algorithm will provide earlier detection and treatment of B12 deficiency and prevention of potential significant neurological consequences benefitting patient healthcare. Alternatively for those laboratories unable to access an Holo-TC assay then the combination of TB12 and MMA is also a cost-effective option.

Chapter 1.0 INTRODUCTION

1.1 Anaemia

Anaemia is defined by the World Health Organisation (WHO) as a reduced haemoglobin concentration or number of red cells. As the haemoglobin molecule is essential for the carriage of oxygen in the bloodstream, decreases result in reduced ability to deliver oxygen to tissues causing features such as fatigue, weakness, and shortage of breath. Adequate haemoglobin concentrations change with age. Adult patients are classed as anaemic if haemoglobin concentrations for males are <130 g/L and for non-pregnant females <120 g/L. Causes of anaemia range from inherited blood cell disorders, chronic diseases, infections, pregnancy related, inflammatory conditions and nutritional deficiencies either from inadequate intake or malabsorption. The commonest form of deficiency is iron deficiency however deficiencies in vitamin B12 (B12) and folate are also seen (WHO, 2023).

1.2 Vitamin B12

1.2.1 Vitamin B12 homeostasis

B12, also known as cobalamin because it contains a corrin ring with cobalt at its centre (Figure 1.1), is a water soluble vitamin (Herrmann and Obeid, 2012) that has an essential role in nervous system function and is a coenzyme for methylmalonyl-CoA mutase, and methionine synthase required for the production of red blood cells and deoxyribose nucleic acid (DNA) (Salinas et al, 2018). The corrin ring is a 15 membered ring of four pyrrole subunits connected

by methylene links and two pyrroles are directly joined that surround a central cobalt ion. The cobalt ion is held in place by four nitrogen atoms from the pyrrole groups. The sixth ligand of the cobalt atom confers B12's metabolic activity as it can link to:

1. a methyl group (CH3) - as in methylcobalamin
2. a 5'-deoxyadenosine at the 5' position - as in adenosylcobalamin (coenzyme B_{12})
3. a cyanide group - as in Vitamin B_{12} - as supplied from drug companies (cyanocobalamin).

The cobalt (Co) atom in the cobalamins (Co-corrin complexes) can shuttle between three oxidation states, Co(I), Co(II) and Co(III) which is the enabling factor for all of the activities of B12.

For the methyl transfer reactions involving methylcobalamin (in the cytosol), Co(I) is involved. The reaction catalysed by methionine synthase involves two methyl group transfers:

1. Methylcob(III)alamin + homocysteine ---> cob(I)alamin + methionine
2. cob(I)alamin + methyltetrahydrofolate ---> Methylcob(III)alamin + tetrahydrofolate

The second cofactor role of B12 is for adenosylcobalamin which is involved in rearrangement reactions, e.g. d-methylmalonyl-CoA-mutase in the degradation of odd-numbered carbon skeletons of amino acids (threonine, valine, methionine) and fatty acids (propionyl-CoA). In B12 deficiency methylmalonyl-CoA is hydrolysed to methylmalonic acid and excreted. The formation of adenosylcobalamin takes place in the mitochondria. This requires the central

Co(III) atom to be reduced to Co(I) in a reaction dependent on reduced nicotinamide adenine dinucleotide (NADH/H+) and flavin adenine dinucleotide (FADH2). This results in the conversion of methylmalonyl CoA to succinyl CoA, catalysed by methylmalonyl-CoA mutase, and energy generation for the cell (Gröber, Kisters and Schmidt, 2013).

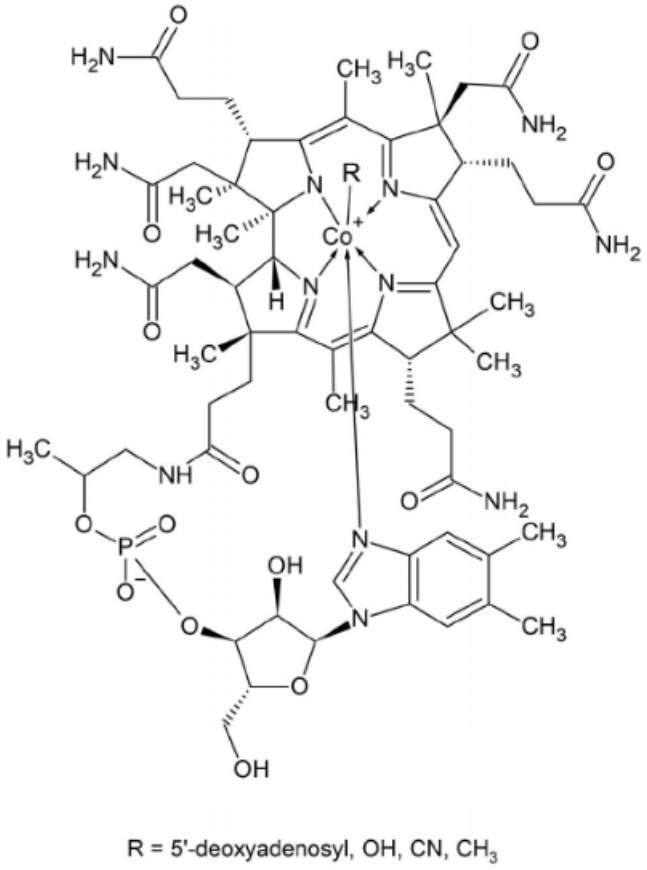

Figure 1.1. The chemical structure of vitamin B12. The corrin ring is a 15 membered ring of four pyrrole subunits connected by methylene links and two pyrroles are directly joined that surround a central cobalt ion. The cobalt ion is held in place by four nitrogen atoms from the pyrrole groups. The corrin ring has multiple ligands which are utilised in vitamin B12's metabolic role (Gröber, Kisters and Schmidt, 2013).

B12 in nature is produced *de novo* by some prokaryotes, singled celled microorganisms which include certain bacteria (e.g. *Lactobacillus rossiae and Lactobacillus reuteri*) and archaea, but not by plants. Both aerobic (bacteria) and anaerobic (archaea) biosynthetic pathways exist as well as a salvage pathway. Approximately 30 enzyme-catalysed steps are involved in converting δ-aminolevulinate (ALA) via uroporphyrinogen III and adenosylcobyric acid to its final form adenosylcobalamin. The central cobalt (catalytic site) is incorporated early (in anaerobic organisms) or late (in aerobic organisms) (Fang, Kang and Zhang, 2017).

The synthesised prokaryotic B12 is transferred and accumulates in animal tissues and can occur in some plant and mushrooms through microbial interaction. Ruminants acquire B12 via a symbiotic relationship with their stomach bacteria (B12-synthesising). The B12 synthesised is absorbed in the intestine, transferred into the blood and stored in the liver and muscles of the animal or secreted in the milk. In aquatic environments B12 produced by bacteria and archaea (*Thaumarchaeota)* is taken up by B12-requiring bacteria as well as eukaryotic phytoplankton which then become food for larval fish and bivalves. Edible plant and mushrooms gain B12 from concomitant B12-synthesising bacteria in the soil and environment (Watanabe and Bito, 2018). Therefore, humans acquire B12, formed by microbial interaction, from ruminants (cattle and sheep) and fish (or shellfish). Other good dietary sources of B12 are liver, kidney, pork, chicken and dairy items which enhances the risk of inadequate intake in those with a vegan or vegetarian diet (Hoffbrand, 2016).

Large scale industrial production of B12 is by microbiological fermentation using *Pseudomonas denitrificans, Propionibacterium shermanii* or *Sinorhizobium meliloti,* requiring complex and expensive media and long fermentation cycles. More recently *Escherichia coli* has been used with genes from *Salmonella typhimurium* in the production of B12 (Fang, Kang and Zhang, 2017).

Body stores of B12 are usually between 1 and 3 mg with a small turn over (0.1%) daily. Deficiency occurs when the store of B12 falls below 300 µg. Dietary deficiency can take many years to manifest as a 1 mg store will last approximately 3 years (Allen, 2008).

1.2.2 Vitamin B12 absorption

The absorption of B12 is complex (Figure 1.2). The recommended daily allowance for adults to ensure absorption of 1 µg/L is 2.4 µg/L to 2.8 µg/L, with the average absorption from food being approximately 50% (Stabler and Allen, 2004). B12 absorption can be passive through the duodenum and ileum however this is inefficient, or active through the ileum (Neilsen et al, 2012).

When eating food, salivary and oesophageal glands produce haptocorrin (HC) (a cobalamin-binding protein) (Allen, 2008). In the stomach, gastric epithelial cells secrete hydrochloric acid and pepsin, which releases the B12 from food which binds to haptocorrin. The parietal cells of the stomach secrete intrinsic factor (IF) which binds B12 to form a B12-IF complex which is used as a transporter via the duodenum to the terminal ileum (O'Leary and Samman,

2010). In the presence of calcium, the B12-IF complex is absorbed in the terminal ileum by the membrane protein cubilin where it is endocytosed, degraded, and recycled (Rizzo, et al, 2016).

In the bloodstream around 80% of B12 is bound to haptocorrin, forming holo-haptocorrin and about 20% is bound to transcobalamin, forming holo-transcobalamin (Holo-TC). Only B12 bound to transcobalamin is available to the cells, hence Holo-TC is also called active B12 (Hunt, Harrington, and Robinson, 2014).

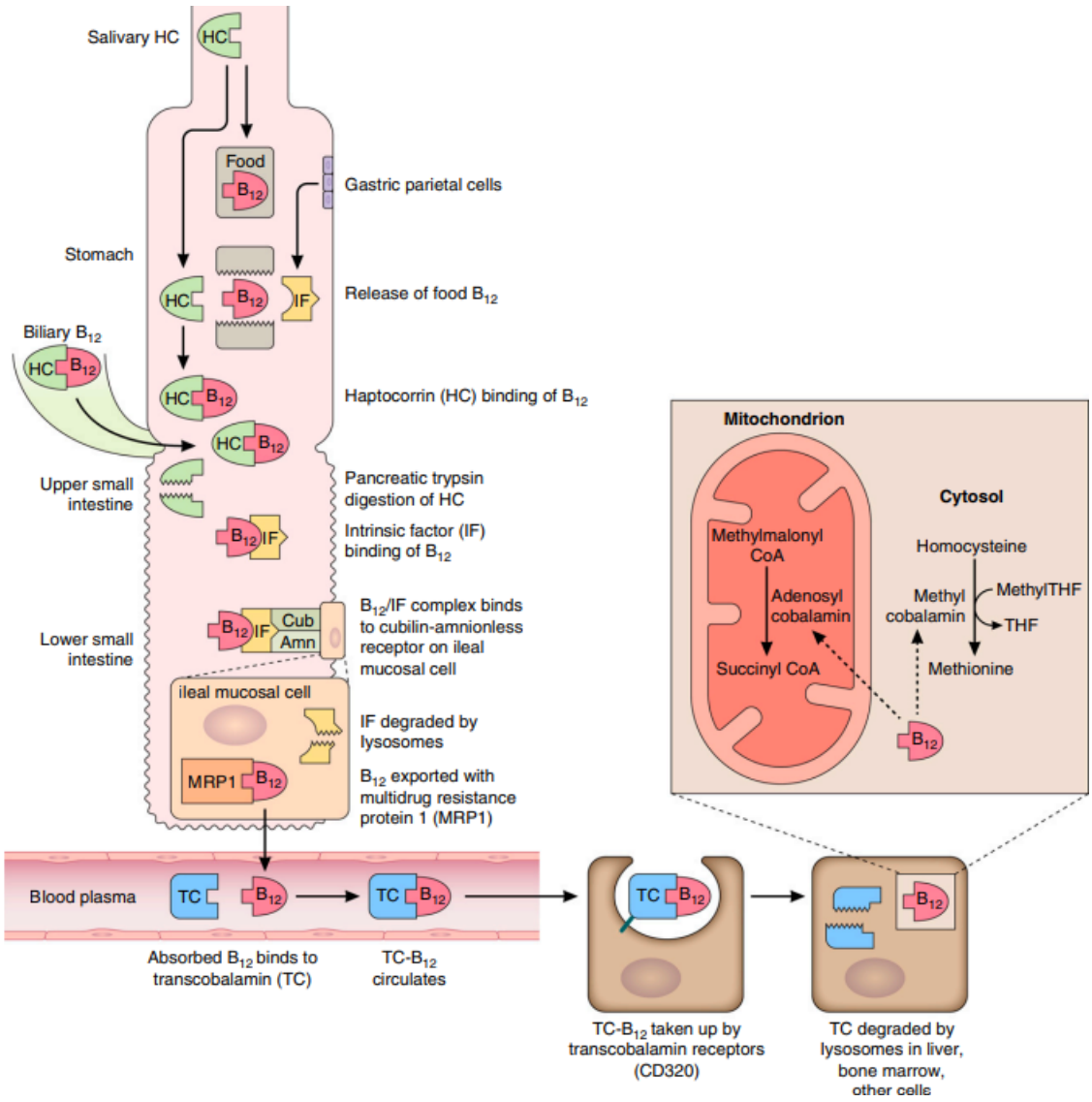

Figure 1.2. B12 absorption and cellular uptake. Food B12 is released in the stomach and binds to salivary haptocorrin (HC). In the small intestine pancreatic proteases release B12 which binds to intrinsic factor (IF). The IF-B12 complex then binds to the cubam receptor in the terminal ileum mucosal cell. B12 is exported with MRP1 to the blood circulation and B12 binds to transcobalamin (TC). TC receptors on all rapidly dividing cells convert B12 to adenosylcobalamin in the mitochondria and methylcobalamin in the cytosol to serve as cofactors for the two B12 dependent enzyme reactions, methylmalonyl-CoA mutase and methionine synthase respectively. CoA, coenzyme A; THF, tetrahydrofolate, MRP1, multidrug resistance protein 1 (Green, 2017).

1.2.3 Vitamin B12 metabolism

Both B12 and folate are required for DNA synthesis and once absorbed they undergo a series of reactions leading to the synthesis of purines and pyrimidines (O'Leary and Samman, 2010). The synthesis of methionine synthase, a vitamin B12 dependent enzyme drives the metabolic action (Figure 1.3). The cobalamin pathway is a series of reactions that involves the conversion of cob(II)alamin (2+) to cob(I)alamin (1+) and then to methylcob(III)alamin (3+), to regenerate the cob(I)alamin (1+) cofactor. B12 is delivered to methionine synthase forming cob(I)alamin (1+), (the most reduced form) and binds to the methyl group of the substrate, 5-methyltetrahydrofolate to form methylcob(III)alamin (3+), which transfers its methyl group to homocysteine (Hcy) to form methionine and tetrahydrofolate (THF) products. Re-methylation of methionine to give homocysteine completes the methionine cycle.

In the folate cycle, folic acid (synthetic form of vitamin B_9) is reduced to dihydrofolate by dihydrofolate reductase (produced in the liver and requires NADPH as a cofactor) with further reductions generating THF (the active co-enzyme form). Methylenetetrahydrofolate reductase (MTHFR) converts THF to 5-methyl-THF (5-MTHF), the active form of folic acid. 5,10 methylene-THF is used in the production of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP) being the rate limiting step in the synthesis of DNA, while 10-formyl-THF is required for new purine synthesis (Froese et al, 2019).

In the mitochondrion methylmalonyl-CoA mutase catalyses the conversion of methylmalonyl CoA to succinyl CoA and this reaction is part of the degradation of odd chain fatty acids, cholesterol and branched chain amino acids. B12, in the form of adenosylcobalamin, acts as the coenzyme for this reaction. Succinyl CoA is then used in the Krebs cycle for energy production in aerobic respiration.

When B12 or folate deficiency occurs the substrates of these enzymes accumulate, with B12 deficiency an increase in is seen in methylmalonic acid (MMA) and Hcy is increased when either B12 or folate deficiency is present (Nagaratnam, Nagaratnam and Cheuk, 2018) and megaloblastic changes in blood cells may be seen.

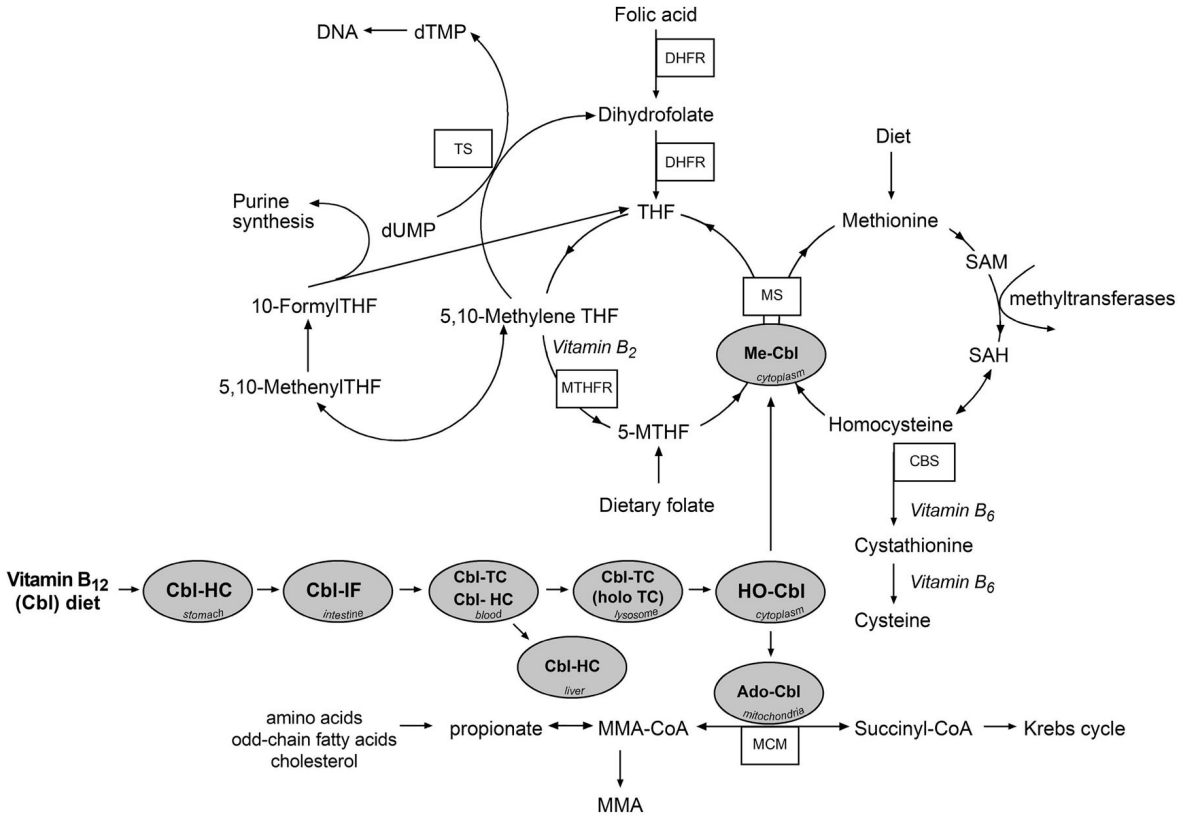

Figure 1.3. Vitamin B12 processing via two enzymatic pathways in production of DNA. Cbl: cobalamin; CBS: cystathionine beta-synthase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; DHFR: dihydrofolate reductase; HC: haptocorrin; holoTC: holotranscobalamin; HO-Cbl: hydroxocobalamin; IF: intrinsic factor; MS: methionine synthase; Me-Cbl: methylcobalamin; MTHFR: methylene tetrahydrofolate reductase; MMA: methylmalonic acid; MCM: methylmalonyl-CoA mutase; Ado-Cbl: adenosylcobalamin; 5-MTHF: 5-methyltetrahydrofolate; SAH: S-Adenosyl homocysteine; SAM: S-Adenosyl methionine; THF: tetrahydrofolate; TS: thymidylate synthase; TC: transcobalamin (Sobczyńska-Malefora et al, 2021).

1.3 B12 Deficiency

1.3.1 Prevalence of B12 deficiency

The reported prevalence of B12 deficiency in the UK population is estimated to be approximately 6% under the age of 60 years but nearer to 20% in the over 60-year-olds. For people with vegan diets, around 11% are B12 deficient (Hunt, Harrington and Robinson, 2014). International data (USA and Netherlands),

propose prevalence if <40 years is around 3%, 4% between ages of 40-60 years, 6% when >60 years or >20% when>85 years (NICE, 2023).

1.3.2 Clinical presentation of B12 deficiency

The clinical presentation of B12 deficiency can be very variable but has high morbidity if untreated. Features are often non-specific and typical of ageing e.g. fatigue, loss of memory and depression. Anaemia may cause mucosal changes such as glossitis or 'beefy red tongue' (Zhou, 2018) as well as angular stomatitis and jaundice, but some patients are asymptomatic until severely anaemic. Others, present with neurological and psychiatric symptoms, such as confusion, delirium and pins and needles. The neurological complications may occur with or without anaemia and, include peripheral neuropathy and subacute combined degeneration (SCD) which arises from demyelination of the dorsal and lateral columns of the spinal cord. SCD presents with an unsteady gait which if untreated can cause irreversible damage of the central nervous system resulting in paralysis and dementia ('megaloblastic madness') (Nagaratnam, Nagaratnam and Cheuk, 2018), however early treatment aids regeneration of damaged axons (Dharmarajan and Norkus, 2001). B12 deficiency is associated with brain atrophy, structural weaknesses in hippocampus, higher brain volume losses than seen in normal ageing and severe white matter lesions (Nagaratnam, Nagaratnam and Cheuk, 2018). Patients with B12 concentrations in the lower third of the reference range are more than four times more likely to have confirmed Alzheimer disease (Clarke et al, 2007b). The loss

of grey matter in Alzheimer's can be slowed with B12 therapy (Iqtidar and Chaudary, 2012, Spence, 2016, Porter et al, 2016).

Patients presenting with the above features to their General Practitioner (GP) are likely to have a Full Blood Count (FBC) test. In moderate to severe cases the FBC can be indicative of B12 deficiency with a raised mean cell volume (MCV) (>100 fL) and accompanying blood film features of oval macrocytes and hypersegmented neutrophils, however folate deficiency cannot be excluded at this stage and the morphological changes are neither sensitive nor specific in early B12 deficiency (Herrmann and Obeid, 2012) and approximately 25% of patients with neurological impairment, owing to B12 deficiency, have a normal MCV (Voukelatou et al, 2016).

Subclinical B12 deficiency is often asymptomatic and is approximately 10 times more common than clinical B12 deficiency, however it is not possible to predict which asymptomatic patients will go on to become symptomatic (Hvas, Ellegaard and Nexø, 2001) however some patients may have subtle neurological symptoms that may respond to B12 therapy (Kumar, 2014).

1.3.3 Causes of B12 deficiency

A wide array of causes of B12 deficiency exist. Although dietary insufficiency (vegetarian/vegan) can result in B12 deficiency the main cause is malabsorption which can be of three types, (1) intestinal malabsorption owing

to, for example, ileal resection, Crohn's disease, stagnant loop syndrome, tropical sprue, fish tapeworm and bariatric surgery (Stabler, 2013), (2) an autoimmune disorder producing IgG antibodies to the B12 transport protein, intrinsic factor (IF), or to gastric parietal cells (GPC) which signifies classical pernicious anaemia (PA) (Shipton and Thachil, 2015); (3) a congenital abnormality as in the case of congenital IF deficiency (Wolffenbuttel et al, 2019) or a genetic defect such as Imerslund-Gräsbeck syndrome (Gräsbeck, 2006, Nielsen et al, 2012), which are often identified in childhood.

The commonest cause of B12 deficiency in the elderly (40-70%) is food-bound cobalamin malabsorption (FBCM) which worsens with advancing age owing to the susceptibility of the elderly to atrophic gastritis, with its associated increase in *H Pylori* infections and reduced gastric secretions, preventing B12 release from food and therefore, its absorption (Wong, 2017).

PA is an autoimmune disorder producing IgG antibodies to transport protein IF and/or GPC, preventing adequate B12 absorption. Anti-parietal cell antibodies occur in 90% patients with PA but only 5% of healthy adults (sensitivity >90%, specificity 50%), similarly, binding and blocking IF antibodies are found in most patients with PA (sensitivity 50%, specificity 98%). Patients with PA have, in addition to the megaloblastic anaemia, gastric parietal cell atrophy, achlorhydria and epithelial cell atrophy. PA was common in North European women >50 years old, familial, associated with the development of other autoimmune diseases including vitiligo, Hashimoto's thyroiditis, Addison's disease (Mohamed et al, 2020) and has been linked to stomach and colorectal cancer (Murphy et

al, 2015, Miranti et al, 2017). In recent NICE guidelines (NG239) (2024), the term autoimmune gastritis is used instead of PA, as life-threatening anaemia associated with chronic severe B12 deficiency occurs rarely. Autoimmune gastritis is defined in NG239 as 'a chronic inflammatory condition that can lead to B12 deficiency, it is associated with auto-antibodies against gastric parietal cells and intrinsic factor, these may or may not be present but if are, can destroy parietal cells reducing secretion of gastric acid preventing release of B12 from food, in addition it can affect the ability of parietal cells to produce intrinsic factor further reducing B12 absorption' (NICE, 2024).

The elderly and women are more susceptible to the development of autoimmune disorders, and in women is thought to be due to having two X chromosomes which can become mutated (Angum et al, 2020).

Many drugs such as corticosteroids, antibiotics, gastric ulcer medications (proton pump inhibitors (PPIs), histamine H2 (HH2) blockers), gout medication, metformin for diabetes, the oral contraceptives pill (OCP) and hormone replacement therapy (HRT) are associated with a reduced concentration of B12. For some of these, such as corticosteroids and antibiotics the mechanisms remain unclear.

PPIs cause B12 deficiency by inhibiting the H^+/K^+-ATPase proton pump of the gastric parietal cell, reducing the number of hydrogen ions released into the stomach and decreasing the production of gastric acid. Since gastric acid is

required to free protein bound B12 for absorption this is the likely mechanism for B12 deficiency (Heidelbaugh, 2013, Mumtaz et al, 2022). Similarly, HH2 blockers inhibit the interaction of histamine with the parietal cell histamine H2 receptor. This blocks a cyclic adenosine monophosphate (cAMP)-dependent pathway that promotes H^+/K^+-ATPase function therefore reducing gastric acid production (Miller, 2018).

For gout medication such as colchicine, a reduction of intrinsic factor receptor levels in the ileum was postulated as the cause of reduced B12 absorption in guinea-pigs (Stopa, O'Brien and Katz, 1979), but there is no information about the cause in humans.

The mechanisms for the reduction in B12 absorption caused by metformin are not fully understood but interference of the calcium dependent binding of the intrinsic factor/B12 complex to the cubam receptor in the terminal ileum has been proposed (Bell, 2022).

Berenson and Rahman (2012) found decreased B12 levels in those using the OCP or HRT, especially in the first 6 months. The decrease was not clinically significant and did not result in B12 tissue depletion. The low total B12 is thought to be caused by a reduction in haptocorrin (transcobalamin I, TCN1) and an increase in transcobalamin III (TCN3).

There has been an increase in B12 deficiency seen in adolescents and younger adults due to increased recreational use of nitrous oxide (N_2O) as an illegal high. N_2O misuse leads to the cobalt ion in B12 becoming rapidly oxidised (from 1+ to 3+ valence state). This results in irreversible inactivation, functional B12 deficiency and impaired metabolic processes leading to neurological deficits. These can often be reversed with aggressive B12 supplementation and cessation of N_2O use but may take months to resolve. Some patients have a low B12 but up to 30% will have normal results (Campdesuner et al, 2020, Harker, Seed and Jogia, 2020, Mahgoub, Deliwala and Bachuwa, 2021, Martinez and Tabaac, 2021). The detailed mechanism for the neurological impact involves the oxidation of the cobalt in cob(I)alamin forming cob(II)alamin Cob(II)alamin is unable to accept methyl groups, decreasing the formation of methylcobalamin (the cofactor for methionine synthase) which results in reduced conversion of Hcy to methionine (Met) and an accumulation of Hcy. Met is required for myelin production and maintenance and therefore, demyelination occurs in the peripheral and central nervous systems. Cob(II)alamin also acts as a cofactor for methionine synthase reductase (MSR) and it is suggested that the accumulation of cob(II)alamin causes increased MSR activity, converting cob(II)alamin to cob(III)alamin, through S-adenosylmethionine, leading to more Hcy production and thereby preventing the in-built reductive recovery system from working. The effect of N_2O on MMA-CoA mutase which converts MMA-CoA to succinyl-CoA is still debated, however the oxidation of cob(I)alamin results in adenosylcobalamin deficiency (cofactor for MMA-CoA mutase) leading to overall B12 deficiency and this causes an accumulation of MMA (Garakani et al, 2016, Gernez et al, 2023). This explains why methionine synthase is impacted initially whilst methylmalonyl-CoA mutase

is impacted later. In November 2023, N_2O became a Class C drug, illegal in the UK to possess, supply, import, export or produce outside its intended purposes (House of Commons, 2023).

A high alcohol intake can result in B12 deficiency due to food intake being replaced by alcohol. Alcohol also irritates the stomach and intestine mucosal lining leading to gastritis which reduces B12 absorption and may result in liver damage impairing its ability to store and release B12 (Fragasso, 2013).

Chronic kidney disease (CKD) increases with age with B12 deficiency a common complication. The kidney plays an important role in Holo-TC metabolism. Holo-TC is filtered at the glomerulus and is reabsorbed in the proximal tubule by megalin. Renal disease therefore causes a functional B12 deficiency due to decreased reabsorption of Holo-TC in the proximal tubule. Furthermore, haemodialysis patients often have poorer diets due to dietary restriction and foods high in B12 often also contain high levels of electrolytes (McMahon et al, 2015).

B12 deficiency is prevalent in pregnancy due to increased requirements and haemodilution effects (Dror and Allen, 2012). Using a cut-off of 150 pmol/L TB12, Sukumar et al (2016a) investigated associations between obesity, gestational diabetes (GDM) and B12 in the UK (West Midlands) and found overall B12 deficiency in 26% of pregnant women (32% and 22% with and without GDM, respectively) in a multi-ethnic cohort. Sukumar et al (2016) in a

meta-analysis of B12 deficiency, involving cohorts from across the world, found B12 deficiency in 21%, 19% and 29% of pregnant women, in 1st, 2nd and 3rd trimesters, respectively using a cut-off value of 150 pmol/L. In 2021, Saravanan et al, reported a prevalence of B12 insufficiency of 42.3% in a multi-centre, multi-ethnic cohort of pregnant women of 26-28 weeks gestation, with lower concentrations seen in those with GDM. However, a B12 cut-off of 220 pmol/L was used in this study and may account for the higher percentage of insufficiency. The assays used to identify deficiency in pregnancy have variable sensitivity, TB12 concentrations fall even when there is no deficiency due to reduced haptocorrin (Koebnick et al, 2002). Haemodilution and hormone changes (increased oestrogen) impact MMA and T-Hcy concentrations (Dimitrova et al, 2002). T-Hcy concentrations are much lower in pregnancy, due to reduced plasma albumin, but increase throughout the trimesters (Walker et al, 1999). The assay recommended by NICE (NG239) to identify B12 deficiency in pregnancy is Holo-TC which appears to give an accurate assessment of B12 status (NICE, 2024). This study will not investigate B12 deficiency in pregnancy as these patients are excluded.

It is known that genetic variations can cause differences in B12 status with at least 59 B12-related gene polymorphisms from 19 genes reported (Sunendran et al, 2018). These single-nucleotide polymorphisms (SNPs) can be categorised into six groups based on their roles in B12 metabolism:

- co-factors or regulators for transport e.g. fucosyltransferase 2 (FUT2), fucosyltransferase 6 (FUT6), methylmalonic aciduria and homocystinuria cblC type (MMACHC), TCN1, transcobalamin II (TCN2),

- membrane transporters e.g. ATP binding cassette subfamily D member 4 (ABCD4), cubilin (CUBN), CD320 molecule (CD320),
- enzymes in the one-carbon cycle e.g. cystathionine beta synthase (CBS), methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR),
- cell cycle regulation e.g. membrane spanning 4 domains A3 (MS4A3),
- mitochondrial proteins e.g. citrate lyase beta like (CLYBL), methylmalonic aciduria (cobalamin deficiency) cb1A type (MMAA), methylmalonyl-CoA mutase (MUT),
- other genes with unknown function (actin like 9 (ACTL9), carbamoyl-phosphate synthase 1 (CPS1), DNA methyltransferase/TRNA aspartic acid methyltransferase 1 (DNMT2/ TRDMT1), paraoxonase 1 (PON1).

These genetic differences can contribute to individual variability in vitamin B12 status and susceptibility to deficiency. It has also become evident that some SNPs are more prevalent in certain ethnic groups whilst the same SNP has been found to have a different effect on B12 concentration in different populations. Some examples of this are discussed.

The MMACHC Gene (chromosome 1p34.1) encodes a chaperone protein which binds to B12 in the cytoplasm catalysing cyanobalamin into cob(II)alamin (Kim, Gherasim and Banerjee, 2008). In Icelandic populations SNP rs12272669 (A>G, R206Q) is associated with higher vitamin B12 levels in 'A' allele carriers (Grarup et al, 2013). In North European populations SNP rs10789465 shows associations with B12 levels (Andrew et al, 2013) however it is unclear how these variants affect gene regulation.

The FUT2 Gene (chromosome 19q13.33) encodes fucosyltransferase 2, an enzyme involved in the synthesis of the Lewis blood group antigens (Das and Haloi, 2014). These antigens mediate the attachment of gastric pathogens (H pylori) to the gastric mucosa which can affect the absorption of B12 (Azevedo et al, 2008) which has been postulated as the mechanism for the variation. However, some investigators have found that H pylori status does not influence FUT2 and B12 (Oussalah et al, 2012). Velkova et al (2017) found that the FUT2 SNP (rs601338) influences B12 concentrations via HC glycosylation but does not affect Holo-TC explaining some of the disparity seen between TB12 and Holo-TC test results.

Seven SNPs have been associated with the FUT2 gene and some are more common in certain ethnic groups. In European populations, for SNP rs601338 (G428A, W143X), the 'A' allele is common and associated with blood group non-secretor status, which is linked to higher vitamin B12 levels (Hazra et al, 2009). In Indian populations the 'G' allele is more common and is associated with lower B12 concentrations, which may partly explain why Indians are found to have lower B12 results (Tanwar et al, 2013). In East Asian populations the rs601338 variant is rare (Hu et al, 2016) but SNP rs1047781 (A385T) is common, associated with non-secretor status and higher vitamin B12 levels but this SNP is not seen in Caucasian populations (Kudo et al, 1996).

For SNP rs602662 (A>G, G258S) the 'A' allele is associated with a lower risk of B12 deficiency, this SNP is seen in Caucasians and Indians (Tanwar et al, 2013,

Zinck, de Groh and MacFarlane, 2015). For SNP rs492602 (A428G) the 'A' allele is associated with lower B12 concentrations in Europeans (Hazra et al, 2009) In Canadians 'G' alleles are more prevalent and associated with a lower risk of B12 deficiency (Zinck, de Groh and MacFarlane, 2015).

The FUT6 Gene (chromosome 19p13.3) encodes fucosyltransferase 6 with same function as FUT2. For the SNP rs3760776 (A>G) the 'A' allele is associated with higher B12 concentrations in Chinese (Lin et al, 2012), Indian (Nongmaithem et al, 2017) and Icelandic (Grarup et al, 2013) populations.

The TCN2 Gene (chromosome 22q12.2) encodes transcobalamin 2 (holo-TC) which binds to B12 in enterocytes. This complex enters the portal circulation ready for use in target tissues. In Caucasian populations, the SNP rs1801198 (776 C>G, P259R) shows mixed associations with vitamin B12 levels. Some studies report lower vitamin B12 levels in 'CC' genotype carriers (Stanislawska-Sachyadyn et al (2010), while others find no significant association (Reidel et al, 2011). In Portuguese populations, the 'G' allele of rs1801198 is associated with lower Holo-TC concentrations (Castro et al, 2010).

The CUBN Gene (chromosome 10p13) encodes the intestinal receptor cubilin which recognises the B12-IF complex and binds to Amnionless to internalise B12 into intestinal cells (Fyfe et al, 2004). For the same SNP conflicting data is seen. In Nordic and European populations, the 'G' allele is associated with higher vitamin B12 concentrations (Grarup et al, 2013) however, in Canadian

populations the 'G' allele of rs1801222 (G>A, F253S)) is associated with a higher risk of vitamin B12 deficiency (Zinck, de Groh and MacFarlane, 2015).

The ABCD4 gene (chromosome 14q24.3) encodes for a transporter protein, which moves B12 from lysosomes to the cytosol. Once in the cytosol B12 can be converted to methylcobalamin and adenosylcobalamin (Fettelschoss et al, 2017). In Nordic populations the ‘T’ allele of the rs3742801 (T>C, E368K) and ‘C’ allele of the rs4619337 variants were associated with higher B12 concentrations (Grarup et al, 2013).

The CD320 gene (chromosome 19p13.2) encodes the Holo-TC receptor. Variants may alter the receptor's ability to bind and internalise transcobalamin bound B12, influencing cellular B12 levels. In Canadians the ‘C’ allele of the rs2336573 (T>C, G220R) variant was associated with a lower risk of B12 deficiency (Zinck, de Groh and MacFarlane, 2015) however in adults from Iceland and Denmark the ‘T’ allele was associated with higher B12 concentrations (Grarup et al, 2013).

The MTHFR gene (chromosome 1p36) codes for a critical enzyme involved in homocysteine remethylation. MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in an irreversible reaction. Two MTHFR SNPs are the rs1801133 (C677T) and rs1801131 (A1298C) variants both associated with reduced enzyme activity. Thuesen, et al (2010) reported that ‘T’ allele carriers of the C677T genotype variant were

associated with an increased prevalence of low B12 concentrations in Danish adults.

The MS4A3 gene (chromosome11q.12.1) encodes a haematopoietic cell cycle regulator and may have a role in renewal of intestinal and gastric epithelial cells involved in absorption of B12 (Li et al, 2005). Lin et al (2012) found Chinese adults with the 'T' allele for SNP rs2298585 had higher B12 concentrations.

The MMAA gene (chromosome 4q31) encodes a protein that transfers B12 to the mitochondria. In Indian and Nordic populations, the ‘C’ allele for rs2270655 (G>C, Q363H) is associated with lower concentrations of B12 (Nongmaithem et al, 2017, Grarup et al, 2013).

The MUT gene (chromosome 6p12.3) encodes for the enzyme which converts methylmalonyl-CoA to succinyl-CoA using B12 as cofactor (Adenosylcobalamin). 'T' allele carriers for rs1141321 (C>T, R532H) and 'C' allele carriers for rs9473555 both have lower B12 concentrations in Chinese (Lin et al, 2012), Nordic (Grarup et al, 2013) and Caucasian (Hazra et al, 2009) populations.

The CLYBL gene (chromosome 13q32.3) encodes for the enzyme citramalyl-CoA lyase located in the mitochondrion converting citramalyl-CoA into acetyl CoA and pyruvate, essential intermediates in energy production. Approximately, 5% of humans have a stop codon SNP which is associated with low

concentrations of B12, however the mechanism is unknown (Strittmatter et al, 2014). The association between SNP rs41281112 (C>T, R259X) and B12 concentrations has been studied in two different populations. Lin et al (2012) found that the 'T' allele was associated with lower B12 concentrations in Chinese adults and Grarup et al (2013) found the same in Nordic adults.

Genetic studies provide some evidence that ethnicity may impact B12 status Most genetic studies, however, have been conducted in Caucasian populations with little research undertaken in Black populations. Health inequalities may occur if a single unified reference range for B12 status is utilised. This study will look at the effect of ethnicity on B12 status.

1.3.4 Treatment of B12 deficiency

Until recently treatment of B12 deficiency in primary care was dependent on the presence or absence of anaemia (pernicious) or neurological symptoms. In 2023, NICE recommended, if present, 1 mg hydroxocobalamin injected intramuscularly on alternative days until no further improvement and then hydroxocobalamin 1 mg injected intramuscularly every 2 months (lifelong). If there were no neurological symptoms then treatment was intramuscular 1 mg hydroxocobalamin three times a week for 2 weeks followed by 1 mg every 3 months (lifelong) if non-dietary cause. If dietary insufficiency was thought to be the cause, then oral cyanocobalamin (1 mg) tablets or 1 mg hydroxocobalamin injected every 6 months should be used.

Following the release of NICE guidance 239 in March 2024, treatment options remains the same although more emphasis is given to who should receive treatment based on whether the cause is irreversible or reversible with IM treatment recommended (in addition to those with neurological symptoms), in patients with confirmed autoimmune gastritis, those following gastrectomy or ileal resection and for those with medicine induced B12 deficiency. If oral B12 replacement is required then the supplement dosage should be 1 mg containing cyanocobalamin, methylcobalamin or adenosylcobalamin (NICE, 2024).

1.4 Laboratory diagnosis of Vitamin B12 deficiency

If B12 deficiency is suspected then history, examination and investigations should be undertaken including a FBC and blood film and tests to measure the concentration of B12 in the serum.

1.4.1 FBC changes and morphology

As discussed in section 1.3.2. the FBC is used as the first line test for investigation of anaemia and whilst a raised MCV with associated low haemoglobin concentration can be indicative of B12 deficiency it is not sensitive nor specific. Furthermore, blood film morphology changes are not seen until deficiency is well established. Platelets and white blood cells can be low and occasional primitive blast cells can be present in severe cases leading to the name megaloblastic anaemia. The reticulocyte count is also low in relation to the degree of anaemia.

The role of B12 in the production of DNA has been outlined in Figure 1.3. and deficiency affects all dividing cells especially proliferating cells of the bone marrow. When B12 deficiency occurs, ribonucleic acid (RNA) synthesis progresses unhindered in the cytoplasm, the red cells develop nuclear-cytoplasmic imbalance (asynchrony) with abundant basophilic cytoplasm and enlarged nuclei. The slowed synthesis of DNA leads to prolonged cell cycling and the cells released into the circulation without their normal number of divisions. Thus, the red cells are enlarged and oval shaped and the neutrophils hypersegmented due to retention of surplus nuclear material leading to the typical morphological features seen (Aslinia, Mazza and Yale, 2006) (figure 1.4).

Neutrophils are called hypersegmented if the neutrophils have six or more lobes or more than 3% of neutrophils have at least five lobes (Bain, 2017).

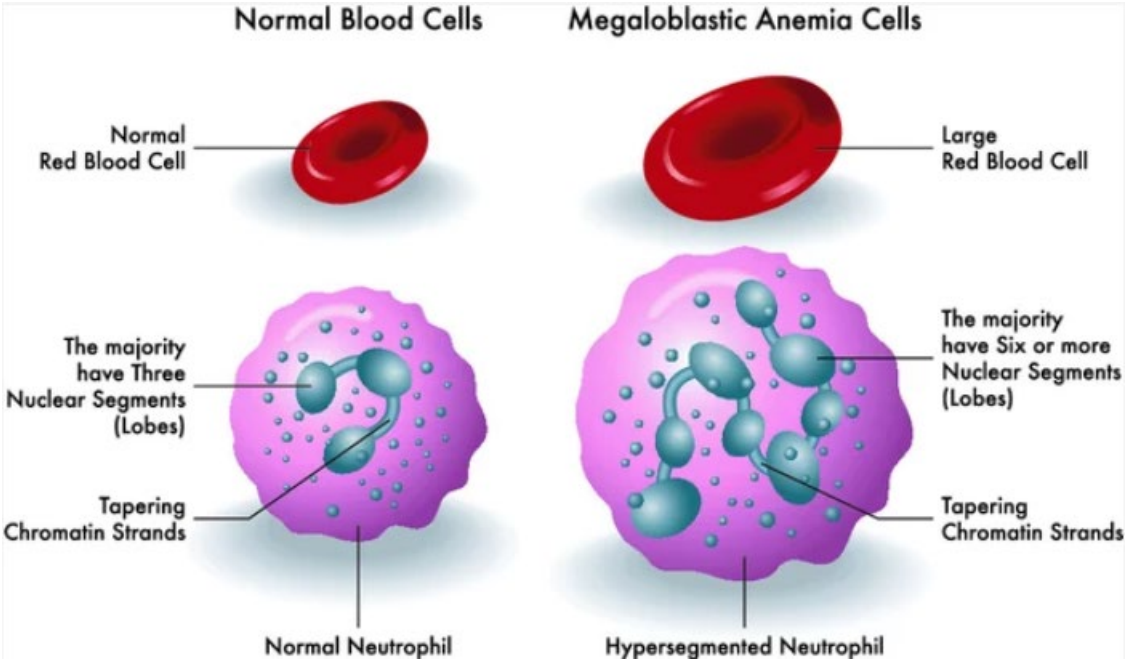

Figure 1.4. Comparison of normal and megaloblastic anaemia cells, showing enlarged oval macrocytes and neutrophils with hypersegmented nuclei (image downloaded from Stock Adobe, 2024).

Due to the lack of specificity and sensitivity of the FBC in identifying patients with B12 deficiency, tests to measure the concentration of B12 in the body are used and the most common test is the serum Total B12 assay.

1.4.2 Serum Total B12 (TB12) assay

Competitive protein binding (CPB) immunoassays widely used in the UK for measurement of TB12, are inexpensive and a range of kit suppliers exist.

The British Society of Haematology 2014 guideline for the diagnosis and treatment of Cobalamin and Folate disorders document the lack of specificity and sensitivity of TB12 for robust diagnostic testing (Devalia, Hamilton and Molloy, 2014).

The main limitation of TB12 assays is their lack of specificity for the active form of B12, Holo-TC, which is available for metabolic functions (Hunt, Harrington, and Robinson, 2014) as the TB12 assays measure both the Active B12 and inactive haptocorrin bound B12.

Some haematological malignancies (chronic myeloid leukaemia, polycythaemia vera, myelofibrosis, acute leukaemia's) and liver cancer have increased HC (inactive B12) thereby giving high TB12 results (Harrington, 2016). In haematological malignancies HC is released from the proliferating leucocytes whilst in liver disease there is reduced hepatic clearance of HC plus increased

HC release from damaged hepatocytes. In lymphoproliferative disorders, such as multiple myeloma and lymphoma, both HC and Holo-TC are increased in the circulation. The HC is release from neoplastic cells but the mechanism for the raised Holo-TC is not fully understood but may relate to macrophage activity. In solid organ tumours of the lung, breast, intestine and kidney increased release of HC, from these tissues to the circulation, is thought to be the cause of the high TB12 results (Arendt and Nexø, 2013).

There is currently no gold standard test for measuring B12 deficiency, different reference ranges and cut-off values are used to identify deficiency which impacts the sensitivity and specificity of the assays and different units are used by laboratories which can confuse users. Furthermore, other variables such as age, sex, ethnicity and drug therapy may affect the reference ranges (Sobczyńska-Malefora et al, 2023) and therefore using a single unified reference range may cause health inequalities for patients.

Due to the variation in platforms and assays for TB12, laboratories are advised to determine their own reference range (Devalia, Hamilton and Molloy, 2014). There is little harmonisation between different TB12 assays, with large biases in results seen, in external quality assurance (EQA) surveys, between assays and variation in assay performance over time. Beckman (DxI) has a large negative bias across the whole measurement range compared to Abbott (Architect and Alinity) assays and Roche (Cobas) when assessed against the all laboratory trimmed mean (ALTM) (expected result). Some suppliers do not have a consistent bias across the measurement range, the Siemens Atellica has a

negative bias for samples with TB12 results within or above the reference range but a positive bias for samples with results in the deficient range (RWT EQA data, Birmingham Quality, 2023). The reason for the difference is due to the use of supplier calibrants rather than an international reference standard (IS). A B12 and folate IS (03/178) was developed in 2005 which replaced the first IS (81/563) as the original was calibrated using a microbiological *Euglena* assay (a manual assay, very different to the immunoassays used today). The 03/178 IS assigned value for TB12 was calibrated by CPB assays from different supplier's platforms (Thorpe et al, 2007). If the current CPB assays were calibrated against the IS, then better concordance would be seen between suppliers. At present assay specific reference ranges and deficiency cut-off values are needed resulting in difficulties comparing results between laboratories, and potential for misdiagnosis.

The clinically replete concentration for TB12 is unclear and concentrations do not correlate well with clinical symptoms leading to a lack of a distinct 'disease state' however TB12 <200 ng/L (<148 pmol/L) should diagnose 97% of true B12 deficient patients however no similar cut-off concentration is provided for subclinical deficiency (Devalia Hamilton and Molloy, 2014).

Conversely, Smelt et al reported, clinically significant vitamin B12 deficiency may be present even with B12 concentrations in the reference range as TB12 assays used to diagnose deficiency are a poor predictor of functional concentration with a failure rate of 22–35% (Smelt et al, 2016).

Patients with TB12 concentrations of <100 ng/L (<75 pmol/L) usually have clinical or metabolic B12 deficiency. In the elderly, low TB12 concentrations of 100-160 ng/L (75-118 pmol/L) may occur in the absence of anaemia or macrocytosis (MCV <105 fL) (Devalia, Hamilton and Molloy, 2014).

CPB assay interpretation has large indeterminate range or 'grey zone' often reported as 200-350 ng/L (148-258 pmol/L), but will vary by assay, in which patients, clinically suspected of deficiency, should be further tested with a functional assay, for example MMA or total Hcy (T-Hcy) (Iqtidar and Chaudary, 2012). NICE (2024) have suggested the indeterminate range of 180-350 ng/L should now be used.

Some CPB assays may give higher results when a large concentration of IF antibody is present thereby causing missed diagnoses of B12 deficiency (Carmel and Agrawal, 2012). In addition, macro-B12, an immune complex between B12 and Immunoglobulin G (IgG) antibodies, which is biologically inactive may lead to missed diagnoses of B12 deficiency (Belkhouribchia, 2023).

Stabler (2013) reported that up to 50% of patients with low TB12, who do not respond to treatment, have normal MMA concentrations so these are false positive results.

In order to provide better diagnostic testing for B12 deficiency other biomarkers can be utilised such as Holo-TC, and the functional markers, T-Hcy and MMA. Each of these assays have their own limitations and no one test can provide a definitive diagnosis. The limitations of these biomarkers will be discussed together with how they may be combined in diagnostic testing algorithms.

1.4.3 Serum Active B12 / Holotranscobalamin (Holo-TC) assay

The first Holo-TC assay used magnetic beads coated with vitamin B12 to precipitate apo-transcobalamin (apoTC) and the Holo-TC present in the supernatant was then measured by enzyme linked immunosorbent assay (ELISA) (Nexø et al, 2002, Ulleland et al, 2002). The second was a radioimmunoassay, marketed by Axis Shield in 2006, who later worked with Abbott Diagnostics to produce a microparticle enzyme immunoassay for their high throughput immunoassay analysers. Other companies such as Siemens, Beckman and Roche have developed kits to run on their automated immunoassay platforms. There is variation between the assays of different manufacturers with the Roche method having a positive bias when compared to the Abbott test, with a corresponding higher reference range for Roche (37-188 pmol/L) compared to 25-165 pmol/L for Abbott (Heil et al, 2019).

The Holo-TC test is usually more expensive than the TB12 assay and few laboratories in the UK have it in routine use and at the time of writing, only the Nutristasis Unit, St Thomas' Hospital has an ISO 15189 accredited method (UKAS website, 2022) although the laboratory at Royal Wolverhampton NHS

Trust, New Cross Hospital will be seeking accreditation for the Holo-TC assay verified during this study.

Lack of standardisation has also been reported for Holo-TC assays, which led to the assignment of Holo-TC values to the International Reference Standard previously developed for TB12 and folate (Thorpe et al, 2016). Some assays such as Abbott Diagnostics Architect (in use in author's Trust) and Alinity Active B12 assays have been calibrated to IS 03/178 (Abbott Diagnostics kit inserts, 2023) however some suppliers kits, have not, leading to the variability seen in EQA results and requirement for supplier specific reference ranges and cut-off values. Siemens (Atellica) and Roche (Cobas) EQA results are negatively biased, Roche significantly so, when compared to the Abbott (Architect and Alinity) assays (RWT EQA data, Birmingham Quality, 2023).

Like TB12, Holo-TC assays have an indeterminate 'grey' zone range with various ranges quoted by investigators of anywhere between 25-75 pmol/L leading to questions about the diagnostic accuracy of the test (Herrmann and Obeid, 2013, Golding, 2016a). Holo-TC concentrations <25 pmol/L are regarded as deficient for the Abbott kits.

Schrempf et al (2011) concluded that neither Holo-TC nor TB12 assays could be recommended to diagnose deficiency in neuropsychiatric patients as positive predictive values were low, as a result of too many false positive results. A case of subacute degeneration missed by Holo-TC was reported by Ulrich et al

(2015) as the patient had taken supplements containing B12 before admission, the MMA was very high confirming true functional deficiency. However, Salinas et al (2018) suggested that Holo-TC may be a better indicator of intracellular B12 as the association of cognitive impairment with Holo-TC is stronger than with TB12.

Knoepfel et al (2018) described twenty seven cases of replete Holo-TC with low TB12 in patients with confirmed B12 deficiency (by MMA or Hcy), the index case had PA, the other cases were from heathy seniors or routine laboratory samples. The authors suggest the inconsistent results may be due to autoantibodies interfering with the Holo-TC assay; the use of oral B12 supplements; a decrease in haptocorrin expression or polymorphisms in the transcobalamin gene (TCN2) leading to increased Holo-TC. However, the actual frequency of these 'falsely normal' results were found to be very low in the populations investigated (0.1-0.2%). Sobczyńska-Malefora et al (2016) also documented two patients with a genetic variant of TCN2 leading to very low Holo-TC results but replete TB12 which, if Holo-TC were used alone, would falsely label these patients as deficient. This was thought to be due to the variant interfering with the monoclonal antibodies used in the assay preventing detection of Holo-TC.

Herrmann et al (2003, 2005) noted that concentrations of Holo-TC can be affected by renal dysfunction so it may not be a suitable test of B12 status in renal patients. Holo-TC is a small molecule (42 kDa) which is filtered by the kidney, therefore Holo-TC may be increased as kidney function decreases due

to reduced renal clearance (Nexø and Hoffmann-Lucke, 2011). However other authors have reported that Holo-TC can be used in these patients (Loikas et al, 2007a).

Golding (2016b) summarised the problems raised by authors with Holo-TC in his review which included the lack of agreement of the quoted half-life of Holo-TC, the disparity of reported correlations with TB12, the variation in reported sensitivity of the assay to recent oral intake and the lack of defined the cut-off values used to identify clinical and subclinical deficiency and implied that Holo-TC may not be a dependable marker of early deficiency.

Al Aisari et al (2010) found little difference in the accuracy of Holo-TC and TB12 assays to diagnose B12 deficiency in most cases and Clarke et al (2007a) found a slightly improved diagnostic accuracy but felt that they could not endorse Holo-TC nor TB12 to screen older asymptomatic patients due to a higher false positive rate in this group. Carmel (2011) also expressed concerns around its use for patients with sub-clinical B12 deficiency however 30–40% of sub-clinical B12 deficiency may be transient, resulting from FBCM (Shipton and Thachil, 2015).

Many authors have reported that the Holo-TC assay has better diagnostic accuracy and is a more sensitive, early marker of B12 deficiency than TB12 (Herrmann et al, 2003, 2005, Hvas and Nexø, 2005, Čabarkapa et al, 2007,

Obeid and Herrmann, 2007, Nexø and Hoffmann-Lucke, 2011, Heil et al, 2012 and Bondu et al 2020).

In 2015, the National Institute for Health and Clinical Excellence (NICE) published a MedTech innovation briefing supporting the use of Holo-TC assays to improve the diagnostic accuracy of B12 deficiency detection.

This study will assess whether Holo-TC has a greater diagnostic accuracy for B12 deficiency compared to TB12 when used as part of a new diagnostic testing pathway in conjunction with MMA.

1.4.4 Functional assays for B12 deficiency confirmation

Two functional assays exist for the confirmation of B12 deficiency, MMA and T-Hcy as both of these analytes accumulate when B12 is deficient, as previously described.

1.4.4.1 Serum Methylmalonic Acid (MMA) assay

MMA is reported to be the proxy ‘gold’ standard marker (the earliest and most representative) of metabolic B12 insufficiency. In B12 deficiency the concentration of MMA increases. Interpretation requires age related ranges, Nutristasis Unit, St Thomas' Hospital quote a range of 0-280 nmol/L for ≤65 years and 0-360 nmol/L for >65 years (Nutristasis Unit, St Thomas' Hospital, 2023).

There are various published methods for analysis of MMA, using gas chromatography mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry methods (LCMS/MS) and these are robust providing stable internal standards are utilised and interference from succinate (isomeric to MMA) is excluded (Appelblad and Schneede, 2008, Garg and Hammett-Stabler, 2010, Yuan et al, 2012, Lakso, Mineva et al 2015, Obeid, Geisel and Herrmann, 2015). GC-MS methods have largely been replaced with LCMS/MS as the latter is simpler and quicker to perform because derivatisation is no longer required. Other methods of sample extraction include ultracentrifugation and solid phase (Garg and Hammett-Stabler, 2010).

Like Holo-TC, MMA testing is more expensive and requires more complex equipment and expertise than the TB12 assay and at the time of writing, only the Nutristasis Unit, St Thomas' Hospital have an ISO 15189 accredited method (UKAS website, 2022).

Renal impairment, common in the elderly, has been reported to increase MMA (Loikas et al 2007a, Harrington, 2019). In renal disease MMA levels increase to 300-700 nmol/L however patients with megaloblastic anaemia or myelopathy have results above 500 nmol/L or even 1000 nmol/L (Stabler, 2013, Risch et al, 2015).

Elevated MMA also occurs in cases of hypovolemia, bacterial overgrowth of the small intestine and with inborn errors of metabolism (Fu et al, 2013, Ambati et al, 2017).

Herrmann and Obeid (2013) suggested that MMA is a poor indicator of B12 deficiency, with reduced sensitivity, as their study found 63% of people with low Holo-TC had normal MMA results while 9% of patients with replete Holo-TC had elevated MMA results.

Hvas, Ellegaard and Nexø (2001) reported that only 16% of untreated asymptomatic individuals with a raised MMA (>280 nmol/L), who were followed up for almost 4 years, had a significant increase in MMA during this period and MMA declined in 44% of patients indicating that MMA cannot be used to predict who will go on to become symptomatic.

Many studies indicate that MMA is a good test for diagnosing B12 deficiency. MMA predicted B12 deficiency in end stage renal failure in patients receiving B12 injections during haemodialysis (Iqbal et al, 2013); was found to be a better predictor of B12 deficiency than TB12 in cancer patients when using Fedosov quotient as the comparator test for receiver operating characteristic (ROC) analysis (Vashi et al, 2016), and a useful biomarker for monitoring sub-clinical B12 deficiency in population studies (Carmel, 2011).

Ambati et al (2017) concluded that elevated MMA is a relatively specific and sensitive test for B12 deficiency that decreases with treatment and Harrington (2019) reported, that as a metabolic marker of B12 deficiency, it is often referred to as the ‘gold’ standard test with values >750 nmol/L indicating ‘definite’ B12 deficiency (Harrington, 2017).

The BSH guideline recommend MMA as a confirmatory test for indeterminate cases of B12 deficiency (Devalia Hamilton and Molloy, 2014).

Some SNPs have been shown to affect serum MMA concentration. The HIBCH gene encodes 3-hydroxyisobutyryl-CoA hydrolase, a mitochondrial enzyme involved in amino acid valine, leucine and isoleucine metabolism. SNP rs291466 causes a missense change in the initiator methionine codon increasing MMA production which is independent of B12 status. Homozygotes for the variant allele (CC) have, on average, 46% higher MMA concentrations compared to those homozygous for the wild-type methionine-encoding allele (TT). The use of MMA as a confirmatory biomarker of B12 deficiency in these patients could result in false-positive diagnoses of B12 deficiency, particularly in older adults where concentrations are already increased. Therefore, it is important to consider the presence of the HIBCH rs291466 polymorphism when interpreting MMA levels, especially in cases where MMA is elevated but other B12 biomarkers do not indicate deficiency (Molloy et al, 2016).

Evidence for the potential for MMA to act as a tumour marker may also limit its utility as a biomarker for functional B12 deficiency. Various authors have studied a raised MMA's association with tumour progression and aggressiveness. Gomes et al (2020a,2020b) found that MMA induced the expression of SRY-box transcription factor 4 (SOX4) leading to a transcriptional reprogramming that resulted in cancer cells with aggressive phenotypes and poor prognosis. This included epithelial-to-mesenchymal transition (EMT), increased resistance to chemotherapeutic drugs, and increased metastatic potential. Gomes et al (2022) went on to study the increased metastatic potential in breast and lung cancer cells. They found metastatic signalling through extracellular signal-regulated kinase 2 (ERK2) drives the transcriptional switch that downregulates methylmalonyl-CoA epimerase (MCEE), leading to increased levels and accumulation of MMA which promotes cancer cell invasiveness.

Hu et al (2023) found that the increase of MMA with age promoted colorectal carcinoma (CRC) progression. MMA activated the Wnt/β-catenin signalling pathway (involved in cell proliferation, migration, differentiation and apoptosis) in CRC cells, leading to increased expression of β-catenin and decreased expression of p-GSK-3β, promoting EMT and cancer cell aggressiveness by enhancing their migratory and invasive capabilities. Hu et al (2024) also demonstrated pancreatic neuroendocrine neoplasm (PanNEN) progression, due to MMA increasing the expression of inhibin subunit beta A protein gene (INHBA) by the neuroendocrine-specific transcription factor FOX2, to induce microphthalmia-associated transcription factor (MITF) mediated EMT. PanNENs often present with metastases at diagnosis with poor prognosis and

high mortality. INHBA is aberrantly expressed in many other tumour types including breast, ovarian, lung and colon.

Tejero et al (2025) found MMA induces metabolic abnormalities in CD8+ T cells, leading to their exhaustion and suppression of anti-tumour immunity. CD8+ T cells function declines with age and increased MMA levels induce a dysfunctional phenotype with immunosuppressive transcriptional reprogramming and increased expression of exhaustion markers like TOX, PD-1 and CD38. MMA-induced CD8+ T cell exhaustion is linked to defects in mitochondrial function and suppression of NADH-regenerating reactions in the Krebs cycle.

Li et al (2022) studied MMA as a oncometabolite produced by aggressive cancer cells, involved in promoting cancer-associated fibroblast (CAF) activation in the tumour microenvironment (TME) and driving metastatic progression. The CAFs secrete IL6 loaded extracellular vesicles (EVs) which activate JAK/STAT3 and TGFβ signalling pathways in tumour cells, promoting aggressive behaviours.

Many of these pathways are linked and focussing on MMA could provide novel therapeutic targets for cancer. If circulating MMA is raised in patients with cancer it may restrict the utility of MMA as a functional biomarker for B12 deficiency. The association of MMA with cancer was unknown when this study began and if known about may have altered the testing algorithm proposed.

1.4.4.2 Serum Total Homocysteine (T-Hcy) assay

In B12 deficiency T-Hcy is raised (>15 μmol/L) and this has historically been reported to be associated with the development of cardiovascular disease in which endothelial damage, inflammation and atherosclerotic plaque formation leads to blood clots, strokes and heart attacks (Ganguly and Alam, 2015) however more recent studies have found little evidence that the association is causal (Miao et al, 2021).

T-Hcy can be measured in serum or plasma by a variety of methods (Alam, Kumar and Ganguly, 2019) including LC MS/MS (Poele-Pothoff et al, 1995, Magera et al, 1999) or automated analytical methods including fluorescence polarisation immunoassay and chemiluminescent microparticle immunoassays (Abbott Diagnostics, 2024), competitive chemiluminescence immunoassay and nephelometry (Siemens Healthineers, 2024), enzymatic assay (Beckman Coulter, 2024) and enzymatic cycling assay (Roche Diagnostics, 2024). Poor agreement is seen between LC MS/MS and automated analytical techniques with mass spectrometry methods regarded as producing more accurate results than immunoassay methods as the latter can be subject to interference and bias depending on the assay (Nexø et al, 2000, Brunelli et al, 2001). However, immunoassays are faster and do not require sample pre-treatment nor expensive mass spectrometry equipment. Results obtained by different techniques are not interchangeable (Ubbink et al, 1999), assay-specific reference ranges should be calculated with an assay that has been calibrated to the homocysteine international reference plasma (NIST 1955) (National Institute

of Standards and Technology (NIST), 2024). The cost per test for T-Hcy is still high, as the automated assays are not widely available in all laboratories.

At the time of planning this study it was felt that MMA was more specific than T-Hcy for the confirmation of B12 deficiency as MMA is raised in B12 deficiency whereas T-Hcy is also increased in folate deficiency which often coexists especially in older patients or those with poor diets (Shane and Stokstad, 1985). In addition, T-Hcy is affected by other vitamin deficiencies B2 (riboflavin) and B6 (pyridoxine) because they are involved as cofactors in its metabolism, B2 in the conversion of methylenetetrahydrofolate to methyltetrahydrofolate which is essential for the remethylation of homocysteine back to methionine and B6 acts in the transsulfuration pathway converting homocysteine to cysteine (Bajic, Z et al, 2022).

Physiological factors affect T-Hcy. These include diurnal variation (higher in the evening) (Lavie and Lavie, 2004); a supine position at venepuncture (10% lower values than if sitting) (Rasmussen, Moller and Lyngbak, 1999); a postprandial rise of 10-15% 6-8 hrs after a high protein meal. Furthermore, the intra-individual biological variation is up to 8% in healthy individuals and 25% in those with raised T-Hcy (Refsum et al, 2004). Other life-style choices increase T-Hcy such as smoking due to oxidative stress affecting the body's antioxidant defence systems and reduction of folate, B12 and B6 through altered metabolism (O'Callaghan et al, 2002) and high coffee intake due to a combination of caffeine (B6 antagonist) and chlorogenic acid with similar associated mechanisms to smoking (Verhoef et al, 2002).

SNPs have an impact on T-Hcy concentrations. such as the MTHFR mutation C677T which causes elevated homocysteine due to faulty folate metabolism already discussed in section 1.3.3, as does classical homocystinuria (Kraus et al, 1999), an inherited metabolic disorder of methionine amino acid metabolism due to deficiency of cystathionine beta synthase (CBS) or methionine synthase, however this is normally detected early by newborn bloodspot screening (NHS.UK, 2024).

Like MMA, T-Hcy rises with age mainly due to malabsorption of B12 and folate (Refsum et al, 2004), renal dysfunction (due to a combination of reduced clearance, altered T-Hcy metabolism and dietary restriction) (van Guldener, 2006), hypothyroidism (due to lowered folate levels and a lower glomerular filtration rate) (Diekman et al, 2001), SLE (associated with inflammation, renal dysfunction, nutritional deficiencies and medication) (Sam et al, 2020), malignant neoplasms/myeloproliferative disorders (due to treatment with folate antagonists) and diabetes (due to metformin therapy).

Sex differences are seen in T-Hcy with higher concentrations in males and post-menopausal females, due to lower oestrogen levels which have a regulatory role in T-Hcy metabolism (Dimitrova et al, 2002). Consequently, a unified reference range is inappropriate, and laboratories should set their own ranges for age and sex.

Whilst there are some ethnic differences seen with T-Hcy, there is little difference at the upper limit of normal for populations with similar diets living in the same areas (Refsum et al, 2004).

Some medications cause elevated T-Hcy. These include lipid lowering drugs such as fibrates and bile acid sequestrants, the mechanism does not appear to be associated with vitamin status but may involve impaired renal function or changes in creatine metabolism (Dierkes et al, 2003, Lent-Schochet and Jialal, 2023), methotrexate because it is a folate antagonist (Friedman and Cronstein, 2019), levodopa since its metabolism by catechol-o-methyltransferase requires B12, B6 and folate cofactors (Ahlskog 2023), anti-seizure medication (Diaz-Arrastia, 2000) and metformin which reduces both B12 and folate by increased metabolism and reduced absorption in the gut (Bell, 2022).

T-Hcy was not measured in this study owing to preanalytical sample requirements. Blood samples should be centrifuged within 1 hour or kept cold on ice until centrifugation as T-Hcy continues to be released from red cells with an increase of T-Hcy of 1 µmol/L per hour at room temperature (Ueland et al, 1993). As the study was utilising surplus serum samples, the serum would not have been prepared appropriately for analysis of T-Hcy.

Extended T-Hcy stability prior to centrifugation may be achieved by the use of blood stabilisation tubes such as Drew Scientific containing 3-deaza-adenosine (3-dad) (Bowron and Stansbie, 2003), Greiner Vacuette containing sodium

citrate/citric acid solution (Greiner bio-one, 2024) and Sarstedt Monovette containing a proprietary stabiliser (de Graaf et al, 2008) These have been reported to increase stability at room temperature to 6 hours, 6 hours and 8 hours, respectively, with extensions to 72 hours, 72 hours and 96 hours, respectively if stored 2-8^{o}C.

The recent evidence around the potential pitfalls of MMA as a functional marker of B12 deficiency indicate that perhaps T-Hcy was dismissed too readily and may be an area for future investigation.

In addition, the UK government plans to fortify non-wholemeal flour with folic acid (already in place in other countries) by the end of 2026 to reduce neural tube defects (NTD) by 20% (Gov.UK, 2024), although some authors feel the fortification levels could be set higher to reduce up to 80% of NTD (Wald, 2022). This would also help reduce the number of folate deficient patients and T-Hcy could then be more effectively used to confirm B12 deficiency.

T-Hcy may be the best marker for patients using nitrous oxide as T-Hcy levels increase before MMA, however MMA may be a better marker of clinical severity (Grzych et al, 2023).

It was considered, when planning this study, that MMA had fewer limitations as a functional marker of B12 deficiency than T-Hcy. This may be an area to revisit in the future given current evidence.

1.4.5 Diagnostic testing algorithms

Most authors agree that TB12 measurement, alone, does not reliably detect B12 deficiency and either combinations of tests of TB12 with a metabolic marker of deficiency, such as MMA or T-Hcy or replacement of TB12 with Holo-TC (Hughes and McNulty, 2018) is required. Herrmann et al (2003) favoured Holo-TC in combination with MMA, as did Obeid and Herrmann (2007), Herrmann and Obeid (2008, 2013) and Hannibal et al (2016) in adults, the latter preferring T-Hcy to Holo-TC in neonates. Hvas and Nexø (2006), Carmel (2011), Devalia, Hamilton and Molloy (2014), suggested TB12 and MMA, and Miller et al (2006) both TB12 and Holo-TC.

Berg and Shaw (2012) proposed cascade testing of MMA and IF antibodies when TB12 is low (<160 ng/L, <118 pmol/L). Sukumar and Saravanan (2019) recommended TB12, then Holo-TC, if TB12 is indeterminate, with MMA as follow-up.

A more mathematical approach toward establishing B12 deficiency was proposed by Fedosov et al (2015). Fedosov developed the combined B12 (cB12) marker to improve diagnostic precision, utilising TB12, Holo-TC, MMA and T-Hcy results, where: cB12 = log 10[(Holo-TC X TB12)/(MMA X T-Hcy)] – (age factor). The cB12 can also be calculated when one or two markers are missing with acceptable results (Fedosov et al, 2015). Golding (2016b)

expressed concern that cB12 may be useful in population studies but not be suitable for individuals, concluding that large trials are required to assess its utility.

One such trial was undertaken by Campos et al (2020) who published a diagnostic accuracy study using TB12, Holo-TC, MMA and T-Hcy, utilising cB12 as the 'gold standard' in a large mixed central European population (>11000 samples). They found the detection of subclinical B12 deficiency was highest for Holo-TC, followed by MMA, TB12 and T-Hcy. However, even though for women ≥50 years Holo-TC was the best predictor, for women <50 years and men no test was superior to TB12. The study had some limitations, such as failure to collect the haemoglobin concentration, details on renal function, B12 intake and clinical symptoms, particularly as renal function affects MMA, T-Hcy and possibly Holo-TC.

In the author's laboratory if a result falls into the indeterminate range for TB12 it is left to the requestor to assess the patient clinically, for signs and symptoms of deficiency and to request further testing. For patients with deficient results intrinsic factor may be requested but very few requests are received for functional B12 assays such as MMA or T-Hcy and these tests are often not commissioned by Integrated Care Boards (ICB). This study aimed improve the diagnosis of B12 deficiency in its patient population. Whilst cB12 has been proven to be the most diagnostically accurate method for the identification of B12 deficiency and especially subclinical deficiency, it also has the highest cost and is unsuitable for large scale testing. Holo-TC has been reported to have a

higher diagnostic accurate than TB12, and therefore, a testing algorithm was proposed with Holo-TC as the first line test (with high sensitivity to reduce missed diagnoses of B12 deficiency) followed by a confirmatory test, MMA (with high specificity to prevent unnecessary treatment for replete patients). The aim was to provide patients with a better clinical service in order to begin B12 deficiency treatment earlier and prevent disability later in life.

In Fedosov and colleagues (2015) assessment in the use of two, three and four marker combinations they found that Holo-TC and MMA used together gave the smallest analytical error in missing B12 deficiency when using just two tests rather than all four. In Harrington's 'Laboratory assessment of vitamin B12 status' review (2017), the author suggested measuring Holo-TC and if it was in the indeterminate range (25-70 pmol/L, for the Abbott assay), approximately 25% of patients, then to measure MMA. This is the algorithm used in this study except a Holo-TC indeterminate range of 25-50 pmol/L was used as originally proposed by Abbott Diagnostics. Figures 1.5 and 1.6 show the current and proposed B12 testing algorithms for this study.

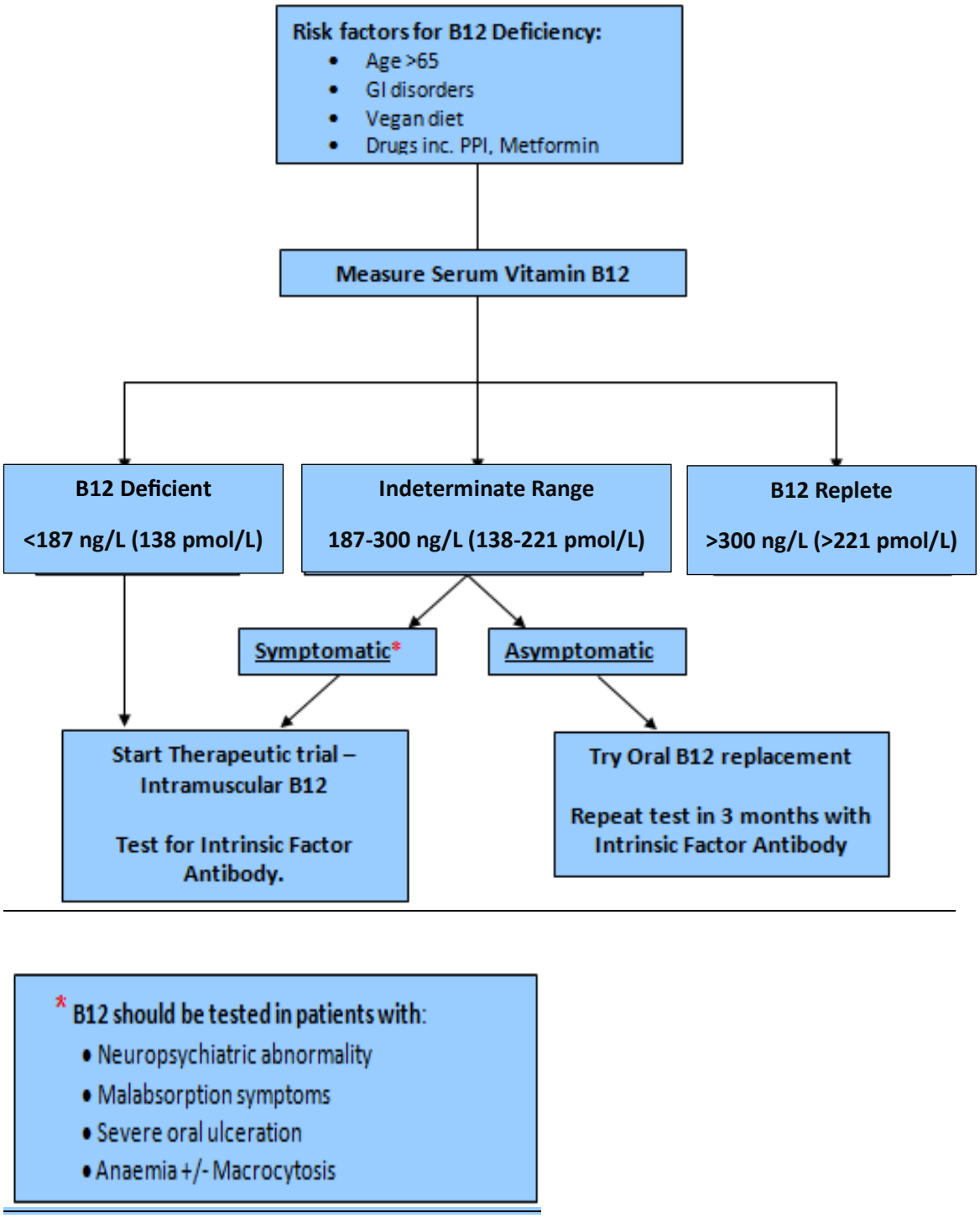

Figure 1.5. Current laboratory B12 testing algorithm using TB12 assay.

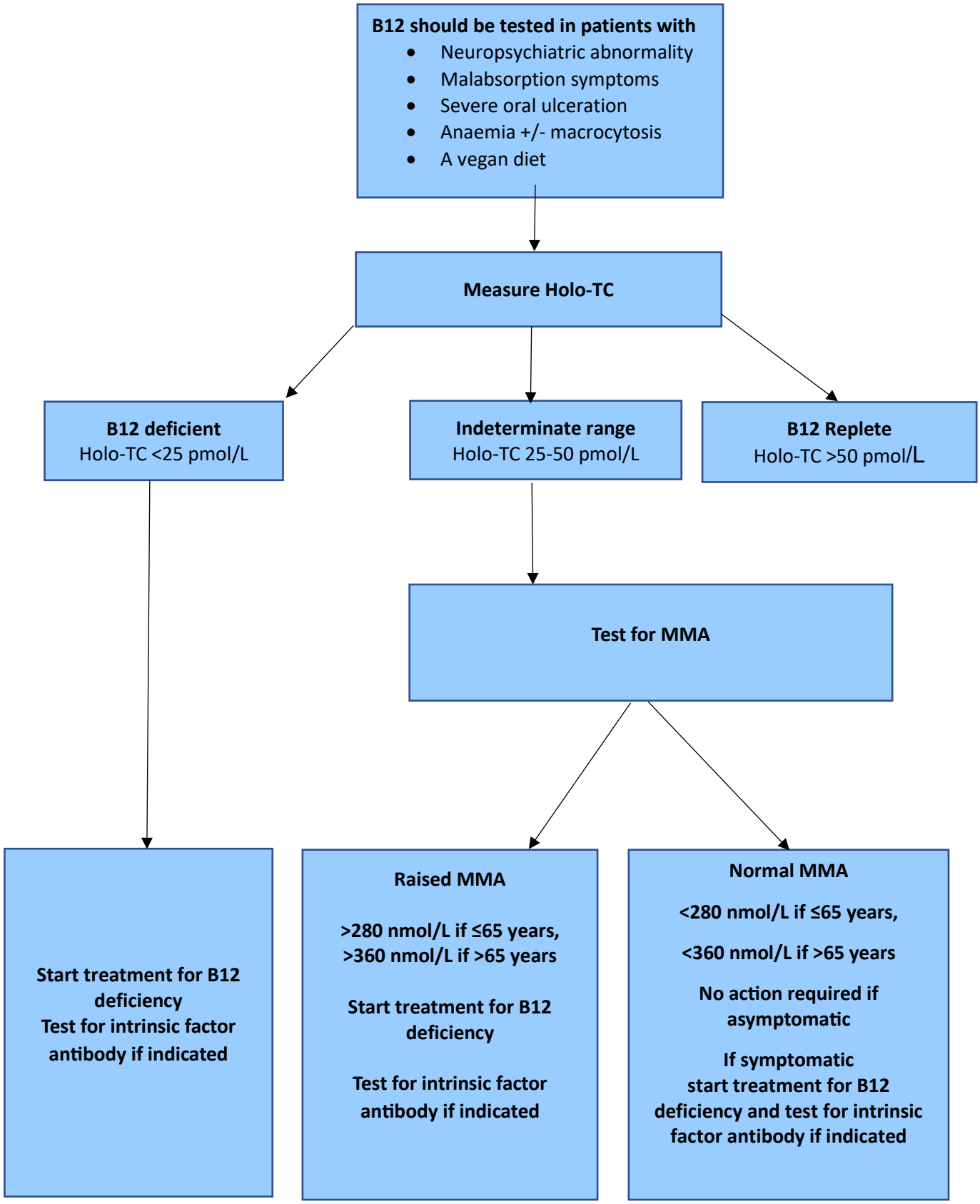

Figure 1.6. Proposed B12 testing algorithm using Holo-TC ± MMA assay.

1.5 Health economics evaluation of B12 deficiency

In the UK, Public Health England fund the National Diet and Nutrition Survey which looks at the diet and nutritional status of a cross-section of the UK general population. TB12 and Holo-TC concentrations are measured as part of the survey. There have been reports on both iron and folate deficiency but no reports on B12 deficiency (UK Government, 2020).

Health economic benefits are about improving healthcare outcomes involving interactions with individuals, healthcare providers and clinical settings in an effective and efficient way. NICE (2011) recommend a cost-effectiveness analysis for economic evaluation of diagnostic tests.

The main focus of this study was to develop a diagnostic testing algorithm that would give value for money and was clinically effective. At commencement of this study no health economic evaluations had been performed in the UK adult patient population and only a limited number of cost-effectiveness studies had been undertaken worldwide. A Dutch study estimated that using MMA in addition to TB12 to guide intramuscular (IM) B12 treatment saved 91 euros per person per year and switching to oral B12 would save a further 39 euros (Jacobs et al, 2013).

A study by Vidal-Alaball, Butler and Potter (2006) compared the costs of IM (4 injections per annum) and high dose oral (1 tablet per day, actual dose not stated) vitamin B12 replacement in a UK primary care setting. At the time of the study, it was calculated that the cost of treating a patient with IM B12 was between £56-100 per year. For high dose oral B12 treatment the cost was £36 per year with large savings in nursing time. The health economic evaluation analysis in this thesis will assess if these findings are still valid.

The BSH guideline suggested that oral B12 could be used for maintenance therapy, correction of asymptomatic deficiency or in cases of FBCM however this is not standard practice in the UK, only Sweden and Canada routinely prescribe oral vitamin B12 (Shipton and Thachil, 2015).

The National Health and Nutrition Examination Surveys (NHANES) in the USA, reported that more trials were required before advocating B12 fortification to prevent subclinical deficiency (Berg and Shaw, 2012). Limitations of the NHANES data include variation in cut-off values used for B12 deficiency which affects prevalence (ranges from 3-25%, across the USA) and lack of consistency in measurement techniques in different countries making comparisons difficult (Yetley and Johnson, 2011).

Silverstein et al (2018) conducted a large population study on elderly Canadian patients who had received a prescription for IM B12 and found that nearly two-thirds (65.6%) of patients received an IM B12 injection with either a replete B12

concentration or without a B12 result in the year prior to treatment. This study highlights possible misdiagnoses and importance of appropriate follow-up.

Since the inception of this study, NICE published their own health economic evaluation (NG239) (NICE, 2024) which enabled some health economic comparisons to be made.

1.6 Summary of literature review

The literature review found there is currently no ‘gold’ standard test for measuring B12 deficiency. For the TB12 test variable reference cut-off values are used to identify B12 deficiency which impacts the sensitivity and specificity of the assays leading to under and over reporting of deficiency. Different reporting units are used by laboratories which can confuse users. The TB12 assays have a ‘grey area’ where up to 30% of results may fall. Clinically B12 results do not always correlate with symptoms with many subclinical patients seen. Symptoms can be vague and non-specific leading to delayed diagnosis however if B12 deficiency is left untreated poor outcomes ensue with increased morbidity and neurological deficits common.

Many diagnostic accuracy studies have been performed on different test combinations for detecting B12 deficiency and generally conclude that Holo-TC and MMA are superior to TB12, with both improved sensitivity and specificity.

The Holo-TC (immunoassay) and MMA (LCMS/MS) assays are not routinely available in most UK laboratories, owing to the additional cost and complex set-up respectively, and many MMA assays are for research use only.

The review found, despite B12 deficiency being a well-researched area, there are still significant gaps in knowledge with little evidence of cost-effectiveness analysis in B12 deficiency diagnosis being undertaken. There have been limited evaluations in some countries but no health economics benefit analysis of B12 diagnosis and treatment has been performed in the UK and this forms the focal point of this research.

1.7 Aim, Objectives and Hypotheses of project

1.7.1 Aim

The overall aim of this project was to assess the cost-effectiveness of the current TB12 algorithm (Figure 1.5) compared to an algorithm using Holo-TC and for indeterminate cases MMA (Figure 1.6).

Following the recommendation in Harrington's 'Laboratory assessment of vitamin B12 status' review (2017), this project assumed a 'gold standard' testing regime of Holo-TC as the first line test, with MMA as a second line test, when Holo-TC is indeterminate, and therefore, assumed that this strategy will have greater diagnostic accuracy for B12 deficiency than TB12, the test in use at the

author's Trust at the start of this research. The project assessed whether the introduction of the new testing regime would have a health economic benefit.

As the new testing algorithm is more expensive, this must therefore be balanced against potential savings from a reduction in the under and over-diagnosis of B12 deficiency such as prevention of unnecessary additional testing and treatment.

A decision tree model (Figure 1.7) was used to compare costs and health utilities between current practice (TB12) and Holo-TC ± MMA. The model considered patient health measured in terms of Quality Adjusted Life Years (QALY's). The expected costs and QALY's incurred in the first year following the initial TB12 test were calculated. This data was used to compare the cost-effectiveness of the current algorithm for determining B12 deficiency to the proposed new algorithm.

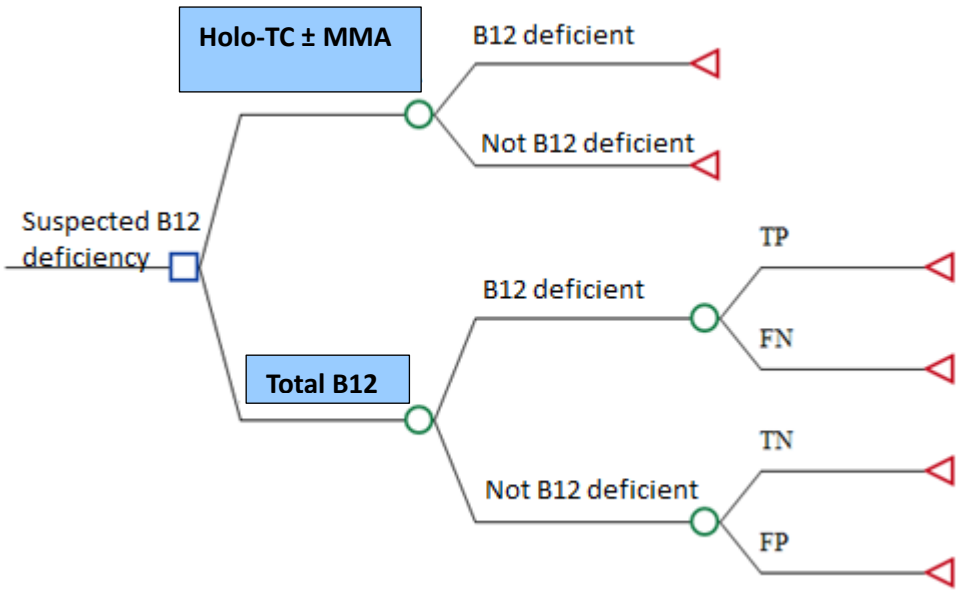

Figure 1.7. Example decision tree for the cost-effectiveness analysis of TB12 versus Holo-TC ± MMA to diagnose vitamin B12 deficiency. Key: TP true positive, FN false negative, TN true negative, FP false positive.

1.7.2 Objectives

In order to achieve the aim, the following objectives were required:

- Set up and verify the Holo-TC immunoassay on the automated chemistry immunoassay platform, the Abbott ARCHITECT i2000sr analyser using methods and reagents supplied by Abbott diagnostics, chosen to enable routine analysis of a large volume of samples. As Holo-TC is a CE marked assay used as per manufacturer's instructions only verification of the assay was required: trueness (bias) using External Quality Assurance samples (EQA) and patient comparisons; imprecision, linearity, recovery and levels of detection and quantitation. Reference intervals were taken

from manufacturers kit insert to assess suitability for the local patient population.

- Set up and verify a liquid chromatography tandem mass spectrometry (LCMS/MS) assay for MMA using an appropriate extraction method and mass spectrometry analyser, to enable routine analysis of a large volume of samples. Verification of the MMA assay included: trueness (bias), imprecision, recovery, measurement uncertainty, analytical specificity and sensitivity, interfering substances, linearity, measuring range and reference intervals. The evaluation included patient and EQA comparisons.

- Identification and collection of surplus serum samples on primary care patients with suspected B12 deficiency, after TB12 and other routine tests had been completed.

- Collection of FBC data, any blood film morphology comments if performed to assess anaemia, urea and electrolytes (U+E) results to assess renal function, thyroid stimulating hormone (TSH) to assess thyroid function, serum folate and TB12 result. Other important data such as age, ethnicity, symptoms of B12 deficiency, known gastrointestinal disorder/surgery, diabetic status, medications and vegetarian/vegan diet were obtained from the laboratory information system (LIMS), clinical portal or GP electronic records.

- Follow-up of patients identified by TB12 test as deficient or indeterminate 12 months after initial TB12 test to collate data on further or duplicate testing undertaken and any treatment given.

- Analysis of anonymised serum samples for Holo-TC and MMA. All patient samples had an Holo-TC and MMA run to assess 'true positives' rather than just test those that were low or in the 'grey, indeterminate area' of 25-50 pmol/L (Abbott kit insert).

- Calculation of diagnostic accuracy of the TB12 test versus the Holo-TC test and the diagnostic accuracy of the current testing algorithm (TB12) versus the proposed new testing algorithm using Holo-TC ± MMA to assess superiority of tests and algorithms.

- Review published cut off values for B12 deficiency for Holo-TC and MMA to assess suitability for use in the local patient population.

- Compare the cost-effectiveness of the current test for determining B12 deficiency to the proposed new testing algorithm.

1.7.3 Hypotheses

The null hypotheses were:

- There will be no difference in diagnostic accuracy between the current testing algorithm using the existing test, TB12 and the new algorithm using a combination of Holo-TC and MMA to identify B12 deficiency in a primary care population.

- There will be no health economics benefits seen using the new testing algorithm over the existing one.

Chapter 2.0 MATERIALS AND METHODS

2.1. Ethical approval, consent and copyright approvals

Ethical approval for the study was deemed not required by NHS Health Research Authority (appendix 1) as the utilisation of surplus serum only was not classed as research but as service improvement. Local approval for the study was obtained from the local research and development team for capacity and capability to deliver the study at New Cross Hospital, part of the Royal Wolverhampton NHS Trust (appendix 1). The ETHOS approval form for Manchester Metropolitan University was completed on Moodle (appendix 1). The service evaluation used excess biological material collected during routine clinical care in patients with suspected B12 deficiency. It involved no deviation from standard clinical care and the subsequent data obtained (Holo-TC and MMA) was not used in the clinical management of patients. All collected material was anonymised for the purpose of the study.

Written consent was not sought from the patients under study with implied consent provided at venepuncture. Patient details were required to identify B12 deficient patients for follow-up however all data was pseudo-anonymised throughout sample collection and fully anonymised as soon as possible prior to data analysis.

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from the relevant journals for use of copyrighted figures. For all figures the original authors/source were credited in the respective citation.

2.2 Sample population

A cohort of 1100 adult primary care patients from Wolverhampton Clinical Commissioning Group (CCG) having a routine TB12 test for suspected B12 deficiency were selected for inclusion in this study. The inclusion criteria were, the patient should be ≥18 years old, suspected of having B12 deficiency and have a TB12 result. Any patients that were <18 years old or were known to be pregnant were excluded.

The sample size chosen was consistent with the work of Bujang et al (2016) who suggested that with a sample of more than 300 subjects the estimated statistics for sensitivity and specificity derived from the sample are likely to be the same as the true values within the intended population. However it was felt by increasing the sample population to over 1000 would further enhance the power of the study and see if the local study population was consistent with published prevalence levels of B12 deficiency in the UK, approximately 6% if <60 years of age and 20% if >60 (Tyler et al, 2022).

2.3 Sample and data collection

2.3.1. Identification of samples

Before the start of this project, an ‘active B12 pilot’ meeting was held with three GP’s from Wolverhampton CCG (including the CCG Lead GP) and laboratory

staff including Consultant Clinical Scientists, Consultant Haematologists and the thesis author to outline the project objectives. After gaining buy-in from the three GP's, all GP's in the Wolverhampton CCG were emailed, inviting them to be involved in the project and to contact the laboratory if interested. From the replies visits to ten GP surgeries were undertaken in person to explain the procedure and the data to be collected. The ten GP surgeries were spread over Wolverhampton (Central Wolverhampton, Wednesfield, Low Hill, Warstones, Tettenhall, Bilston, Willenhall and Penn) representing 25% of Wolverhampton's GP surgeries including some areas with high deprivation.

To aid data collection, a requesting profile set was devised on the laboratory information system (LIMS), TD NexLabs Livextens v22.11A (Technidata Medical Software, Warrington, UK) which using the Electronic requesting system requested a TB12, serum folate, U+E, TSH, FBC, Holo-TC and MMA tests and captured relevant data from GP's requesting TB12 tests on their patients (Section 2.3.3).

In October 2020 the laboratory LIMS changed to Winpath Enterprise vs 7.24 (CliniSys Inc, Chertsey, UK), therefore the testing profile was no longer viable as an option and from then on Wolverhampton CCG B12 test lists were run to capture the B12 requests sent, two days after arrival to ensure all routine tests had been completed. Samples were randomly selected from these lists, and samples with sufficient surplus sample were included for aliquoting.

Approximately 400 samples were collected using the original LIMS method and 700 samples from the new LIMS. A number of patient samples were excluded

from analysis for various reasons (section 2.3.3) including age criteria not met and duplicate patient requests, with a final test cohort of 1003 patients.

2.3.2 Sample types and preparation

Routine peripheral blood samples for FBC and blood film analysis were collected in 3 mL K3EDTA anti-coagulant vacuettes (Greiner, catalogue number 454086). Serum was derived from blood samples collected in 5 mL CAT Serum separator clot activator vacuettes (Greiner, catalogue number 456010) and used for analysis of TB12, folate, TSH, U+E, Holo-TC and MMA. Samples were centrifuged, within six hours of collection, in one of the three centrifuges on the Inpeco Track (Abbott Diagnostics, Chicago, Illinois, USA) at 3500 rpm for 10 minutes.

FBC analysis was performed on the Sysmex XN-10 or XN-20 FBC analyser (Sysmex Corporation, Kobe, Japan) within 24 hours of sample collection and blood film made if required according to laboratory protocols.

Serum TB12, serum folate, Holo-TC and TSH were performed using an Abbott Architect i2000 Immunoassay analyser and U+E using the c16000 Chemistry analyser (Abbott Diagnostics, Chicago, Illinois, USA).

Serum MMA was tested on an AB Sciex 6500 LC/MSMS analyser (Sciex, Danaher Corporation, USA).

Where routinely requested, the TB12, folate, U+E and TSH were processed on the day of receipt.

Two days after the completion of routine tests the residual samples were retrieved from the refrigerated storage unit, transferred into three, 2 ml micro tubes, pseudo-anonymised and frozen at -80^{o}C until required for analysis of Holo-TC assay and MMA.

2.3.3 Data collection

Routine test results were extracted from the LIMS. Some requests were missing routine test results, in order to obtain complete datasets, 54 U+E, 117 TSH and 7 folate tests were analysed using the spare frozen aliquot. There were 21 requests with either no FBC sample received, insufficient sample or clotted sample.

Fifty-nine samples were excluded from the study, some due to the age of the patient (<18 years); duplication of patient requests; samples with no TB12 result due to pre-analytical errors and a few that were unable to be matched to a patient record. Due to technical breakdowns of the Sciex mass spectrometer and backlogs of routine work the final batch of MMA samples was not analysed and the data for these samples removed. The final study cohort was 1003 samples.

Clinical information was collected from the LIMS and Royal Wolverhampton Trust Clinical Web Portal, which links to primary care electronic records. The information collected, where available, included:

- Symptoms of B12 deficiency

- Medication history specifically B12 replacement, metformin, thyroxine, oral contraceptives, hormone replacement therapy, proton pump inhibitors
- History of gastrointestinal disorder/surgery
- History of Diabetes
- Smoking history
- Vegan or vegetarian diet
- Ethnicity

Relevant follow-up routine testing in patients who had deficient or indeterminate TB12 results were recorded for one year post the initial sample request including repeat TB12, IF antibody, GPC antibody, coeliac screening via tissue transglutaminase IgA (TTG-IgA) or endomysial antibodies, Holo-TC, MMA or T-Hcy along with the results of these tests and details of any surgical procedures that may have affected B12 concentrations (weight loss procedures, stomach resection, etc).

Patients who had received B12 treatment, either oral or IM, in the same one year period were identified from the LIMS, clinical portal or GP electronic records.

The follow-up tests and treatment resulting from the initial TB12 result were costed and used to compare the cost-effectiveness of the current algorithm for determining B12 deficiency to the proposed new algorithm to identify health economic benefits (Section 2.6).

2.4 Methods used including principles of measurement

2.4.1 Full Blood Count (FBC) and blood film

2.4.1.1 FBC

FBC was performed using the Sysmex XN-9100 tracked system which consists of automated FBC analyser's XN-10 or XN-20 (Sysmex Corporation, Kobe, Japan). The Sysmex XN-10 or XN-20 FBC analyser performs analysis of peripheral blood cells on a small volume of whole blood (88 µl), generating thirty FBC parameters including red cell enumeration (RCC), sizing (mean cell volume, MCV) and haemoglobin (Hb) measurement using three main methods, fluorescence flow cytometry, hydrodynamically focussed impedance and cyanide free sodium lauryl sulphate (SLS) Hb measurement (Sysmex UK website, 2023). The impedance method follows the Coulter principle (Figure 2.1) which is enhanced by using hydrodynamic focussing of sheath flow to ensure only a single stream of red cells pass through the aperture (Figure 2.2). As a cell passes through the aperture it acts as an electrical insulator causing a break in the circuit, the pulse height being proportional to the size of the cell passing through. Therefore, the number of red cells were counted and the size of red cells measured. The MCV calculation requires another measured parameter, the haematocrit (Hct) which is the cumulative value of the individual cell pulse heights.

Thus, the MCV (fL) = Hct (L/L)/RCC ($x10^{12}$/L) x1000.

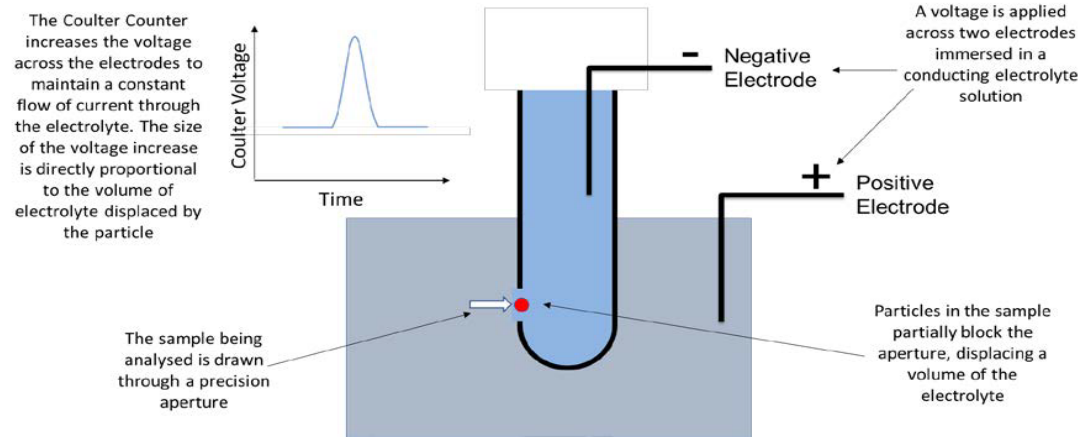


Figure 2.1. Coulter Principle, cells in an electrolyte pass through an aperture disrupting the current, the size of the pulse is proportional to the size of the cell and the number of pulses can be counted to give a total cell count (Beckman Coulter website, 2023).

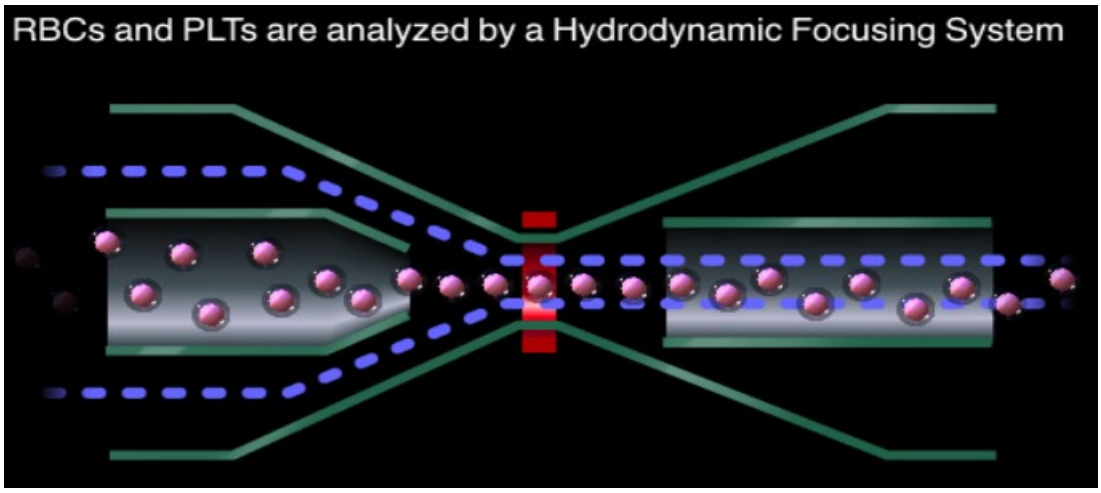


Figure 2.2 Hydrodynamic focussing used on Sysmex XN analysers, sheath fluid ensures a single stream of cells pass the detector and prevents multiple cells being counted or recirculated. (Sysmex UK website, 2023).

For Hb measurement the SLS reagent lyses the red and white blood cells, alters the globin molecule and oxidises the haem group. The hydrophilic groups of the SLS bind to the haem, which forms a coloured SLS-Hb complex. This was then measured photometrically by light absorbance at 555 nm with the amount of light absorbed proportional to the Hb concentration in the sample, Figure 2.3 (Sysmex, 2023).

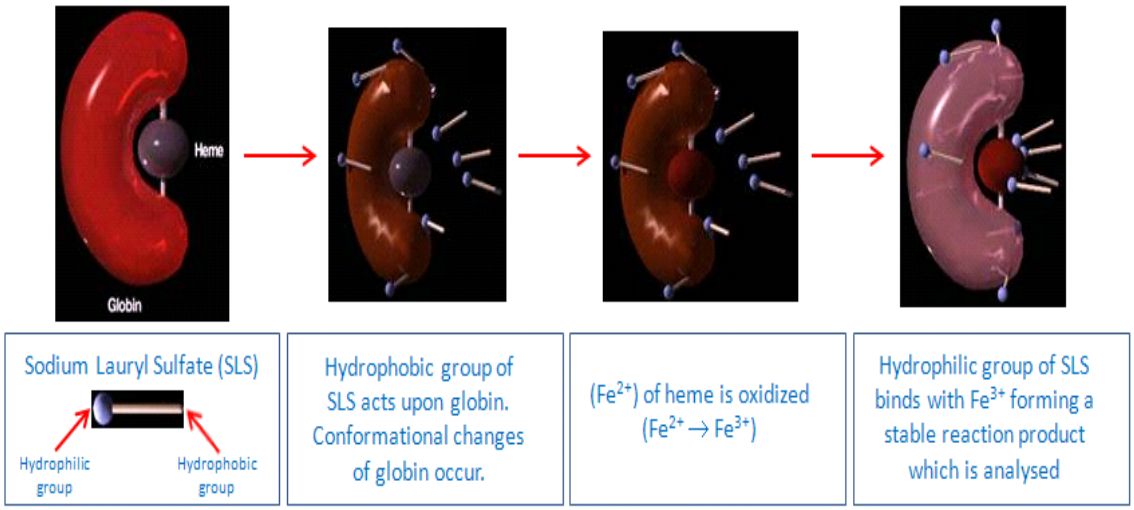


Figure 2.3. Haemoglobin measurement, the SLS reagent modifies globin and oxidises haem to create a coloured complex that can be measured photometrically (Sysmex UK website, 2023).

XN analysers were calibrated by Sysmex engineers (Sysmex XN CAL product number BC553492) and Sysmex trilevel (Low, Normal and High) XN Check Levels 1, 2, 3 (Sysmex XN check, product numbers AL359722, CU248646, AE474952 respectively) internal quality control (IQC) were run three times per day following laboratory protocols and acceptable performance checked before patient samples were analysed (Sysmex XN Check product inserts, 2023).

2.4.1.2 Blood films

The Sysmex middleware, extended information processing unit (EPU) has film making criteria installed to automatically add on films, where clinically indicated.

For our patient population films were made if:

Hb < 90 g/L

MCV <70 or > 107 fL

White Blood Cells (WBC) < 1.0 $x10^9$/L

Platelets <100 x 10^9/L

Nucleated Red Blood Cells (NRBC) >1.1 $x10^9$/L

FBC XN analyser generated results flags :- ‘Blast’, ‘Fragments‘, ‘WBC abnormal scatter‘.

Where indicated wedge-shaped blood films were automatically spread and stained using May Grunwald Giemsa (Romanowsky stain) at pH 7.0 by the SP50 analyser (Sysmex Corporation, Kobe, Japan). Romanowsky stains are neutral stains composed of a mixture of methylene blue (azure) dyes and eosin Y. The azures are basic dyes that bind acid nuclei and result in a blue/purple colour. The acid dye eosin is attracted to the alkaline cytoplasm producing a red/pink colour (Table 2.1) (Bain, 2006).

Table 2.1. Characteristic staining of different cell components with Romanowsky stain (Bain, 2006).

Cellular Component	Colour
Nucleus and Chromatin (including Howell – Jolly bodies)	Purple
Promyelocyte granules and Auer rods	Purple – red
Cytoplasm of neutrophils	Pink
Cytoplasm of lymphocytes	Blue
Cytoplasm of monocytes	Blue – grey
Cytoplasm rich in RNA (i.e. 'basophilic cytoplasm')	Deep blue
Specific granules of neutrophils, granules of lymphocytes.	Light purple or pink
Specific granules of basophils	Deep purple/black
Specific granules of eosinophils	Orange/salmon pink
Red cells, haemoglobin	Red / Pink
Platelets	Purple

The blood films were then passed to the DI60 digital imager (Sysmex Corporation, Kobe, Japan) and images captured for later review by the laboratory staff, to ensure accurate results were reported and abnormalities detected. In patients with B12 deficiency these included checking for enlarged red cells (oval macrocytes, high MCV), hypersegmented neutrophils, other red cell shape changes such as tear drop poikilocytes and fragments, a lower WBC or platelet count and occasionally the presence of blast cells in severe deficiency.

2.4.2 Principle of measurement and processing of Serum Urea and Electrolytes (U+E)

Requests for TB12 routinely have a U+E profile sent to assess patients' renal function.

A U+E profile included the following tests Urea, Sodium, Potassium, Creatinine and the calculated parameter estimated glomerular filtration rate (eGFR).

Serum U+E were measured using Inpeco tracked, fully automated Abbott Architect System c16000 chemistry analysers (Abbott Diagnostics, Chicago, Illinois, USA).

The Urea Nitrogen assay (Abbott Architect System Serum Urea Nitrogen kit, product number 7D75-32) is a modification of an enzymatic procedure. The test is a kinetic assay in which the initial rate of the reaction is linear for a limited period of time. Urea in the sample was hydrolysed by urease to ammonia and carbon dioxide. The second reaction, catalysed by glutamate dehydrogenase (GLD) converted ammonia and α-ketoglutarate to glutamate and water with the simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD). Two moles of NADH are oxidised for each mole of urea present. The initial rate of decrease in absorbance at 340 nm was proportional to the urea concentration in the sample. The assay was calibrated as per laboratory protocols (Product number 1E65, Architect Multi-constituent calibrator) (Abbott diagnostics serum Urea Nitrogen kit insert, 2023). Three levels of IQC samples were analysed daily following laboratory protocols and acceptable performance checked before samples were processed

(Multichem S Plus controls, Levels 1,2,3 for Abbott Architect, product numbers OSP78-10, OSP78-11, OSP78-12 respectively) (Technopath Clinical Diagnostics, 2023).

The c16000 employs Integrated Chip Technology (ICT) to determine simultaneously the sodium and potassium electrolyte concentrations, via potentiometry (Abbott Architect System ICT kit, product number 2P32). The ICT system used solid state ion-selective electrodes (ISE) contained within a single ICT module. Electrodes used were:-

Sodium A crown ether ionophore incorporated into an ion-selective plastic membrane.

Potassium Valinomycin incorporated into an ion-selective plastic membrane.

Reference A silver/silver chloride electrode in a potassium chloride gel inner solution, separated from sample by a porous ceramic disk.

An electrical potential (voltage) was generated across the membranes between the reference and measuring electrodes in accordance with the Nernst equation. The voltage was compared to previously determined calibrator voltages and converted to ion concentration. The assay was calibrated as per laboratory protocols (Product number 1E46, Architect ICT calibrator) (Abbott diagnostics ICT kit insert, 2023). Three levels of IQC samples were ran daily following laboratory protocols and checked before samples were processed (Multichem S Plus controls as before).

In the enzymatic serum creatinine assay (Abbott Architect System, Enzymatic serum creatinine kit, product number 8L24-41), creatinine in the sample was hydrolysed by creatininase to creatine. Creatine was in turn hydrolysed by creatinase to sarcosine and urea. Sarcosine from this reaction was oxidised by sarcosine oxidase to glycine and formaldehyde, with resulting production of hydrogen peroxide. The hydrogen peroxide reacted with 4-aminoantipyrine and N-ethyl-N-sulfopropyl-m-toluidine in the presence of peroxidase to yield a quinoneimine dye. The change in absorbance at 548 nm was proportional to the creatinine concentration in the sample. The assay was calibrated as per laboratory protocols (Product number 6K30, Architect Clinical Chemistry calibrator) (Abbott Architect System, Enzymatic serum creatinine kit insert, 2023). Three levels of IQC samples were ran daily and checked before samples were processed (Multichem S Plus controls as before).

The eGFR is calculated using the CKD-EPI (CKD Epidemiology Collaboration) 2009 equation (Figure 2.4). It is recommended for adults and utilises serum creatinine, age, sex and race in the calculation. It is designed for serum creatinine methods that are traceable to isotope dilution mass spectrometry (IDMS) methods.

Expressed as a single equation:

$eGFR_{cr} = 142 \times \min(S_{cr}/\kappa, 1)^{\alpha} \times \max(S_{cr}/\kappa, 1)^{-1.200} \times 0.9938^{Age} \times 1.012$ [if female]

where:

S_{cr} = standardized serum creatinine in mg/dL

κ = 0.7 (females) or 0.9 (males)

α = -0.241 (female) or -0.302 (male)

$\min(S_{cr}/\kappa, 1)$ is the minimum of S_{cr}/κ or 1.0

$\max(S_{cr}/\kappa, 1)$ is the maximum of S_{cr}/κ or 1.0

Age (years)

Figure 2.4. eGFR calculation using the CKD-EPI (Levey et al, 2009).

2.4.3 Principle of measurement and processing of Serum Thyroid Stimulating Hormone (TSH)

Serum TSH was measured using the Abbott Architect i2000 Immunoassay analyser (Abbott Diagnostics, Chicago, Illinois, USA). TSH was measured using a two-step (sandwich) immunoassay using chemiluminescent microparticle immunoassay (CMIA) and protocols called chemiflex (Abbott Architect System TSH kit, product number 7K62). Figure 2.5 for CMIA principle. In the first step sample, anti-β TSH antibody (mouse) coated paramagnetic microparticles and diluent were mixed. TSH present in the sample binds to the anti-TSH antibody coated microparticles. After washing, the second step was addition of anti-α TSH acridinium labelled conjugate. Pre-Trigger and Trigger Solutions were then added to the reaction mixture; the resulting chemiluminescent reaction was measured as relative light units (RLU) by the optics system. The amount of TSH in the sample was proportional to the RLU when read off a multi-point calibration curve (Product number 7K62-01 Architect

TSH calibrators) (Abbott diagnostics TSH kit insert, 2023). Three levels of IQC samples were ran daily following laboratory protocols and checked before samples were processed (Multichem IA Plus Controls for Abbott Architect, product number OSP76-10) (Technopath Clinical Diagnostics, 2023).

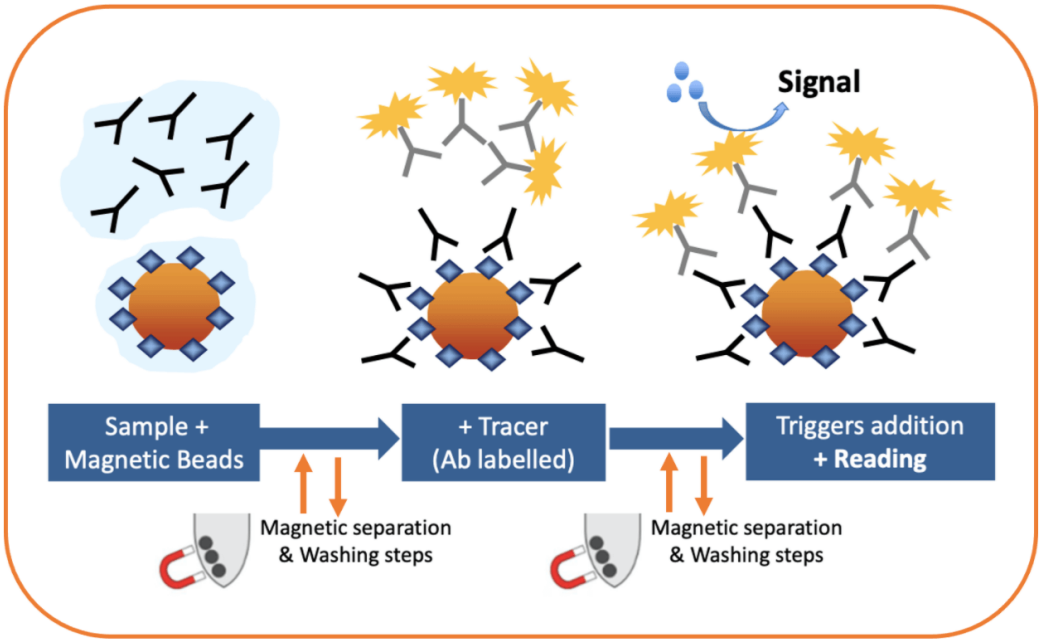


Figure 2.5. Principle of chemiluminescent immunoassay (CMIA) using magnetic beads. Sample containing analyte (antigen) mixed with beads coated with antibody which bind. After washing, labelled tracer and trigger solutions are added to generate chemiluminescence which is compared to a calibration curve to calculate amount of analyte present (Sepmag, 2024).

2.4.4 Principle of measurement and processing of Serum Total B12 (TB12)

Serum TB12 was measured using the Abbott Architect System i2000 Immunoassay analyser (Abbott Diagnostics, Chicago, Illinois, USA). Total vitamin B12 was analysed using CMIA and chemiflex for the quantitative determination of vitamin B12 (Abbott Architect System B12 kit, product number 7K61). The immunoassay is two step (competitive) with an automated sample pretreatment. The pretreated sample aliquot was transferred to a reaction vessel (RV), with pre-treatment reagents 1,2 and 3. Diluent and intrinsic factor

(porcine) coated paramagnetic microparticles were mixed with the sample. B12 present in the sample binds to the intrinsic factor coated microparticles. After washing, B12 acridinium labelled conjugate was added in the second step (binds to the remaining unbound intrinsic factor). Pre-trigger and trigger reagents were added to the reaction and the resulting chemiluminescence was measured in RLU. The amount of B12 in the sample was inversely proportional to the RLU measured by the Architect optical system and was read off a multi-point calibration curve (Product number 7K61-01 Architect B12 calibrators) (Abbott diagnostics B12 reagent kit insert, 2023). Three levels of IQC samples were ran daily following laboratory protocols and checked before samples were processed (Multichem IA Plus Controls as before).

2.4.5 Principle of measurement and processing of Serum Folate

Serum folate was measured using the Abbott Architect System i2000 Immunoassay analyser (Abbott Diagnostics, Chicago, Illinois, USA). The folate assay is similar to the TB12 assay as it is a two-step assay utilising CMIA and chemiflex (Abbott Architect System Folate reagent kit, product number 1P74). Two pre-treatment steps allow the release of folate from endogenous folate binding protein. In Pre-Treatment Step 1, sample and Pre-Treatment Reagent 2 (Dithiothreitol) were pipetted into a RV. In Pre-Treatment Step 2, an aliquot of this mixture was added to Pre-Treatment Reagent 1 (potassium hydroxide). Once the pre-treatment was complete Folate Binding Protein (FBP) coated paramagnetic microparticles (mouse) and assay specific diluent were added, and any folate present in the sample binds to the FBP coated microparticles. After washing, pteroic acid-acridinium labelled conjugate was

added and binds to unoccupied sites on the FBP-coated microparticles. Pre-trigger and trigger Solutions were then added to the reaction mixture; the chemiluminescent reaction was measured as RLU. The amount of folate in the sample was inversely proportional to the RLU measured by the Architect optical system and was read off a multi-point calibration curve (Product number 1P74-01, Architect Folate calibrators) (Abbott diagnostics Folate reagent kit insert, 2023). Three levels of IQC samples were ran daily following laboratory protocols and checked before samples were processed (Multichem IA Plus Controls as before).

2.4.6 Principle of measurement of Holotranscobalamin (Holo-TC /Active B12)

As for the serum TB12 assay the serum Holo-TC was measured using Abbott Architect System i2000 Immunoassay analyser (Abbott Diagnostics, Chicago, Illinois, USA) using a two-step CMIA and chemiflex for the quantitative assessment of Holo-TC (Abbott Architect System Active B12 kit (Holotranscobalamin), product number 3P24-25).

In this assay, the sample and anti-holotranscobalamin (mouse) coated paramagnetic particles were mixed and the Holo-TC in the sample bound to the coated microparticles. After washing, anti-transcobalamin acridinium labelled conjugate was added to the reaction vessel. Following a further wash, pre-trigger and trigger reagents were added and the chemiluminescent reaction measured as RLU however in this assay the amount of RLU was directly proportional to the amount of Holo-TC in the sample which was read off a multi-point calibration curve (Product number 3P24-01, Active B12 Calibrators). (Abbott diagnostics Active B12 (Holotranscobalamin) kit insert 2023).

2.4.6.1 Verification of Holo-TC method

As the Holo-TC kit was CE marked and being utilised as per kit insert with no modification to the method, full validation of the method was not required for ISO 15189 accreditation purposes (UKAS, 2022) therefore only the simpler method verification was required. The following verification procedures were undertaken to ensure the kit was operating as per the supplier performance criteria.

2.4.6.1.1 Imprecision studies

Intra-batch imprecision was evaluated by running replicates (n=10) of three concentrations of the kit calibrators (Calibrator B – 8 pmol/L, calibrator D – 32 pmol/L, calibrator E – 64 pmol/L).

Inter-batch imprecision was assessed by analysing replicates (n=10) of three sample pools prepared by diluting the kit controls and calibrators, over five different days. The pools were prepared as follows:

Sample 1 - Low QC diluted to 8 pmol/L using multi-assay diluent

Sample 2 - High QC diluted to 32 pmol/L using multi-assay diluent

Sample 3 - Calibrator F diluted to 90 pmol/L using multi-assay diluent.

Imprecision was calculated as %CV.

2.4.6.1.2 Bias

Assay Comparison

Thirty surplus serum samples were analysed for Holo-TC at New Cross Hospital, Wolverhampton and also at the Nutristasis Unit, St Thomas' Hospital, London, UKAS accredited laboratory (range of results 7.4-93.3 pmol/L).

Clinical and Laboratory Standards Institute (CLSI) guideline EP9-A2 states the following acceptance criteria for bias (CLSI, 2002).

i) $r \geq 0.975$ ($r^2 \geq 0.950$)

ii) Maximum allowable bias: $<0.375 \times (CV_I^2 + CV_G^2)^{½}$

when CV_I = within subject variation

CV_G = between subject variation

Comparison of EQA

UKNEQAS has a Holo-TC scheme provided by birminghamquality.org.uk that distributes on a monthly basis 3 samples with unknown Holo-TC to participant laboratories. The serum samples were tested, and results returned and compared with other users of the same technology/kits and against all respondents. No scoring system was originally set for this scheme due to small numbers of participants, with a target of <10% bias reported as acceptable however for most assays and EQA schemes ± 2SD is considered acceptable.

Later the A, B, C scoring was introduced with acceptance criteria set. 'A' stands for accuracy (total error), 'B' for bias and 'C' for consistency of bias and were

calculated over a rolling time window combining information derived from many specimens (4 survey, 12 samples). The 'A' score for Holo-TC at the time was a proto-A score set at a flat 10% degree of difficulty until enough data points could be accumulated (UK NEQAS, 2012).

EQA samples (n=25) from UK NEQAS, Birmingham Quality were analysed and compared to the all laboratory trimmed mean (ALTM) to determine accuracy. Acceptable performance was assessed against the EQA provider's criteria where available.

2.4.6.1.3 Stability studies

Three serum samples were chosen, one with an indeterminate Holo-TC result (50 pmol/L), one with a replete result (75 pmol/L) and one with a raised result (>128 pmol/L). Each sample was separated into multiple aliquots, one aliquot was analysed immediately, and the remaining aliquots were stored at room temperature, 4^{o}C and at -80^{o}C. Due to volume of sample obtained, stability in the sample with replete, raised and indeterminate Holo-TC results were tested for one week, four months and eight months, respectively. Each day an aliquot was tested a previously untested aliquot was used.

The kit insert specified serum samples are stable for up to 16 hours at room temperature or three days when stored at 2-8^{o}C. For longer storage serum should be removed off clot and stored at -20^{o}C or colder for up to 6 months. The evaluation covered this time period and conditions.

2.4.6.1.4 Analytical range (Linearity) (CLSI EP06-A Method, CLSI, 2003)

Linearity studies were performed using patients' samples, one with a high Holo-TC (112 pmol/L) as the standard and one with a low Holo-TC (5.8 pmol/L) as the diluent. A series of dilutions were made to maximally cover the measurement range and analysed. The results were plotted on a graph of assigned values versus measured values with a calculation for the slope, intercept and standard error. Manufacturers quoted linear measurement range is 5 pmol/L to 128 pmol/L (Abbott Diagnostics, Holo-TC kit insert).

2.4.6.1.5 Limit of blank (LoB), Limit of detection (LoD), Limit of quantification (LoQ)

LoB: Highest concentration of analyte that would be detected in samples that contain no analyte i.e. a blank

$$\mathbf{LoB = Mean_{blank} + 1.645SD_{blank}}$$

LoD: Lowest concentration that can be reliably distinguished from noise.

$$\mathbf{LoD = LoB + 1.645SD_{low\ concentration\ sample}}$$

LoQ: Actual concentration of analyte that can be reliably detected. If %CV of LoD is acceptable (<20%) then LoD =LoQ

LoB was determined by analysing 10 replicates of the zero calibrant.

LoD was determined by diluting a patient sample to give a Holo-TC concentration of approximately 1.5 pmol/L and analysing 20 times.

The LoQ was determined by analysing six replicates from ten patient samples with Holo-TC concentrations spanning the measurement range (6.6 to 86.9 pmol/L). The LoQ is usually defined as the lowest measurable concentration with a CV ≤ 20%. However, the manufacturer's LoQ is ≤5 pmol/L where LoQ is defined as inter-assay imprecision of ≤10% CV (Abbott Holo-TC kit insert).

2.4.6.1.6 Recovery

An EQA sample (frozen at -20^{o}C after results submission, stored for <6 months), with a known Holo-TC concentration of 85.5 pmol/L was used to spike patient serum samples with a range of Holo-TC concentrations (deficient to raised), with only 10% EQA sample added. A set of blank tubes with 10% stripped serum added to the four patient samples were used for comparison. All tubes were tested in duplicate and percentage recovery calculated using the formula.

(Experimental yield/Theoretical yield) x100.

A recovery greater than the allowable error for bias or imprecision is deemed acceptable for these analytes (Burnett, 2013).

Allowable error = bias + (1.65 x CV_A) where CV_A is imprecision (CLSI, 2002) was used to calculate the allowable recovery range.

The difference between the mean recovery and 100% provides an estimate of the proportional error.

2.4.6.1.7 Acceptance criteria summary (Table 2.2) for Holo-TC

Table 2.2. Acceptance criteria for Holo-TC derived from a combination of Abbott Diagnostics validation data and biological variability data.

Performance Characteristic	Parameter	Target
Bias %	EQA	<10% ± 2SD A score <200 B score <± 15 C score <15
	Method comparison PB regression	$r^2 \geq 0.950$ Bias 10.4%
Imprecision %	%CV for samples with Holo-TC ≥ 20 pmol/L	≤8%
	SD for samples with Holo-TC ≤ 20 pmol/L	≤1.6
Accuracy (% Recovery)		±22.3%
LoB	pmol/L	<0.4
LoD	pmol/L	<1.90
LOQ	pmol/L	≤5.0
	CV%	≤10%
Linearity	Range (pmol/L)	5.0-128.0

2.4.6.1.8 Results range's for B12 deficient, indeterminate and B12 replete

The Abbott Holo-TC kit reportable values for B12 deficient, indeterminate zone and B12 replete patients were verified against the 'proxy gold standard' functional MMA assay for suitability of use in the local population.

2.4.6.2 Analysis of patient samples for Holo-TC

Two levels of IQC samples were run daily and checked before samples were processed and at the end of day (Product number 3P24-10, Active B12 Controls) (Abbott diagnostics Active B12 (Holotranscobalamin) kit insert 2023). No third party IQC was available at the time of kit verification and patients sample processing.

The frozen serum samples were thawed at room temperature for five minutes, mixed and analysed as described in section 2.4.6.

Long term stability of Holo-TC has been investigated previously by Loikas et al (2003) with samples frozen for 16 months at -70ºC with no statistically significant difference seen between initial concentrations and those measured after storage at −70°C (mean of differences, 4 pmol/L; *p*= 0.56). All patient sample aliquots were tested for Holo-TC within this time period.

2.4.7 Principles of measurement of Methylmalonic Acid (MMA)

MMA was measured by HPLC tandem mass spectrometry (HPLC-MS/MS), using Shimadzu Dual pump Quad gradient HPLC system, Applied Biosystems (AB) Sciex 6500 Q-Trap tandem mass spectrometer with Electrospray Ionisation (ESI) source and Peak Scientific Nitrogen gas generator and filter and using the MassChrom® MMA reagent kit for LCMS/MS (CE-IVD) which included a proprietary column, pre-prepared mobile phase solutions A and B, a rinsing solution and clean-up tubes for extraction (Chromsystems, Gräfelfing, Germany, Europe, product number 64000) (Figures 2.6, 2.7 and 2.8).

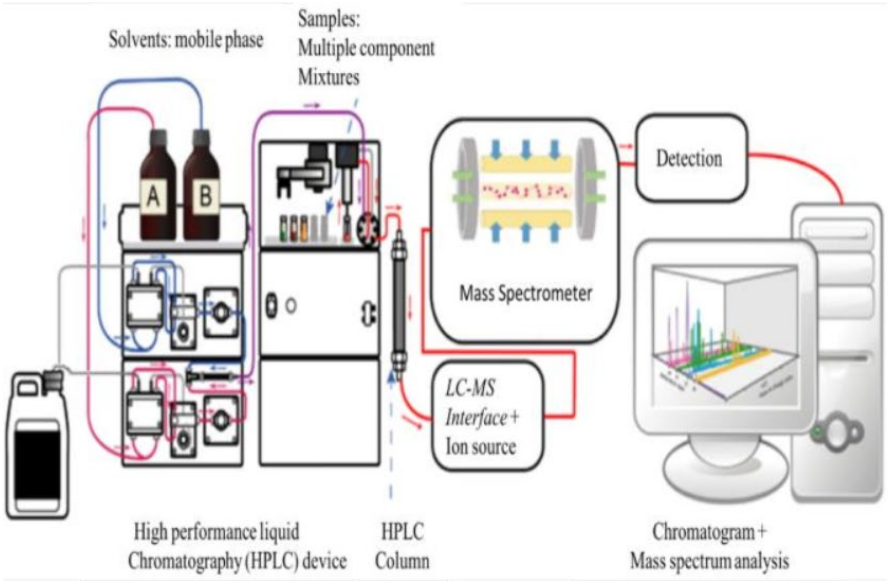


Figure 2.6. Simplified overview image of analytical units involved in HPLC-MS/MS and sample flow (Kavya et al, 2024).

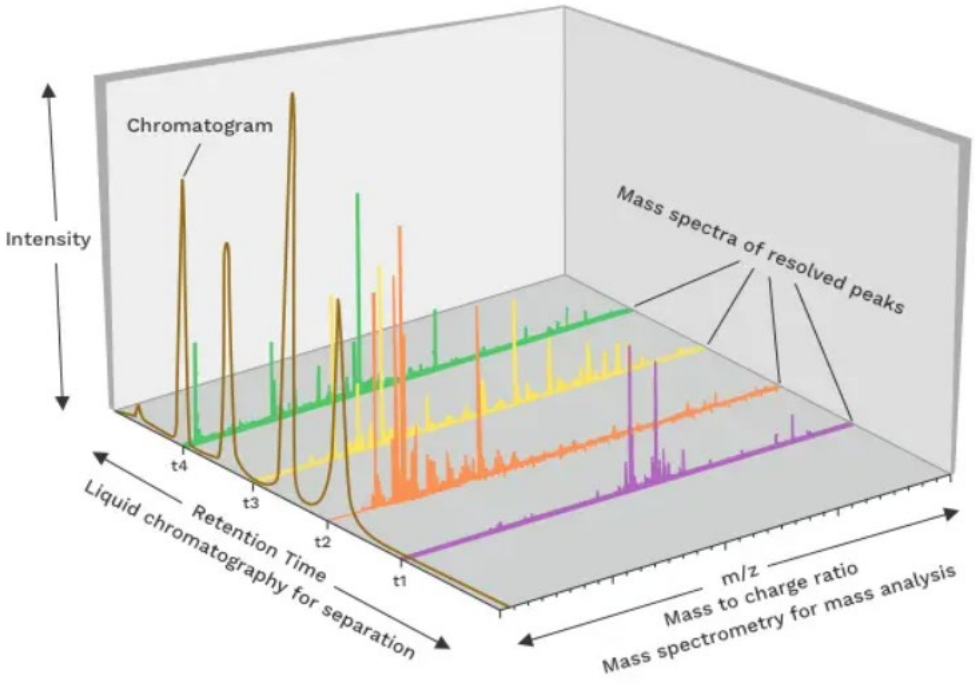


Figure 2.7 Expanded view of chromatogram and mass spectrum analysis showing how mass to charge ratio relates to chromatogram produced (Norena-Caro, 2017).

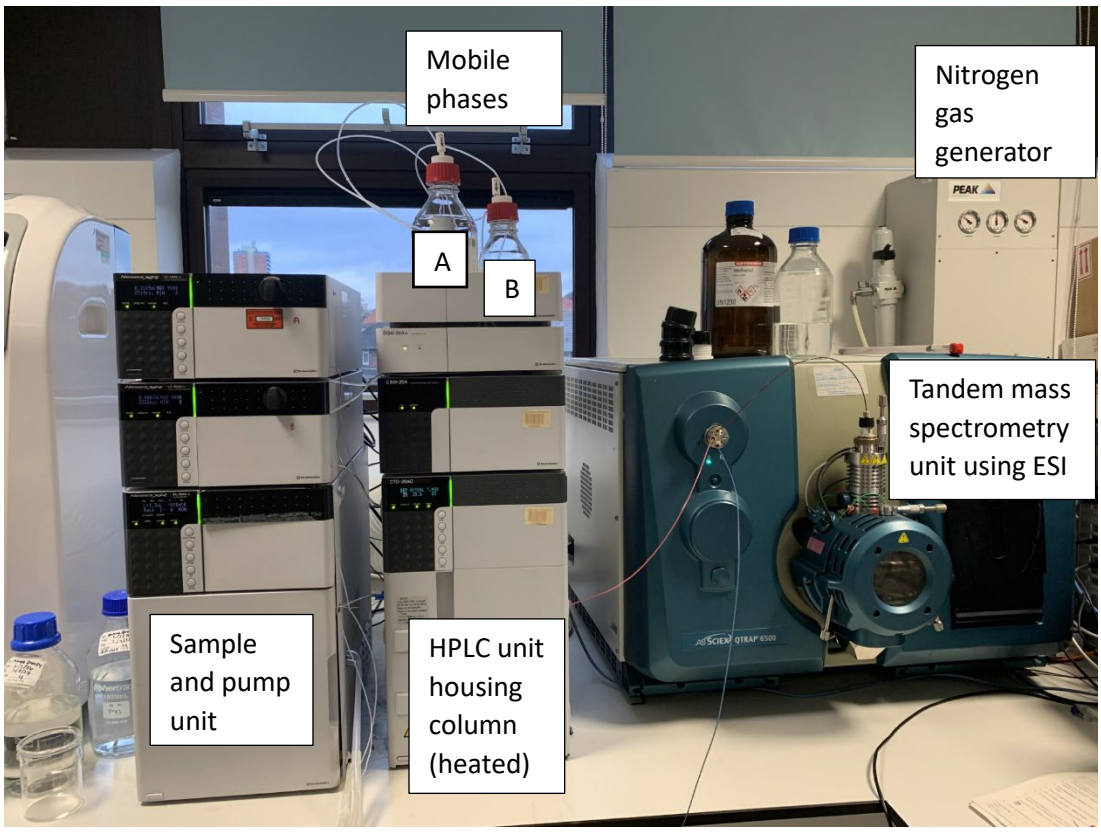


Figure 2.8. Photograph of actual HPLC-MS/MS equipment used, Sciex 6500 tandem mass spectrometer (AB Sciex Technologies, Toronto, Canada) and Shimadzu Nexera X2 UHPLC system, in situ at Sandwell General Hospital, West Bromwich, Birmingham (Parkes, 2024). ESI = Electrospray Ionisation.

The assay involved extracting MMA from serum, this can be undertaken by various methods however the final method, utilised proprietary clean-up tubes supplied as part of the MassChrom® MMA kit. Deuterated MMA Internal standard for serum/plasma (Chromsystems, Gräfelfing, Germany, Europe, product number 64004) was added to account for systemic losses during the course of the assay. The filtrate was injected through a proprietary analytical column (MassChrom® MMA Analytical Column equilibrated with test chromatogram, Chromsystems, Gräfelfing, Germany, Europe, product number 64100) that resolves MMA from its isobar succinic acid. The eluted sample flowed into the MS/MS source where it was ionised by ESI- (to generate negatively charged ions). In Quadrupole 1 (Q1) the MMA quantifier, qualifier and

internal standard parent-ions were selected based on the mass to charge ratio (m/z). In the collision cell (Q0) nitrogen gas caused them to fragment into product ions. In Quadrupole 2 (Q2) the daughter ions were selected to pass through to the detector. When the ions reached the detector, a current created the mass spectrum (Figure 2.9). Sciex Analyst software (version 1.7.2, Sciex, Danaher Corporation, Toronto, Canada) was used to calculate the peak area to quantitate the concentration of MMA in the sample using a multipoint calibration curve (3PLUS1 Multilevel Plasma Calibrator set MMA, Chromsystems, Gräfelfing, Germany, Europe, product number 64028).

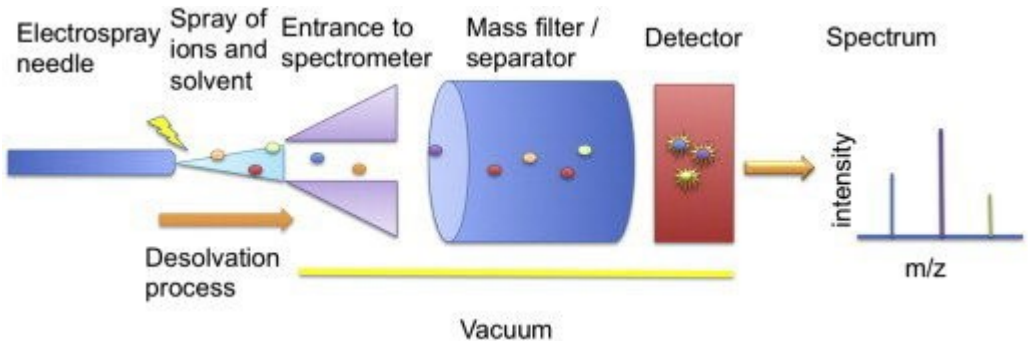


Figure 2.9. Simplified diagram of a mass spectrometer. Sample eluted from HPLC is sprayed using a charged needle and nitrogen gas into the mass spectrometer. The ions of interest are separated and detected with a spectrum produced showing m/z ratio versus the amount of each ion present (Rankin et al, 2014).

2.4.7.1 MMA extraction methods and HPLC/Mass spectrometry combinations investigated

The first method and extraction process trialled used the Acquity UPLC I-class system coupled with a Waters™ Xevo TQ-S quadrupole mass spectrometer

(Waters Corporation, Milford, Massachusetts, USA). The separation was performed with a Acquity™ UPLC CSH (charged surface hybrid) C18 column (100 mm x 2.1 mm) packed with 1.7 µm, 130 Å particles (Waters Corporation, Milford, Massachusetts, USA, product number 186005297). The method involved reverse phase chromatography with a gradient mobile phase of water and acetonitrile and a flow rate of 0.45 mL/min. Samples were extracted using an Ostro™ phospholipid removal plate (Waters Corporation, Milford, Massachusetts, USA, product number 186005518). Sample and deuterated MMA internal standard were pipetted into the plate and formic acid in acetonitrile was forcibly added, after mixing, a vacuum was applied to the plate and the clear filtrate collected and tested. Due to initial problems with the mass spectrometer, this method was also tested on a Waters™ Xevo TQ-S micro quadrupole mass spectrometer (Waters Corporation, Milford, Massachusetts, USA), utilising the same column, method and extraction process described above.

The second method and extraction process' explored was performed on the Shimadzu Nexera X2 UHPLC system coupled with a Sciex 6500 tandem mass spectrometer (AB Sciex Technologies, Toronto, Canada). The separation was performed with a PEEK ZIC® -HILIC column (C18, 100 mm x 2.1 mm) packed with 3 µm, 100 Å particles (Merck KgaA, Darmstadt, Germany, product number 1504410001). An isocratic mobile phase of acetonitrile containing 100 mM ammonium acetate was employed at a flow rate of 1 mL/min. Samples were deproteinated with formic acid and acetonitrile 'crash solution' containing a deuterated MMA internal standard.

The final method tested, and the verification was completed on the Chromsystems Masschrom® CE-IVD kit (Chromsystems, Gräfelfing, Germany, Europe, product number 64000) using the Sciex 6500 tandem mass spectrometer (AB Sciex Technologies, Toronto, Canada) and Shimadzu Nexera X2 UHPLC system and a proprietary analytical column (MassChrom® MMA Analytical Column equilibrated with test chromatogram, Chromsystems, Gräfelfing, Germany, Europe, product number 64100). A gradient mobile phase was employed at a flow rate of 0.7 mL/min (set-up detailed below). The kit included proprietary clean up tubes for sample extraction (product number 64008), sample and deuterated MMA internal standard (product number 64004) were added before vortex mixing and centrifugation with the resultant filtrate tested.

2.4.7.2 Chromsystems MMA analytical set up details

2.4.7.2.1 Liquid chromatography (LC) configuration

The LC was configured to direct the sample to the guard column and elute to waste for 1.6 minutes. The switching valve was used to redirect the flow to the analytical column at 1.6 minutes, then back to waste after the elution of MMA at 2.2 minutes.

2.4.7.2.2 Liquid chromatography mass spectrometer (LCMS/MS) method

Figures 2.10, 2.11, 2.12 and 2.13 show the final verified mass spectrometer and LC parameters respectively as per manufacturer's instructions (Chromsystems, 2019). The injection volume was 20 µL, the oven temperature was set to 25 °C and the starting pressure was 2000-2500 psi.

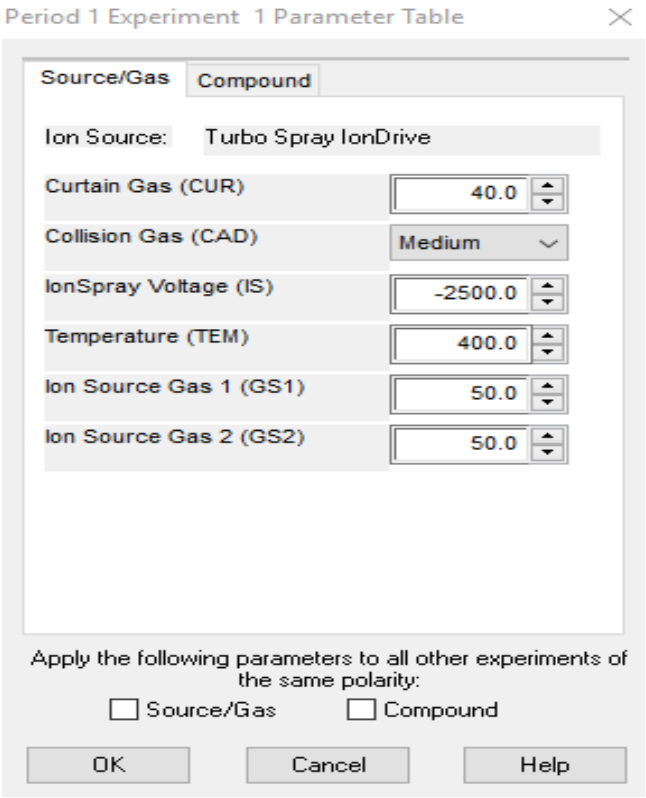


Figure 2.10. MS ion source settings used to ionise the sample gas and focus the beam into the analyser (Screenshot taken from system data manager software).

The mass spectrometer was operated in Electrospray ionisation negative (ESI-) mode with the capillary set at 0 mm vertical, 5 mm horizontal and 1 mm protrusion of the capillary tip. ESI- was the stated method from the supplier however ESI is a softer ionisation technique, causing minimal fragmentation

and is suitable for moderately polar molecules. MMA is a small hydrophilic molecule (118 Da) with a single functional group which when ionised (through deprotonation) forms single negatively charged ions, the method has high sensitivity and accuracy although ESI- is more difficult to maintain than ESI positive as higher voltages are necessary to electrospray the liquid, acidic conditions can suppress ionisation and other background matrix contaminants can suppress ionisation (Ho et al, 2003).

The valve was set up to direct flow to the mass spectrometer only for the window in which the MMA peak was expected to elute (1.6-2.2 minutes).

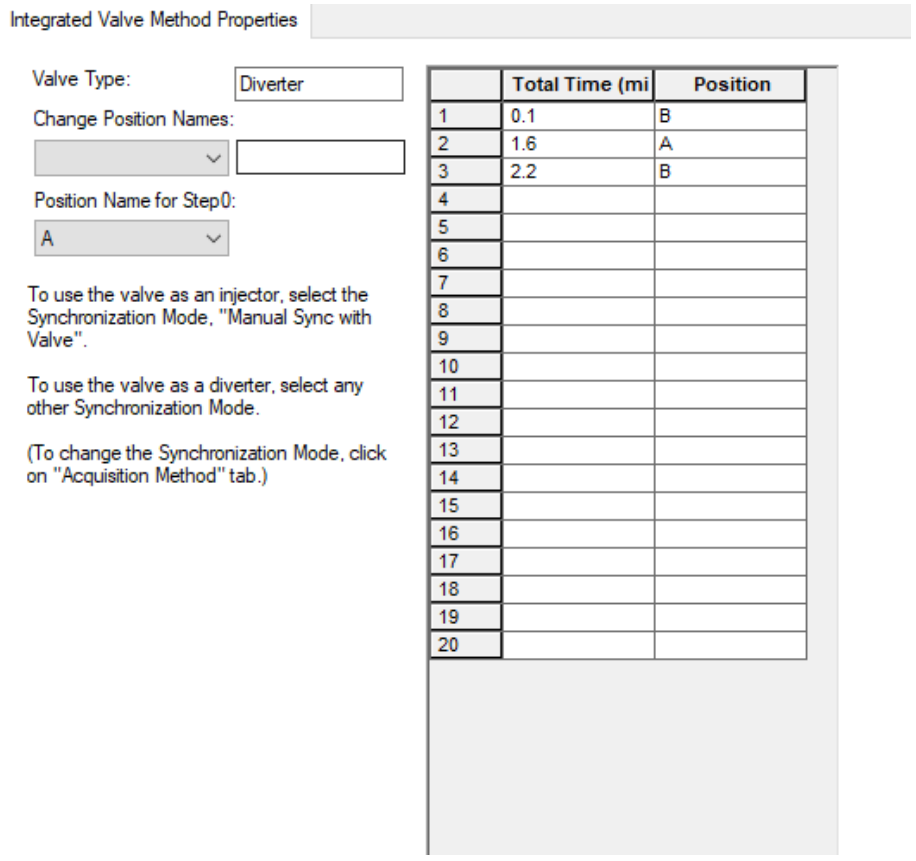
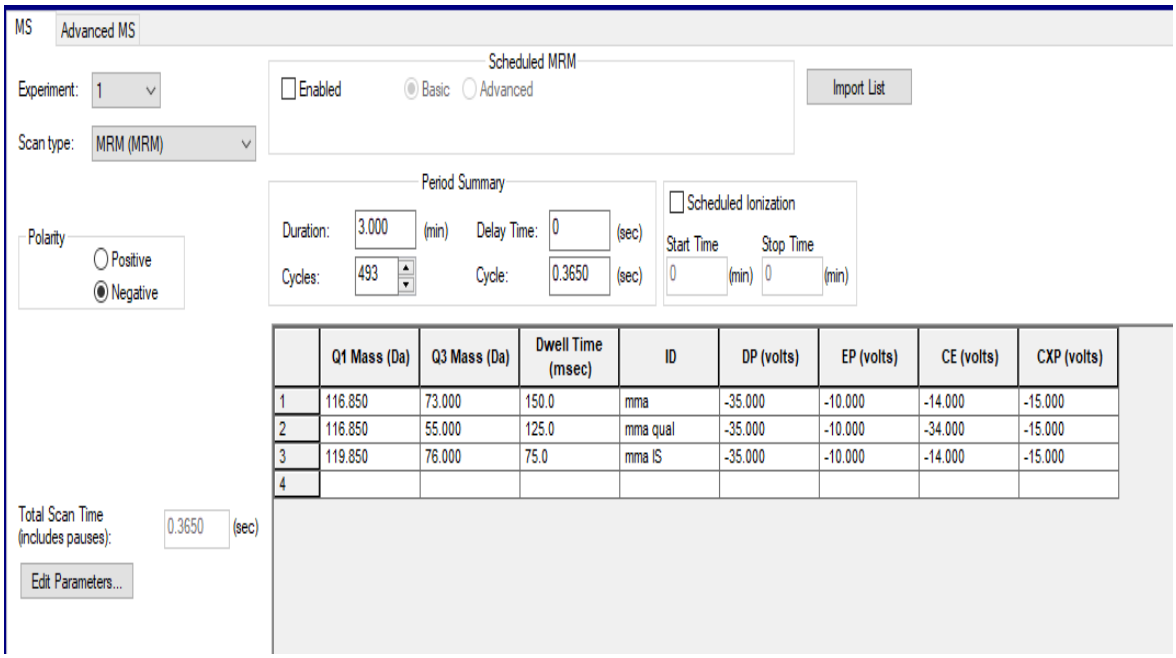


Figure 2.11. Valve setting showing times at which switching occurs (Screenshot taken from system data manager software).



	Q1 Mass (Da)	Q3 Mass (Da)	Dwell Time (msec)	ID	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
1	116.850	73.000	150.0	mma	-35.000	-10.000	-14.000	-15.000
2	116.850	55.000	125.0	mma qual	-35.000	-10.000	-34.000	-15.000
3	119.850	76.000	75.0	mma IS	-35.000	-10.000	-14.000	-15.000
4								

Figure 2.12. Mass spectrometer parameters showing mass of the analytes of interest. The MMA qualifier ion was used to help identify MMA analyte to ensure the measured peak was MMA, not matrix or anything else. The MMA internal standard (IS) was used to check for systematic losses during the assay (Screenshot taken from system data manager software).

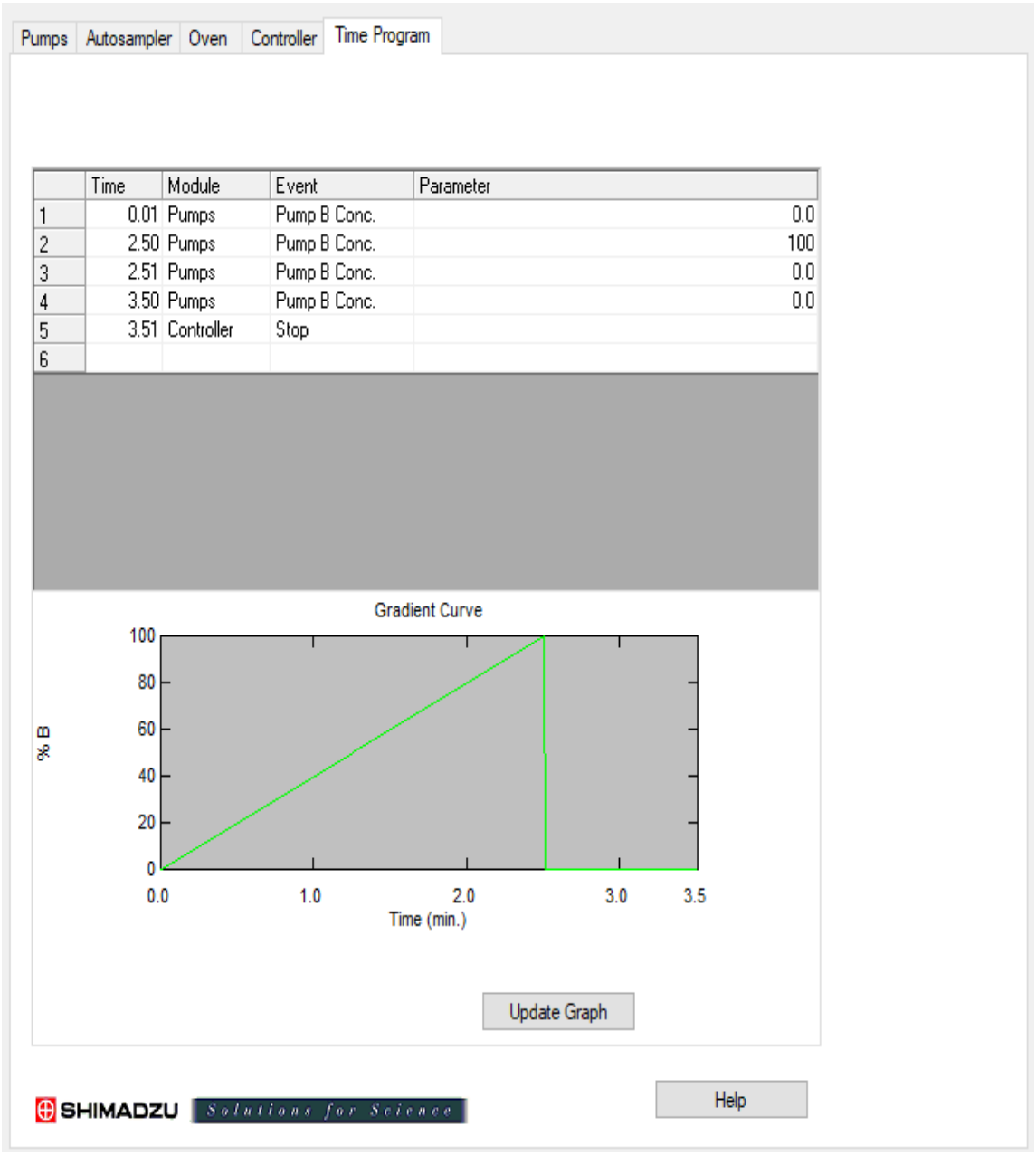


Figure 2.13. LC settings showing the gradient of mobile B during the analysis time (Screenshot taken from system data manager software).

2.4.7.2.3 Quantitation method

Sciex Analyst software (version 1.7.2, Sciex, Danaher Corporation, Toronto, Canada) was used to calculate the peak area ratio to quantitate the concentration of MMA in the sample using a multipoint calibration curve

(3PLUS1 Multilevel Plasma Calibrator set MMA, Chromsystems, Gräfelfing, Germany, Europe, product number 64028). A smoothing factor of 9 was applied. The calibration employed a linear fit and 1/x weighting as recommended by Chromsystems. The assay time from injection to result was 3.5 minutes per sample. For laboratory processing the intention would be to prepare sample batch and run the assay overnight. Figure 2.14 showed an example mass chromatogram on a patient with a normal MMA concentration that would be used to calculate the peak area ratio.

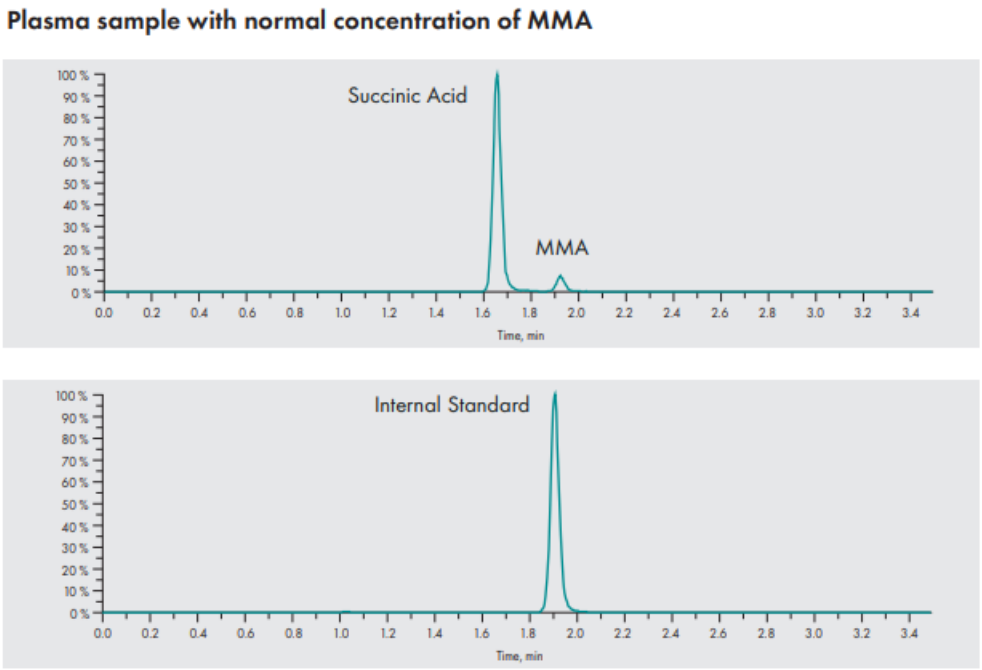


Figure 2.14. Example mass chromatograph of a sample with a normal concentration of MMA (Chromsystems, 2019).

2.4.7.3 Verification of MMA method

The final MMA method established and adopted was using a CE marked kit from Chromsystems as stated above which was introduced as per suppliers' instructions with no modification to the method. Therefore, full validation of the method was not required for ISO 15189 accreditation purposes (UKAS, 2022) and only the simpler method verification was required.

Performance characteristics were assessed in accordance with the appropriate CLSI guideline where possible. Where adjustments had to be made to suggested methods due to availability of materials and feasibility of work in the laboratory setting, this was documented and justified.

The following verification procedures were undertaken:- inter-batch imprecision, bias, sample stability, linearity, analytical sensitivity, recovery, carryover and measurement uncertainty. In addition, as this was a mass spectrometry assay ion suppression testing and isobaric compound interference assessment were also undertaken.

2.4.7.3.1 Imprecision studies

Inter-batch imprecision was assessed at two levels of 3rd party, internal quality controls (IQC) analysed in duplicate over 17 different batches (Recipe

Chemicals, ClinChek® control for MMA, product number MS5082). Two different operators prepared the batches over several weeks.

IQC level 1 had an MMA target value near to the cut-off for B12 deficiency (270 nmol/L). IQC level 2 had a higher concentration of MMA (target 588 nmol/L) within the B12 deficient range.

The manufacturer had assessed imprecision at two different levels on two mass spectrometry systems. However, neither system evaluated (Sciex 4500 and Agilent 6460) was the same as the laboratory mass spectrometer system. The target CVs for level 1 IQC and 2 IQC were 6.7 and 4.8 % respectively (email correspondence with Chromsystems, appendix 2).

2.4.7.3.2 Bias

Assay Comparison

Thirty anonymised patient aliquots covering the measurement range (181-887 nmol/L) for MMA were acquired from the Nutristasis Unit, St Thomas' Hospital, UKAS accredited laboratory results compared with MMA analysis performed at Sandwell Hospital, Birmingham.

CLSI guideline EP9-A2 states the following acceptance criteria for bias (CLSI, 2002).

i) $r \geq 0.975$ ($r^2 \geq 0.950$)

ii) Maximum allowable bias: $<0.375 \times (CV_I^2 + CV_G^2)^{½}$

when CV_I = within subject variation

CV_G = between subject variation

Accuracy was also assessed by analysing pure certified reference material (CRM) (Methylmalonic acid in acetonitrile 1ml, Cerilliant Corporation, Merck, Darmstadt, Germany, product number M-080) at three relevant concentrations and the % bias calculated between expected and measured results.

Comparison of EQA

It was not possible to assess performance against the UK NEQAS, Birmingham Quality holotranscobalamin scheme as there was currently only a single registered user and therefore no acceptable target values.

The laboratory has registered with the ERNDiM scheme (quality assurance in laboratory testing for inborn errors of metabolism). Survey 2024.01 results submitted March 2024. Acceptable performance was assessed against the EQA provider's criteria. The laboratory result was compared to the method mean, with deviation from the mean calculated.

2.4.7.3.3 Stability studies

The literature suggests MMA is very stable, with serum samples stable for up to one week at room temperature, stable for a minimum of 6 months at 5ºC, for 1 year at -20ºC and for 5 years at -70ºC. The samples are also stable for up to 3 freeze thaw cycles (Mineva et al, 2015, Pedersen et al, 2011). All samples for this study had been frozen at -80ºC within 2 days of receipt in the laboratory and MMA testing completed within five years.

Several samples were re-tested on subsequent runs to check freeze thawing of samples.

The stability of extracted samples was quoted by supplier as:-

1 week at 2-8ºC (must be protected from light)

4 days at 20-25ºC (must be protected from light)

A batch of samples was run on the day of extraction, stored at 2-8ºC for 4 days and then rerun. Room temperature storage was not evaluated during the study.

2.4.7.3.4 Analytical range (Linearity)

The linearity assessment was conducted in accordance with CLSI guideline EP6 A **(**CLSI, 2003).

Eleven concentrations spanning the range <30-5080 nmol/L were assessed in duplicate.

A high pool was made up of a serum pool spiked with MMA certified reference material (CRM) (Methylmalonic acid in acetonitrile 1ml, Cerilliant Corporation, Merck, Darmstadt, Germany, product number M-080). It was not possible to create a low pool using patient samples as sufficiently low concentrations could not be found. The low pool was a serum pool diluted with saline to below the LOQ of the assay.

The high and low pools were diluted together in increments to produce a dilution series that spanned beyond the highest and lowest calibrator values.

CLSI guideline EP6A (2003) states the following acceptance criteria for linearity should be no greater than the bias (trueness) goal. This was defined as 8.4%. The linear ranges must also be clinically relevant and achievable according to the manufacturer. The manufacturer target linear range was 30 nmol/L to 5080 nmol/L.

2.4.7.3.5 Analytical Sensitivity - Limit of quantification (LoQ)

The LoQ has previously been described and according to CLSI EP17-A2 method (2004), the allowable imprecision for LoQ is <20%, the LOQ must be clinically useful i.e. lower than lower limit of the reference interval approximately

70 nmol/L (Wolters, Hermann and Hahn, 2003) and LOQ <30 nmol/L is achievable on the laboratory mass spectrometer system according to the manufacturer.

To determine the assay LoQ a patient's sample was diluted with saline to a low concentration below the expected LoQ since there were no samples with very low MMA results. This sample was analysed twelve times and the SD and %CV calculated.

2.4.7.3.6 Recovery (Accuracy)

One pool of serum was spiked with three clinically relevant concentrations of MMA (85, 423, 846 nmol/L respectively) using CRM. An unspiked base-pool sample (176 nmol/L) was also analysed. Recovery was calculated using the following formula:

$$\frac{\text{Spiked concentration} - \text{Base-pool concentration}}{\text{Spike}} \times 100\%$$

A recovery greater than the allowable error for bias or imprecision was deemed acceptable for these analytes (Burnett, 2013).

Allowable error = bias + (1.65 x CV_A) where CV_A is imprecision (CLSI, 2002). An allowable bias of 8.4% and imprecision of 4.1 were applied as previously calculated. Therefore, the allowable recovery was 100 ± 15.2%.

2.4.7.3.7 Analytical Specificity and Interfering Substances

Interferences causing ion suppression in ESI Mass spectrometry can involve the sample matrix or coeluting compounds e.g. succinic acid.

Ion suppression

In order to determine whether there were any compounds present in the method that could interfere with ionisation of the target analytes, an ion suppression test was performed.

A solution of approximately 10 nM MMA and MMA internal standard was infused directly into the mass spectrometer.

The following samples were then injected as per the method:

- Extracted water blank
- System blank (non-extracted Mobile A)
- Blank IS- extraction tube

Ion suppression was defined as a 10% decrease in signal at the retention time (RT) of the respective analytes (1.9-2.1 minutes) (Honour, 2011).

Interferences

Methylmalonic acid and succinic acid are isobaric compounds and must be separated chromatographically. A sample was spiked with succinic acid (Succinic Acid, CRM TraceCERT, Merck, Darmstadt, Germany, product number 49893) *and* analysed using the described method. A resolution of 1.5 or greater indicated full baseline separation.

Resolution (Rs) was calculated using the formula:

Rs = (RT MMA – RT Succinic) / ((peak width MMA + peak width Succinic)/2)

Other interfering substances e.g. lipaemia, icterus, haemolysis had been tested by Chromsystems as part of their validation to see if an effect was seen on analyte peak or retention time, this was not repeated during the verification and any grossly abnormal samples were rejected for testing.

Carryover

A patient sample was spiked with a high concentration of MMA (9900 nmol/L) and a blank calibrator sample was prepared as per the documented extraction process. The blank sample was analysed 6 times, and the mean MMA concentration calculated. The high and low samples were then run alternately, and mean MMA concentration of the blank samples was calculated. The mean

of blanks was compared to mean of the original blank samples using a paired t-test.

2.4.7.3.8 Summary of MMA acceptance criteria (Table 2.3)

Table 2.3. MMA acceptance criteria were derived from a combination of Chromsystems validation data and biological variability data.

Performance Characteristic	parameter	Target
Bias %	**EQA**	Z score <±2
	Sample comparison	$r^2 \geq 0.950$ bias 8.4%
Imprecision % (inter-batch)	**IQC Level 1**	6.7
	IQC Level 2	4.8
Recovery		±15.2%
Carryover	**Paired t-test**	p>0.05
Ion suppression	**% drop in signal**	<10
LOQ	**nmol/L**	<30
	CV%	<20%
Linearity	**Range (nmol/L)**	30-5080
	% non-linearity	<8.4%

2.4.7.3.9 Uncertainty of measurement

The uncertainty of measurement (MU) was calculated as per our laboratory protocol using the formula:

$$u=s/\sqrt{n}$$

where u = standard uncertainty, s=standard deviation, and n= total number of repeats.

For our utility of MU, u = s as we are interested in the MU for a single result for a patient sample.

The overall uncertainty at a stated confidence level was calculated using a coverage factor k. The standard uncertainty (u) or the combined standard uncertainty (u_c) (square root of the sum of squares of contributing standard uncertainties) can be used to determine the overall uncertainty which is also known as the expanded uncertainty which is expressed as:

$$U=ku_c$$

For a 95% level of confidence k=2 and therefore, MU=2SDs)

MU was assessed using seventeen batches of results (34 sets of ClinChek® Recipe IQC data).

2.4.7.3.10 MMA Reference range

Nutristasis Unit, St Thomas' Hospital is the only laboratory with an UKAS f accredited method for MMA by LC-ESI-MS/MS (UKAS, 2022). Their stated reference ranges are age dependent, if ≤65 years then range 0-280 nmol/L and if > 65 years then range 0-360 nmol/L (Nutristasis Unit, St Thomas' Hospital, 2023).

2.4.7.4 Analysis of MMA patient aliquots

Batches of approximately 72 samples were compiled at New Cross Hospital and transported frozen with ice packs to Sandwell General Hospital. The aliquots were thawed at room temperature for 5 minutes, then extracted as previously described in section 2.4.7. Chromsystem calibrators (4 concentrations), were included at the start of each batch (product number 64028) and Recipe IQC (product number MS5082) two levels included at the beginning and end of the batch to rule out assay drift.

2.5 Statistical analysis utilised throughout project

All variables evaluated in the study were analysed using a Kolmogorov-Smirnov D (KSD) statistical test for normality to establish either parametric or non-parametric distribution of the data. As the data did not show parametric distribution, non-parametric methods for analysis of the data were applied e.g. Spearman rank correlations.

The software 'Analyse-it®' for Microsoft®Excel (Version 6.15, Analyse-it® Software Ltd, Leeds, UK) and 'GraphPad Prism' (Version 10.2.3, GraphPad Software, Boston, USA) were utilised for statistical analysis.

2.5.1 Assay verifications

For the verification of the Holo-TC assay and MMA assays the following statistical analysis were undertaken.

Microsoft® Excel was used to calculate percentage coefficient of variation (CV) and standard deviation for accuracy and imprecision analysis.

The paired t-test was used for sample stability and carryover analysis.

Passing Bablok linear regression analysis was used to assess bias and linearity. For an acceptable comparison, the 95% confidence interval (CI) for the slope (proportional bias) should contain 1, and the 95% CI for the intercept (constant bias) should contain 0, with a Passing and Bablok regression analysis r^2 value of ≥ 0.950.

Diagnostic accuracy of TB12 and Holo-TC test for B12 deficiency were calculated producing sensitivity and specificity values by identifying true positive patients (deficient) from false negative or false positive patients and true negative patients (not deficient) with MMA as the gold standard test based on the 2x2 contingency table (Table 2.4), plus negative predictive values (NPV) and positive predictive values (PPV). Receiver operating characteristics (ROC) and area under the curve (AUC) analysis were also performed.

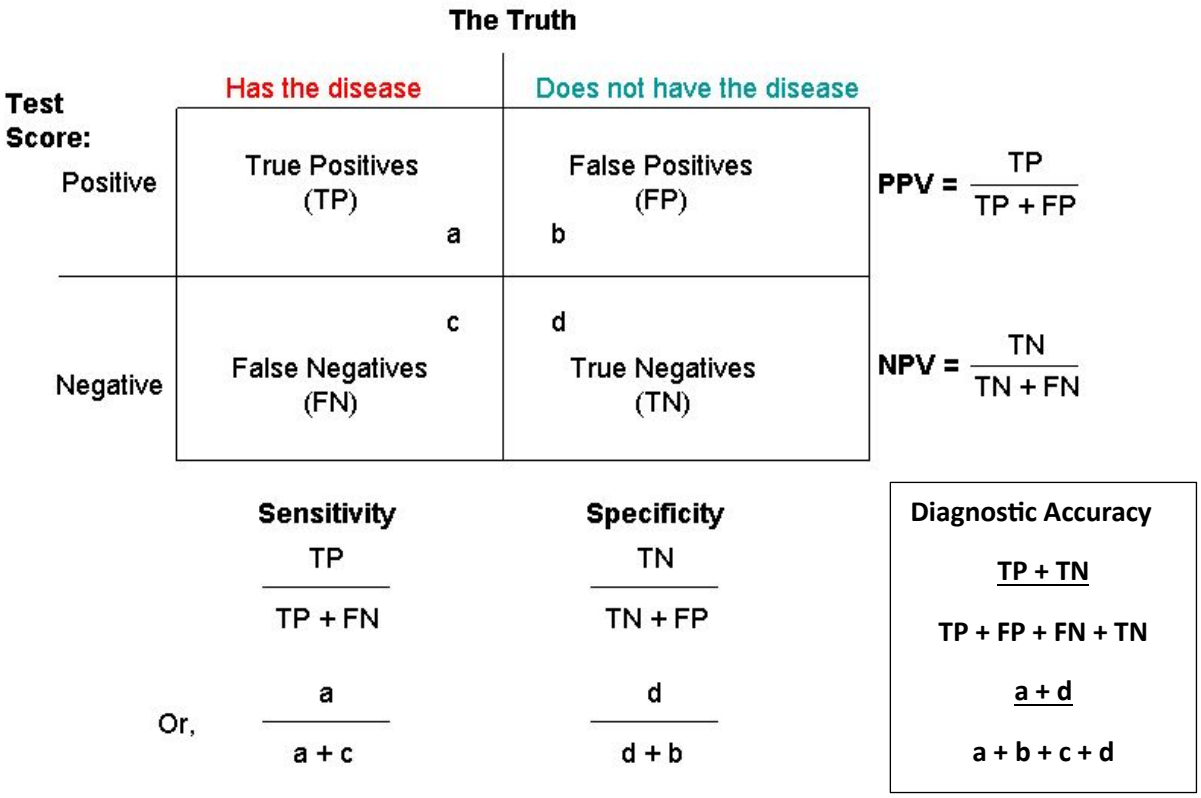


Table 2.4. 2x2 contingency table used to calculate sensitivity, specificity, NPV, PPV and diagnostic accuracy of compared tests (statisnursespitu. blogspot, 2024).

2.5.2 Patient sample analysis

Correlation studies using Spearman's Rank Correlation were conducted to establish associations between haemoglobin, RCC, MCV, TSH, U+E, serum folate, serum TB12, Holo-TC with B12 deficiency utilising MMA as the 'gold' standard assay. The Spearman Rank Correlation measures the linear relationship between two ranked variables. The two variables do not need to be measured on the same scale. Group comparisons were undertaken using one-way ANOVA (analysis of variance). ANOVA tests the null hypothesis that three or more population means are equal versus the alternative hypothesis that at

least one mean is different. As the data was non-parametric, the Kruskal-Wallis test is considered as the non-parametric alternative to the one-way ANOVA comparing three or more group medians. This test was used in addition to ANOVA however the results were not significantly different and therefore only ANOVA results are included. All statistical analysis was performed using a 95% confidence interval and statistical significance defined as $p<0.05$.

2.6 Health Economics evaluation

The cost of a Holo-TC ± MMA test combination is significantly more expensive than the existing serum TB12 test. Potential benefits from the use of Holo-TC ± MMA combination to test for B12 deficiency must therefore be balanced against the additional resources that may be expended, which also extend beyond the increased test cost, and include the cost of all resultant clinical strategies, and the costs of all clinical outcomes.

There are several economic healthcare tools used to evaluate the cost-effectiveness and value of medical and public health interventions including:

- Cost-Benefit Analysis (CBA): Measures both costs and benefits in monetary terms only to determine the overall value of an intervention.
- Cost-Effectiveness Analysis (CEA): Compares the costs and health outcomes of different interventions to determine which provides the best value for money.

- Cost-Utility Analysis (CUA): Is an extension of CEA but uses quality-adjusted life years (QALYs) to measure health outcomes, combining both quality and quantity of life (Gray et al, 2011).

Cost-effectiveness analysis provides a framework to assess the joint clinical and economic impact of new diagnostic strategies however the literature on the economic impact of diagnostic testing for B12 is scant. There was a need therefore to quantitatively compare the cost-effectiveness of the measurement of Holo-TC ± MMA with the current practice used to diagnose B12 deficiency.

For the cost-effectiveness analysis, a decision tree model was used to compare costs and health utilities between current practice (TB12) and Holo-TC /MMA testing algorithm using TreeAge Pro (Healthcare) Software LLC (version 20243 R1.1, Williamstown, Massachusetts, USA).

The model was built and developed as per the guidelines stipulated in the NICE reference case for diagnostic test evaluation (NICE, 2013), using the B12 deficiency prevalence found in the study population. The model considered patient health measured in terms of Quality Adjusted Life Years (QALY's).

A QALY takes into account how a treatment or intervention affects a patient's quantity of life (remaining lifespan) and quality of life (the quality of your remaining years of life). The QALY combines both these factors into a single measure to allow comparison of the health benefits of alternative interventions. A year of perfect health has a QALY of 1, a year of less than perfect health has

a QALY somewhere between 0 and 1 and death has a QALY of 0. Calculating a QALY requires two inputs, one is the utility value associated with a given state of health by the years lived in that state and the second input is the amount of time people live in various states of health. The change in utility value produced by the intervention is multiplied by the duration of the effect to provide the number of QALYs gained. QALYs can then be incorporated with the costs associated with the intervention to calculate the cost per QALY (Gray et al, 2011). The utility values can be obtained from the use of the visual analogue scale (VAS), where patients rate ill health on a scale of 0-100), time trade off (TTO), where patients are asked to choose between remaining in a state of ill health for a period of time or restored to full health with an associated shortened life expectancy, or the EuroQol group, which uses a questionnaire with a standardised descriptive system. The EQ-5D-3L comprises five dimensions: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression each with three response categories. This means there are a total of 243 possible combinations of unique health states (3^5). EuroQol publish tables of responses for states of ill health which can be used for health economic evaluations (Szende, Oppe and Devlin, 2007).

For this study, the costs and QALY's incurred in the first year following the initial TB12 test were calculated. Results were reported as cost-utility analyses, in terms of incremental cost per QALY expressed as the incremental cost-effectiveness ratio (ICER). Patient health utility values were based on EuroQol-5 dimensions (EQ-5D) data.

The health care costs included in the study were: (1)The costs of conducting the tests for both algorithms: equipment, reagents, consumables, calibrators, internal quality controls, external quality assurance and staff resource (2) Patient care within the first year following the initial total B12 test: cost of GP visits; phlebotomy costs; additional test costs e.g. intrinsic factor antibodies or gastric parietal cell antibodies; cost of B12 treatment (medication and administration of treatment and repeat/ follow-up test costs.

NICE's preferred form of ICER is the cost per QALY gained. The ICER was calculated by dividing the difference in costs between the two arms by the difference in health utilities (QALY's) between the two arms. The ICER was compared against willingness to pay (WTP) which is currently £20,000 per QALY gained (NICE, 2013). The net monetary benefit (NMB) was calculated from the effectiveness and WTP. Costs with a positive NMB indicated that the test was cost effective at this threshold.

Following baseline economic health benefits modelling, deterministic sensitivity analysis was performed on the model to evaluate how uncertainty in the model inputs affect the model outputs; to identify which variables affect the model the most and to quantify the overall uncertainty of the model.

Chapter 3.0 RESULTS

Evaluation and verification of Abbott Diagnostics Holotranscobalamin (Holo-TC) assay for the measurement of vitamin B12

3.1 Background

The first line investigation currently for B12 deficiency is Total B12 (TB12), but this is not a sensitive nor specific test and patients with a TB12 result in the reference range may present with symptoms of B12 deficiency. Holo-TC is the biologically available form of B12 and due to its shorter half-life has been reported to be one of the earliest markers for B12 deficiency (Nexø et al, 2002a) and therefore a better indicator of B12 status. This study verified the Abbott ARCHITECT Holo-TC assay and compared its diagnostic accuracy to the Abbott ARCHITECT TB12 assay in the detection of B12 deficient patients. A more accurate test would facilitate more appropriate treatment and therefore, prevention of the longer-term debilitating neurological issues associated with B12 deficiency.

The ARCHITECT Holo-TC assay was chosen to evaluate because Abbott Diagnostic equipment was in use at The Royal Wolverhampton NHS Trust and because it was the first automated Holo-TC assay available for use. The kit has European Conformity in vitro diagnostic approval (CE-IVD) and according to

UKAS ISO15189:2022 (UKAS, 2022) laboratories must verify CE-marked assays for fitness for purpose using objective evidence to assess the manufacturers' performance claims. The clinical and laboratory standards institute (CLSI) issue guidance documents on all aspects of assay verification and the performance characteristics were assessed in accordance with the appropriate CLSI guideline where possible. Where adjustments had to be made to suggested methods due to availability of materials and feasibility of work in the laboratory setting, this was documented and justified.

The verification process consisted of assessment of bias which is a measure of how far the result is from the 'true' result; imprecision which measures the closeness of results on repeated testing and the combination of imprecision and bias give a measure of accuracy. Holo-TC assay bias was investigated using two methods, one using a patient method comparison of two laboratories using the same kit and the other a review of reported EQA results. The verification included assessment of the analytical sensitivity of the assay measuring the lowest concentration of Holo-TC that could be detected; the level able to be measured reproducibly and level quantified accurately and the range at which the assay is linear; recovery studies were conducted to estimate proportional systematic error and accuracy. To complete the verification, the sample stability was investigated over various time periods and temperature storage conditions.

Biological variation data, both within (CV_I) and between (CV_G) subject coefficients of variation may be used to set analytical goals for bias. The European federation of clinical chemistry and laboratory medicine (EFLM, 2024)

publish CV_I and CVG data for a wide range of analytes in. ding TB12, however Holo-TC is not listed. Therefore, the CV_I (13%) and the CV_G (24.6%) was taken from a paper by Brokner et al (2017), CLSI guideline EP9-A2 describe the equations for bias acceptance criteria using this data (CLSI, 2002). Maximum allowable bias = $<0.375 \times (CV_I^2 + CV_G^2)^{½}$ giving a calculated maximum bias target of 10.4% for the Holo-TC assay.

The correlation coefficient r is also an important acceptance criterion for accuracy with r being ≥ 0.975 ($r^2 \geq 0.950$) giving the line of best fit.

The result ranges for B12 deficiency, indeterminate and replete supplied in the Abbott ARCHITECT Holo-TC kit insert were reviewed to assess if appropriate for use with the local population being investigated.

3.2 Aim and Objectives

The aim was to implement and verify the Holo-TC assay and to evaluate whether it improved the diagnosis of B12 deficiency.

Objectives

1. To determine the analytical sensitivity and imprecision (inter and intra) of the Holo-TC assay when compared to Abbott Diagnostics performance claims.

2. To determine the Holo-TC assay bias utilising a patient method comparison study and review of EQA performance.
3. To determine the linearity of the Holo-TC assay and perform recovery studies to assess allowable error to test Abbott Diagnostics' performance claims.
4. To review Abbott's Holo-TC reportable ranges for B12 deficiency and normality to assess if appropriate for use in the local population.
5. To conduct stability studies on patient samples at clinically relevant concentrations to investigate the effect of storage, at different temperatures and time intervals, on Holo-TC results.

3.3 Method

See Chapter 2, section 2.4.6 for principles of measurement of Holo-TC.

The Abbott Diagnostics Holo-TC method was uploaded and installed by an Abbott Service engineer on one of the laboratories Architect i2000sr analysers (serial number iSR51145) using methods and reagents supplied by Abbott diagnostics. The assay was calibrated using Abbott Holo-TC calibrants and two levels of IQC (supplier IQC) were analysed and within acceptable limits before verification work commenced. The assay was used as per manufacturer's kit insert with no modifications.

Refer to section 2.4.6.1 for outlines of verification methods.

3.4 Results

3.4.1 The analytical sensitivity and imprecision of the Holo-TC assay

To determine the analytical sensitivity of the Holo-TC kit limits of blank (LoB), detection (LoD) and quantification (LoQ) were calculated to identity the lowest concentration of sample that could be reliably detected and quantified by the assay in a reproducible manner.

Holo-TC assay LoB (Table 3.1) was found to be 0.103 pmol/L with Abbott Diagnostics quoted LoB of <0.4 pmol/L.

The Holo-TC assay had an LoD (Table 3.1) of 0.216 pmol/L (SD 0.0686 pmol/L, CV of 5.1%) with Abbott Diagnostics quoted LoD of <1.9 pmol/L.

Table 3.1. LOB and LOD measurements for Abbott Architect Holo-TC assay (pmol/L) including mean, SD and %CV calculations.

LoB	Holo-TC pmol/L	LoD	Holo-TC pmol/L
LoB 1	0.06	LoD 1	1.40
LoB 2	0.01	LoD 2	1.30
LoB 3	0.12	LoD 3	1.30
LoB 4	0.03	LoD 4	1.40
LoB 5	0.05	LoD 5	1.30
LoB 6	0.07	LoD 6	1.40
LoB 7	0.04	LoD 7	1.20
LoB 8	0.06	LoD 8	1.30
LoB 9	0.04	LoD 9	1.30
LoB 10	0.05	LoD 10	1.30
Mean	0.05	LoD 11	1.40
SD	0.03	LoD 12	1.40
%CV	60.90	LoD 13	1.40
		LoD 14	1.30
		LoD 15	1.40
		LoD 16	1.40
		LoD 17	1.40
		LoD 18	1.40
		LoD 19	1.50
		LoD 20	1.30
		Mean	1.36
		SD	0.07
		%CV	5.10

The construction of an imprecision profile gave an inter-assay %CV of <4 for the range of Holo-TC concentrations measured (6.6-86.9 pmol/L) and a LoQ of 6.6 pmol/L as shown in Figure 3.1. However, as the LoD CV was <20% it can be postulated that LoD=LoQ therefore LoQ was 0.216 pmol/L.

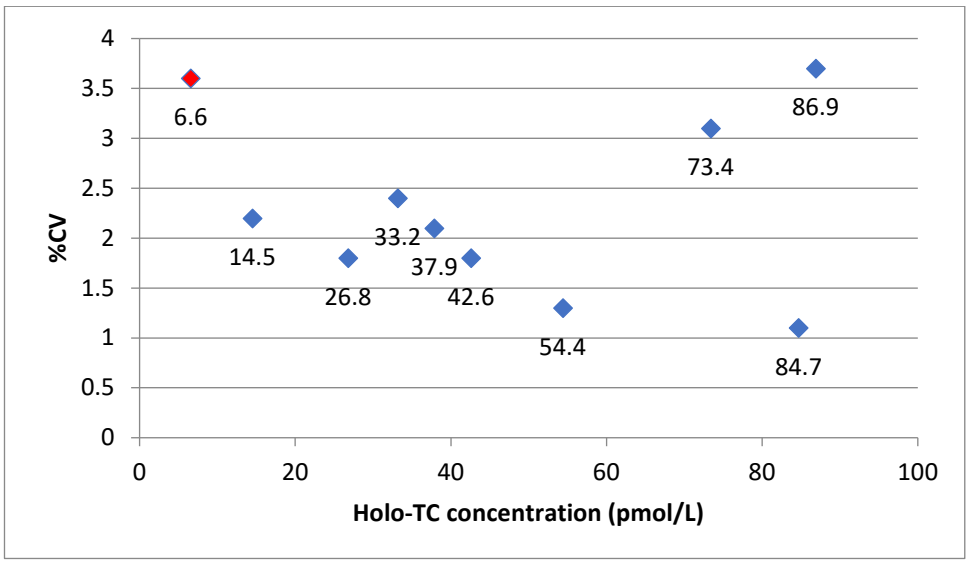


Figure 3.1. Imprecision profile for LoQ for Holo-TC assay showing an inter-assay %CV of <4 for the measurement range 6.6 to 86.9 pmol/L of Holo-TC with LoQ highlighted in red.

Both intra- and inter-batch imprecision were measured using kit controls or calibrators and compared to Abbott diagnostics performance claims. Intra-assay precision was the measure of the variance between data points within an assay in the same batch and inter-assay precision was the measure of the variance between data points of different batches to assess run to run consistency.

Abbott Diagnostics state acceptance criteria for intra-batch imprecision of ≤8% for samples with a Holo-TC ≥20 pmol/L and a SD of ≤1.6 pmol/L for samples with Holo-TC < 20 pmol/L. For intra-assay imprecision, the %CV for Holo-TC were 7.2%, 6.8% and 7.0% at concentrations of 11, 32 and 59 pmol/L respectively and the SD was 0.79 pmol/L for the 11 pmol/L sample, meeting the acceptance criteria (Table 3.2).

Table 3.2. Intra-batch imprecision of Holo-TC assay. Samples 1-3 are three concentrations of Abbott Holo-TC kit calibrators with target values shown. Tabulated results of mean concentrations (pmol/L), SD, %CV and number of replicates tested.

	Mean Holo-TC concentration (pmol/L)	SD (pmol/L)	% CV	Number of replicates (n)
Sample 1 (8 pmol/L)	11	0.79	7.17	10
Sample 2 (32 pmol/L)	32	2.19	6.83	10
Sample 3 (64 pmol/L)	59	4.41	6.97	10

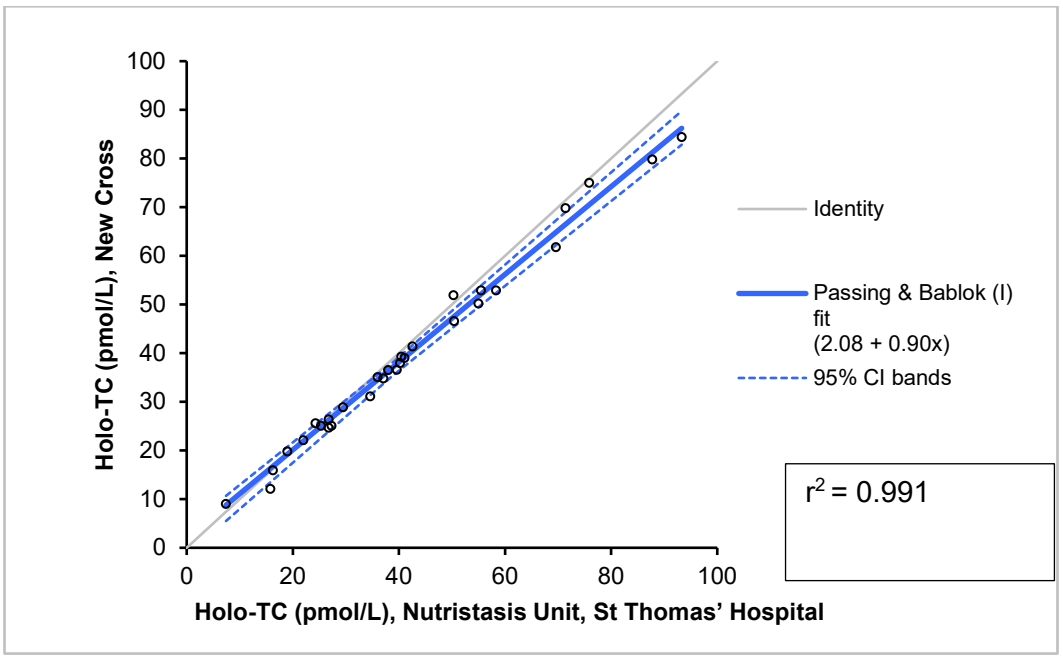
For inter-assay imprecision, the % CV for Holo-TC were 3.6%, 2.4% and 3.1% at concentrations of 7, 24 and 73 pmol/L respectively (Table 3.3). Acceptance criteria for inter-assay imprecision is not provided in the Abbott Holo-TC kit insert.

Table 3.3. Holo-TC Inter-batch imprecision. Samples 1-3 were prepared by diluting kit controls and calibrators to give the target values shown. Tabulated results of mean concentrations (pmol/L), SD and %CV. Samples were analysed ten times on five different days.

	Mean Holo-TC concentration (pmol/L)	SD (pmol/L)	% CV	Number of replicates (n)
Sample 1 (8 pmol/L)	7	0.24	3.58	50
Sample 2 (32 pmol/L)	24	0.58	2.38	50
Sample 3 (90 pmol/L)	73	2.30	3.13	50

3.4.2 Bias of Holo-TC assay derived from a method comparison study using patient samples and a review of EQA performance

Patient samples with Holo-TC results in the range 7.4 to 93.3 pmol/L were analysed at New Cross Hospital, Wolverhampton and then referred to an accredited site using the same Abbott Diagnostics Holo-TC Kit so that a direct comparison of results could be made. Figure 3.2 shows the resulting Passing Bablok method comparison with a r^2 value of 0.991 (acceptance criteria $r^2 \geq 0.950$), the 95% CI of the intercept includes 0 however 95% CI for the slope did not include 1. There was a small negative proportional bias (-2.20 pmol/L) and an absolute bias of 2.08 pmol/L which accounts for the failure of the slope to include 1.



Passing-Bablok fit

Equation	New Cross = 2.084 + 0.9018 Nutristasis Unit, St Thomas'

Parameter	Estimate	Bootstrap 95% CI
Intercept	2.084	-0.5989 to 3.078
Slope	0.9018	0.8707 to 0.9731

CI based on 999 bootstrap samples.

Figure 3.2. Passing and Bablok (PB) method comparison of 30 samples analysed for Holo-TC at New Cross Hospital, Wolverhampton and Nutristasis Unit, St Thomas' Hospital, London, both using the Abbott Diagnostics kit.

The Bland Altman difference plot confirmed that there was a proportional bias (Figure 3.3). The mean absolute bias of the New Cross results compared to the referral laboratory results was -2.20 pmol/L. The bias calculated from these results was 4.1% and the previously acceptable maximum bias target calculated for Holo-TC was 10.4%.

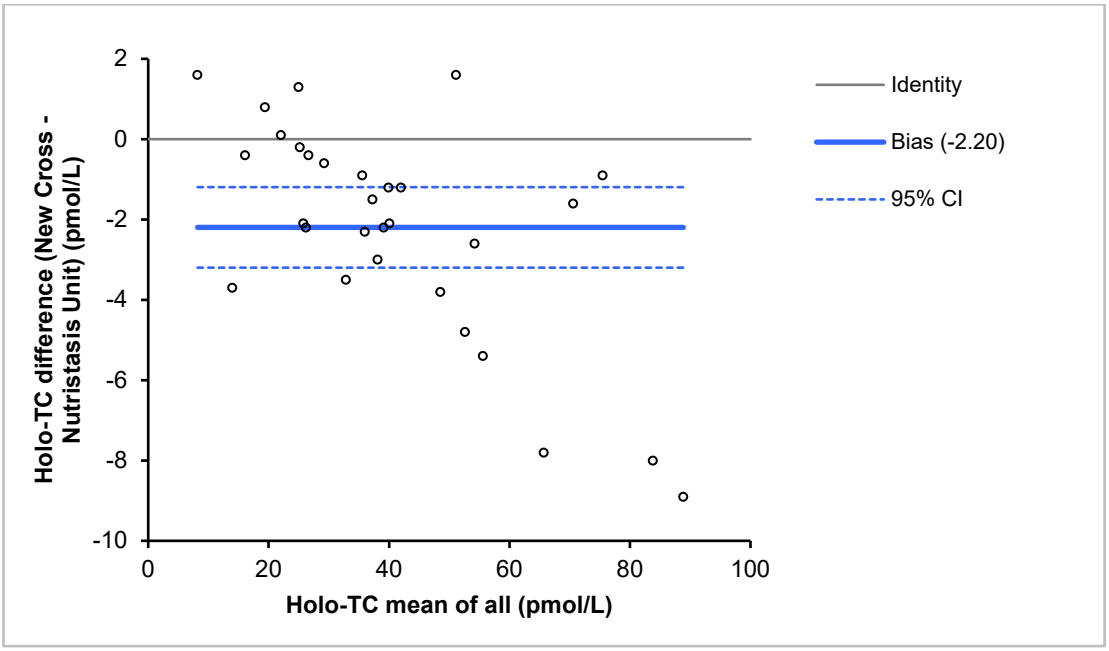


Figure 3.3 Bland-Altman difference plot of 30 *samples analysed for Holo-TC at New Cross Hospital, Wolverhampton and Nutristasis Unit, St Thomas' Hospital, London, both using the Abbott Diagnostics kit.*

The accuracy of the Holo-TC assay was further assessed by comparing the results from 25 UKNEQAS EQA samples to the All Laboratory Trimmed (geometric) Mean (ALTM). Twenty-two results had a bias meeting the acceptance criterion (<10%). All results were within two standard deviations of the ALTM. (Table 3.4). The table also shows the method laboratory trimmed mean (MLTM) which is the geometric mean of the trimmed results for the specimen of all users of the same method i.e. Abbott kit.

The A, B and C scores were available for four UKNEQAS surveys and all were acceptable (A scores of <200 and B and C scores <15) (Table 3.5).

Table 3.4. Holo-TC EQA comparison results. ALTM was the mean trimmed for all laboratories returning results for Holo-TC and was the expected target value. % bias, SD values and ±2SD ranges were determined for the results reported based on distance from the target value. Method trimmed mean (MLTM) for the Abbott Architect Holo-TC group. Results in bold were outside acceptable bias target of 10%.

	Reported Holo-TC result (pmol/L)	MLTM for Holo-TC (pmol/L)	ALTM (target value) for Holo-TC (pmol/L)	% Bias (Acceptable ± 10%)	SD Holo-TC (pmol/L) of ALTM	± 2SD of ALTM Holo-TC (pmol/L) Acceptable range
212A	62.2	58.3	57.9	+7.4	5.2	47.5-68.3
212B	85.4	81.9	81.1	+5.3	8.8	63.5-98.7
212C	17.2	17.16	17.1	+0.5	1.36	14.4-48.6
213A	40.6	41.4	41.6	-2.4	4.7	32.2-51.0
213B	54.2	52.1	52.9	+2.5	5.5	41.9-63.9
213C	116.3	111.4	110.4	+5.3	11.9	86.6-134.2
214A	22.2	21.8	21.6	+2.8	2.45	16.7-26.5
214B	107.4	103.7	102.0	+5.3	11.9	78.2-125.8
214C	65	61.4	60.6	+7.4	6.2	48.2-73.0
246A	73.2	80.5	79.7	-8.2	7.1	65.5-93.9
246B	45.3	48.3	47.9	-5.4	4.1	39.7-56.1
246C	46	48.5	48.0	-4.1	4.6	38.8-57.2
247A	14.2	14.8	14.76	-3.8	1.44	11.9-17.6
247B	70.7	81.8	80.6	**-12.3**	8.4	63.8-97.4
247C	70.7	81.5	80.6	**-12.3**	8.8	63.0-97.6
248A	7.5	7.94	7.98	-6.0	0.95	6.1-9.9
248B	32.7	36.1	35.7	-8.3	3.4	28.9-42.5
248C	48.8	53.8	53.2	-8.3	5.0	43.2-63.2
249A	15.7	17.34	17.53	**-10.5**	1.74	14.1-21.0
249B	42.3	45.7	45.0	-6.1	4.9	35.2-54.8
249C	67.4	73.7	72.3	-6.7	7.3	57.7-86.9
250A	82.4	92.2	90.1	-8.5	8.1	73.9-106.3
251A	72.4	78	75.5	-4.1	6.7	62.1-88.9
251B	52.3	56.2	55.4	-5.6	3.9	47.6-63.2
251C	128	131.3	125.8	+1.8	20.3	85.2-166.4

Table 3.5. EQA survey data results using ABC scoring system. A = accuracy, B = bias, C = consistency of bias, with acceptance criteria. Scores calculated by NEQAS based on returned Holo-TC results and earlier performance over 4 surveys and 12 samples.

Survey	**Method mean** (limit <10%)	**A score** (Acceptable < 200)	**B score** (Acceptable < ± 15)	**C score** (Acceptable < 15)
248	-7.5	75	-7.5	3.9
249	-8.1	78	-7.8	3.4
250	-8.5	78	-7.8	3.1
251	-4.8	72	-7.2	3.1

3.4.3 Linearity and Recovery of the Holo-TC assay

The linearity of the Holo-TC assay was determined using patient samples to approximately cover the measurement range claimed by Abbott Diagnostics (5-128 pmol/L), (Table 3.6 and Figure 3.4). The linearity study showed that the assay was linear between 6.0 to 112.0 pmol/L, with the slope of the plot of measured and assigned (expected) values (0.950 pmol/L) demonstrating acceptable bias (acceptance criterion $r^2 \geq 0.950$) according to CLSI EP06-A guidance (CLSI, 2003). A further study was undertaken to investigate linearity at low concentrations (circa 2.0 - 30.0 pmol/L) which showed an acceptable r^2 value of 1.00 (Table 3.7 and Figure 3.5).

Table 3.6. Holo-TC results of eight dilutions (S01-S08) prepared from a sample with a high Holo-TC (112 pmol/L) and diluted with a low Holo-TC sample (5.8 pmol/L) to cover the quoted measurement range. Analysis performed by Abbott Diagnostics engineer (Appendix 3 for Abbott linearity report).

Sample	% of high pool used	Replicate 1 Holo-TC (pmol/L)	Replicate 2 Holo-TC (pmol/L)	Replicate 3 Holo-TC (pmol/L)	Mean Holo-TC (pmol/L)	Expected values Holo-TC (pmol/L)
S01	Neat	112.8	112.9	110.3	112.0	112.0
S02	0.7	76.3	81.5	79.4	79.1	78.4
S03	0.6	62.0	62.6	62.8	62.5	67.2
S04	0.5	51.2	51.2	51.7	51.2	56.0
S05	0.4	45.9	43.2	48.6	45.9	44.8
S06	0.2	24.3	25.0	23.5	24.3	22.4
S07	0.1	15.0	15.0	Insufficient	15.0	11.2
S08	0.0	5.7	5.8	5.9	5.8	5.8

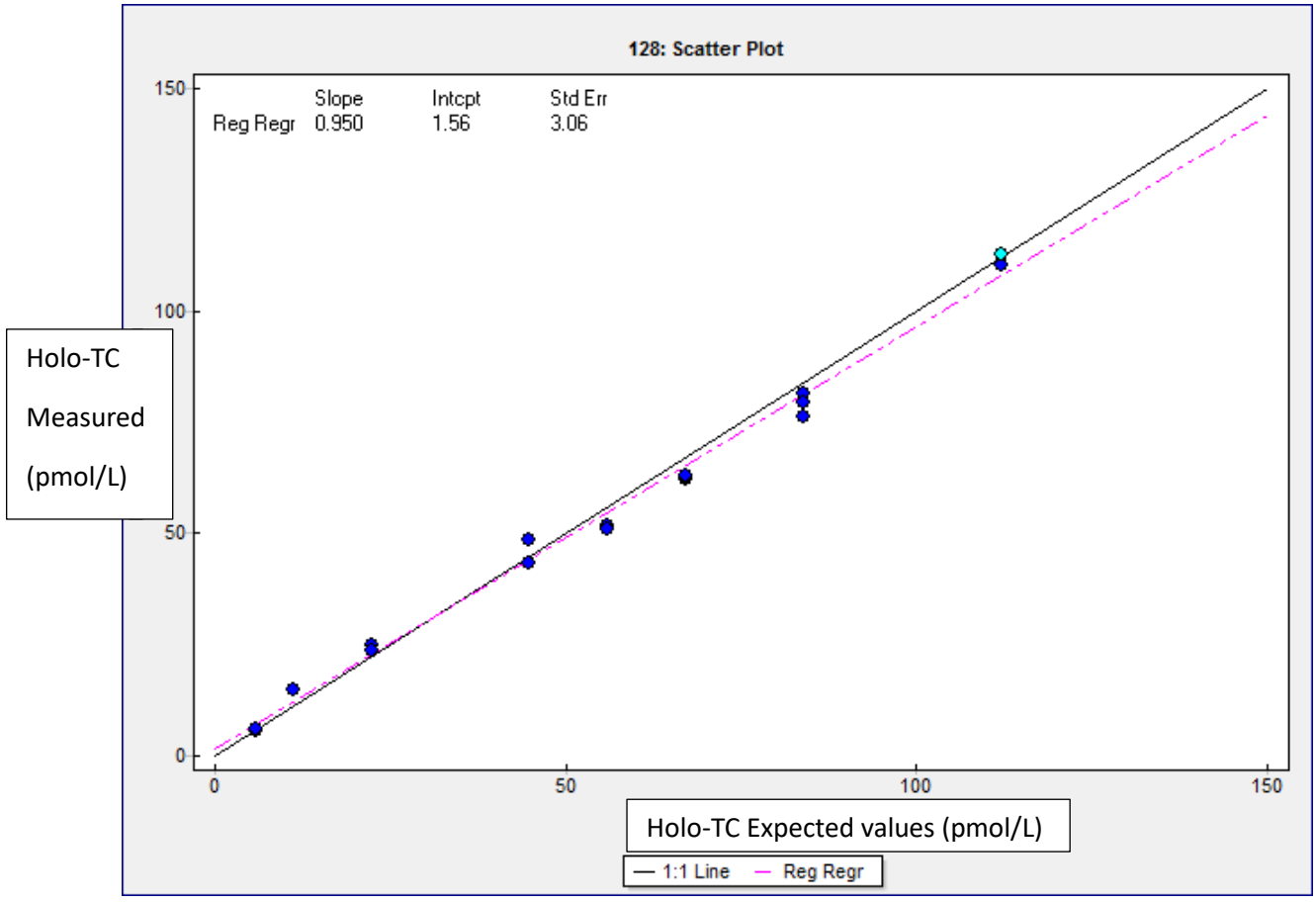


Figure 3.4 Linearity of Holo-TC assay undertaken by Abbott engineer. 8 sample dilutions run in triplicate with assigned values plotted against measured values with an $r^2 = 0.950$ (Appendix 3 for Abbott linearity report).

Table 3.7. Linearity study on patient sample with low Holo-TC (30 pmol/L) and doubling dilutions using stripped serum showing assigned and measured values.

Dilution	Assigned Holo-TC pmol/L	Measured Replicate 1 Holo-TC pmol/L	Measured Replicate 2 Holo-TC pmol/L	Average Measured Holo-TC pmol/L
Neat	30	30	26	28
1 in 2	15	12.4	14.5	13.5
1 in 4	7.5	5.9	6.8	6.4
1 in 8	3.8	3.1	3.3	3.2
1 in 16	1.9	1.6	1.4	1.5

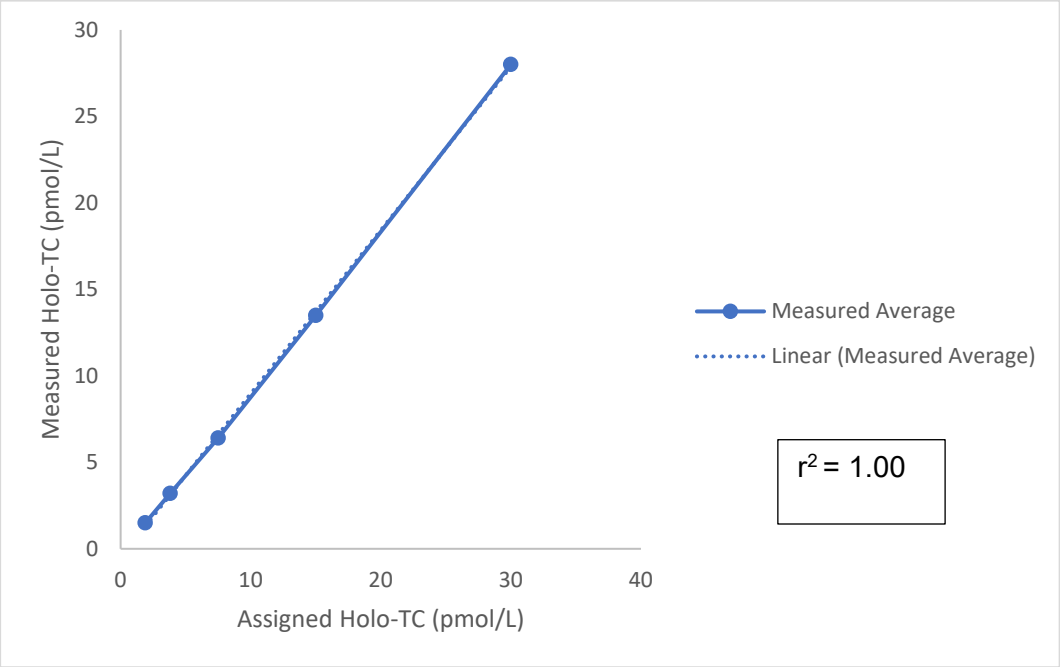


Figure 3.5. Linearity results on low Holo-TC sample with assigned values plotted against measured values $r^2 = 1.00$.

Acceptable recovery for the Holo-TC assay was based on the allowable error, using the bias (10.4%) and imprecision (7.2%) values calculated previously during the verification.

Allowable error = bias + (1.65 x CV_A) where CV_A is imprecision (CLSI, 2002).

This gave an allowable recovery of 100 ± 22.3%. The mean recovery was 100 ± 3.2% for patient samples with four different concentrations of Holo-TC spiked with an EQA sample containing Holo-TC and also with stripped serum not containing Holo-TC (Table 3.8).

Table 3.8. Holo-TC Spiking study (Recovery) where an EQA sample with a Holo-TC concentration of 8.55 pmol/L (denoted as spike in the left hand column) and stripped serum (denoted as blank in the left hand column), were added to patient samples with a range of Holo-TC concentrations (deficient to raised). Samples were tested in duplicate and percentage recovery calculated.

Sample	Unspiked Holo-TC result pmol/L	Spiked Holo-TC result 1 pmol/L	Spiked Holo-TC result 2 pmol/L	Expected Holo-TC spiked result pmol/L	Percentage Recovery
Deficient + spike	**16.2**	**22.3**	**22.4**	**22.5**	**99%**
Deficient + blank	**16.2**	**14.1**	**13.7**	**14.6**	**95%**
Indeterminate + spike	**52.7**	**55.6**	**55.8**	**55.7**	**100%**
Indeterminate + blank	**52.7**	**47.9**	**45.7**	**47.4**	**99%**
Replete + spike	**84.0**	**80.0**	**80.2**	**84.1**	**95%**
Replete + blank	**84.0**	**73.9**	**72.9**	**75.6**	**97%**
Raised + spike	**110.1**	**102.2**	**101.6**	**107.9**	**94%**
Raised + blank	**110.1**	**94.9**	**93.4**	**99.1**	**95%**
Mean recovery					**96.8%**

3.4.4 Assessment of the appropriateness of the Abbott Diagnostics classification limits in the local population under investigation

The patient population studied was not representative of normal therefore the cut off values for deficiency could not be assessed and Abbott Diagnostics cut-off concentrations were used during this study to determine B12 deficiency or sufficiency.

3.4.5 The effects of storage temperature and time on Holo-TC in patient samples

Three patient samples with indeterminate, replete and raised Holo-TC concentrations were stable (results meeting the 'within laboratory' imprecision target of ≤8%) when stored at room temperature for 16 hours, at 4^{o}C for 3 days and 1 month if stored at -80^{o}C confirming the stability claims in the Abbott kit insert (Table 3.9).

Table 3.9 Sample stability for the Holo-TC assay for samples stored at room temperature (RT), 4°C and -80°C. Samples 1,2 and 3 were chosen to represent indeterminate, replete and raised Holo-TC concentrations. Samples were separated into in a number of aliquots. A previously untested aliquot was utilised each time a measurement was made.

Sample Stability Results				
	Holo-TC pmol/L			
	RT	4°C	-80°C	Comments
Sample 1 (50 pmol/L)				
Day 1	51.9			Tested prior to storage
Day 2	51.6	52.4	52.7	
Day 3	51.6	51.6	53.4	
Day 4	50.2	49.8	48.5	
Day 5	49.0	48.3		
Day 7	51.4	48.2	53.7	
Week 2			53.7	
Week 3			53.0	
Week 4			54.0	
Month 2			***57.9**	Recalibration
Month 3			***57.9**	
Month 4			***56.9**	
Month 5			52.8	
Month 6			54.9	New lot - Recalibration
Month 7			54.9	
Month 8			55.3	
Sample 2 (75 pmol/L)				
Day 1	74.8			Tested prior to storage
Day 2	73.3	71.5		
Day 3	70.8	71.7		
Day 7		70.0	71.4	
Sample 3 (>128 pmol/L)				
Day 1	>128			Tested prior to storage
Day 2		127.6		
Day 3		124.8		
Day 7		>128	>128	
Week 2			>128	
Week 3			>128	
Week 4			>128	
Month 2			>128	Recalibration
Month 3			>128	
Month 4			>128	

RT = room temperature storage. *Results for sample 1 for months 2, 3 and 4 (highlighted in bold text) were higher than the initial measured result following recalibration of the assay, the IQC was positively biased at this time.

The verification findings for the Holo-TC assay compared to the acceptance criteria are summarised in Table 3.10.

Table 3.10. Holo-TC method performance achieved compared with published Abbott Diagnostics manufacturer claims, EQA scheme provider acceptance criteria and biological variability data taken from literature.

Performance Characteristic	Parameter	Acceptance criteria	Data from Verification	Acceptance Criteria Achieved
EQA Bias	% Bias	<10% ± 2SD A score <200 B score <± 15 C score <15	<10%[1] ± 2SD A < 200 B < 15 C <15	Yes
Method Comparison	PB regression	$r^2 \geq 0.950$ Maximal bias 10.4%	$r^2 = 0.991$ 4.1%	Yes
Imprecision % (intra-batch)	%CV for samples with Holo-TC ≥ 20 pmol/L	≤8%	≤8% (7%)	Yes
	SD for samples with Holo-TC ≤ 20 pmol/L	≤1.6	≤1.6 (0.8)	Yes
Accuracy (% Recovery)	**%**	± 22.3%	± 3.2%	Yes
LoB	pmol/L	<0.4	0.103	Yes
LoD	pmol/L	<1.90	0.216	Yes
LoQ	pmol/L	≤5.0	0.216	Yes
	CV%	≤10%	<4%	Yes
Linearity	Range (pmol/L)	5-128	2-112	Yes

[1] In 22/25 of EQA results.

3.5 Discussion

Abbott Diagnostics performance claims were met or surpassed as shown in Table 3.10 however there were some issues encountered during the verification process. Initially when undertaking the analyser sensitivity measurements all results for LoB and LoD were recorded as <5.0 pmol/L as the linear range of the assay was set as 5.0-128.0 pmol/L. The lower range on the analyser was set to zero and usable results were then obtained. Both LoB and LoD were lower than quoted Abbott limits with performance deemed acceptable.

When undertaking the LoQ measurement it was not possible to find a patient sample with a 5 pmol/L Holo-TC concentration therefore, the lowest concentration evaluated was 6.6 pmol/L. Sample dilution would have enabled testing to a lower Holo-TC concentration. The highest imprecision in the LoQ profile was < 4%, considerably less than the imprecision used to define the manufacturer's LoQ (stated as <10%). In addition, the definition of LoQ states that if the imprecision of the LoD is acceptable (<20%) then LoD may be used as LoQ. The LoD imprecision was 5.1% giving an LoQ of 0.216 pmol/L. Despite the lower LoQ achieved during the verification, the Abbott Diagnostics' LoQ (<5.0 pmol/L) was used for analysis of patient samples as the Holo-TC assay was used as per manufacturer's instructions.

The intra-assay imprecision was within the Abbott Diagnostics acceptance target of ≤8.0% even for the sample with a concentration of Holo-TC <20

pmol/L. Although Abbott do not provide separate acceptance criteria for inter-assay imprecision, the verification results were within the intra-assay criteria and were therefore, deemed to be acceptable.

For the bias investigations the acceptance criterion was met, the maximal allowable bias using biological variation data was calculated to be 10.4% and the bias calculated during the patient method comparison was 4.1% with the Passing and Bablok regression analysis producing an r^2 value ≥ 0.950 (0.991) with only a small proportional and absolute bias. Passing and Bablok (1985) indicated that thirty patient samples are sufficient for a valid patient comparison assuming that the variables of X and Y are highly correlated, however according to CLSI Guidance (EP9-A2) for method comparison and bias estimation using patient samples (CLSI, 2002), forty patients samples should be analysed for a valid comparison therefore only using thirty patient samples was a limitation of this study although both sites were using the same Abbott Diagnostics Holo-TC kit reducing variability and an even spread of Holo-TC results across the measurement range (seven deficient, 13 in the indeterminate range and 10 in the replete range) were tested ensuring the clinical decision cut-off values could be compared. Samples evaluated covered the Holo-TC concentration range of 7 to 93 pmol/L, further work could involve assessing a number of higher concentration samples however the clinical importance of a high Holo-TC has not been established. Many studies debating the purported association between high TB12 and liver disease or malignancy have not been undertaken for Holo-TC.

For an EQA scheme to be suitable it should supply samples in a near native state and should have sufficient participants (>20) to enable relevant statistics to be performed. These criteria were met by the Birmingham Quality scheme which was only a pilot scheme at the start of the verification with most participants using Abbott Diagnostics Architect Holo-TC kit. For the EQA review, satisfactory performance was achieved, all results were within ± 2SD of the ALTM, with most results (22/25) being within ±1SD, bias was <10% in 22/25 samples and the ABC scoring results were acceptable.

The initial linearity method undertaken in the laboratory consisted of double diluting the 128 pmol/L calibration standard with Abbott sample diluent however this was unsuccessful on three occasions giving approximately 50% of the expected results at all dilutions. The assay was recalibrated after the second failed attempt. The procedure was repeated using stripped serum, as the diluent without any improvement in performance. Stored EQA samples with known values, diluted with stripped serum, were analysed with equivalent results suggesting that there was a calibration issue. Abbott Diagnostics successfully undertook the linearity study, outlined in the methods section. Failure to achieve acceptable results using the calibrant was considered to result from matrix issues. The EQA samples used in the latter procedure had been stored for one year at -20^{o}C and appear to have degraded as newer EQA samples tested gave the appropriate response. The kit insert suggests long-term storage of patient samples at -80^{o}C for 6 months only, therefore the EQA samples had not been stored under optimal conditions and was a reason for the failure.

For the linearity study performed by Abbott Diagnostic the assay was linear across the analytical measurement range of 6.0 to 112.0 pmol/L, samples with higher Holo-TC concentrations were not evaluated. The linearity study utilised a sample with a known high result (112 pmol/L) that could be diluted with a sample with a low Holo-TC concentration rather than a sample that had a result of >128 pmol/L with the true result unknown. The analytical range was wide enough to include the Holo-TC concentration in 95–99% of samples tested. Further evidence that the original linearity problems related to the use of the calibrant came from linearity studies using a patient sample with a Holo-TC of 30 pmol/L which indicated that the assay was linear to 2 pmol/L. Although linearity was not evaluated above 112 pmol/L, a result of >50 pmol/L is classified as replete. Therefore, Abbott Diagnostics quoted linearity of 5-128 pmol/L was used for analysis of patient samples as the Holo-TC assay was used as per manufacturer's instructions.

Recovery of Holo-TC in patient samples exceeded the acceptance criterion at all concentrations assessed.

Stability of Holo-TC in patient samples met Abbott Diagnostics' acceptance criteria. These criteria are suitable for the routine processing of samples as they would normally be analysed on the day of sample receipt, but Holo-TC 'add-on' tests could be accepted up to three days post receipt, if stored refrigerated. Stability of Holo-TC in patient samples stored at -80^{o}C was studied for eight months for an indeterminate sample (longer than the six month manufacturer claim). The sample gave acceptable results at months 5-8 but not for months 2

to 4. The IQC was positively biased at this time owing to recalibration of the assay.

It was not possible to establish local laboratory reportable ranges as the sample population studied was suspected to have B12 deficiency and was therefore not representative of ‘normal’. Each manufacturer supplies a reference range (based on patients tested during their kit validation process) and deficient cut-off concentrations for their assay which are different depending on supplier. Abbott’s reference range was 25-165 pmol/L (Abbott Diagnostics kit insert); Siemens 29-169 pmol/L (FDA.gov, 2024); Beckman Coulter 32.2-152.6 pmol/L (Beckman, 2024) and Roche 37-188 pmol/L (Heil et al, 2019). Like TB12 the literature reports a ‘grey or indeterminate zone’ for Holo-TC of anywhere between 25-75 pmol/L (Golding, 2016a, Hermann and Obeid, 2013) and for the Abbott Architect kit being verified was 25-50 pmol/L. These patients may or may not have B12 deficiency and require further tests to confirm their B12 status.

Further work is required on more appropriate decision limits for treatment and reference ranges which will require Holo-TC assessment in 120 patients without suspected B12 deficiency or other confounding factors e.g. drug treatment known to increase the risk of B12 deficiency. The Black Country Pathology Service (BCPS) network, hosted at Royal Wolverhampton NHS Trust (hub) is ideally placed to undertake this further study once GP work from all four partner Trusts transfers to the hub site. The other partner sites of the BCPS are Dudley Group NHS Foundation Trust, Sandwell and West Birmingham NHS Trust and Walsall Healthcare NHS Trust. The BCPS serves a population of 1.76 million

patients conducting 60 million tests per annum utilising the same analyser platforms and LIMS system. Data collected from the LIMS could inform these decisions without the need for further testing following the replacement of the TB12 test across the network in 2023 with the Holo-TC test.

3.6 Conclusion

The Holo-TC assay was deemed fit for purpose for use in the laboratory as the verification confirmed the manufacturers' performance claims and performed well in the EQA scheme with further work still required on clinical decision making cut-off values and reference ranges.

Chapter 4.0 RESULTS

Verification of the Chromsystems Methylmalonic Acid (MMA) mass spectrometry assay

4.1 Background

There is no agreed laboratory ‘gold’ standard for the diagnosis of B12 deficiency. B12 is a cofactor for the enzymatic conversion of methylmalonyl CoA to succinyl-CoA. B12 deficiency therefore leads to elevated concentrations of methylmalonic acid (MMA) in serum. MMA is a functional marker, widely reported in the literature to be the closest to a ‘gold standard’ test for B12 deficiency (Harrington, 2019).

The rationale for the development of an in-house MMA assay was to use it as an adjunct test on patient samples with indeterminate TB12 (187-300 ng/L) or Holo-TC (25-50 pmol/L) results.

A number of MMA extraction methods and various combinations of mass spectrometry columns, HPLC settings and mass spectrometer analysers were trialled (see method chapter, 2.4.7.1). The final method selected was a CE-IVD approved method from Chromsystems Masschrom® using Shimadzu Nexera X2 UHPLC system coupled with the Sciex 6500 mass spectrometer and the performance characteristics of this assay were verified using CLSI guidelines.

Where adjustments had to be made to CLSI suggested methodology, due to the availability of materials and feasibility of work in the laboratory setting, this was documented and justified.

Selection of a CE-IVD approved kit meant that full assay validation was not required, and verification followed the process outlined in chapter 3 with some additional work performed due to the nature of the assay.

Verification included, imprecision and bias studies, analytical sensitivity (limit of quantitation (LoQ)), linearity, recovery, measurement of uncertainty (MU), sample stability and review of reportable ranges. In addition, also included a carryover study to prove that a high concentration of MMA in one sample does not cause a falsely high result on the subsequent samples and most importantly that the MMA assay was free from interfering factors that could cause ion suppression due to constituents of the sample matrix or coeluting compounds.

4.2 Aim and Objectives

The aim was to establish and verify an MMA method for assessing B12 deficiency in patients with indeterminate TB12 or Holo-TC results.

Objectives

1. To determine the analytical sensitivity and imprecision of the MMA assay when compared to Chromsystems performance claims.
2. To determine MMA assay bias utilising a patient method comparison study and review of EQA performance.
3. To determine the linearity of the MMA assay and perform recovery studies to assess allowable error to test Chromsystems performance claims.
4. To determine the effects of interfering substances on the MMA assay including an ion suppression test and the effect of sample carryover.
5. To conduct sample stability studies of freeze thawing effects on reproducibility of results for the MMA assay.
6. Review the literature for MMA results ranges for B12 deficiency and appropriateness for use in the local population.

4.3 Method

See chapter 2, section 2.4.7 for principles of measurement of MMA which described the final adopted Chromsystems method as well as other methods investigated.

After daily cleaning procedures, the Masschrom® assay was calibrated using Chromsystems 3PLUS1® calibrants (product number, 64028) (appendix 4 for Traceability certificate) with r^2 ≥0.950 as the acceptance criterion. Two levels of

ClinChek® Recipe IQC (product number, MS5082) were analysed and within satisfactory limits (±2SD of target value) before verification work began. The Masschrom® assay was used as per manufacturer's instructions without modification.

Refer to chapter 2, section 2.4.7.3 for outlines of verification methods.

4.4 Results

During the establishment of an in-house liquid chromatography tandem mass spectrometry (LCMS/MS) assay for MMA; for both the Waters reverse chromatography method and the Sciex HILIC methods investigated, no results were obtained due to various issues which are discussed later in this chapter. The Chromsystems method however was established and verified.

4.4.1 Analytical sensitivity and imprecision for the MMA Masschrom assay when compared to Chromsystems performance criteria

The LoQ acceptance criterion is the lowest concentration that can be measured with an imprecision <20% and for the Chromsystems MMA assay is 30 nmol/L. The LoQ should be below the lower limit of the reference interval (~ 70 nmol/L). The imprecision at an MMA concentration of 16.8 nmol/L, based on 12 replicates, was 5.3% meeting the acceptance criterion (Table 4.1).

Table 4.1. LoQ for MMA MassChrom assay obtained from dilution of a sample to give a LoQ below the expected value of 30 nmol/L.

	MMA (nmol/L)
LOQ 1	15.4
LOQ 2	16.7
LOQ 3	17.4
LOQ 4	17.5
LOQ 5	15.9
LOQ 6	17.6
LOQ 7	16.7
LOQ 8	16.8
LOQ 9	16.1
LOQ 10	15.6
LOQ 11	17.9
LOQ 12	17.9
MMA mean nmol/L	**16.8**
SD	**0.9**
CV %	**5.3**

The inter-batch imprecision for the Chromsystems MMA assay was 3.7% for level 1 IQC and 4.1% for level 2 IQC. Acceptance criteria based on manufacturer's claim imprecision were 6.7 and 4.8%, respectively (Table 4.2).

Table 4.2 Inter-batch imprecision for MMA. Two levels of IQC (ClinChek®, Recipe) were tested on 17 different assay runs (run in duplicate) over several weeks, performed by two different operators.

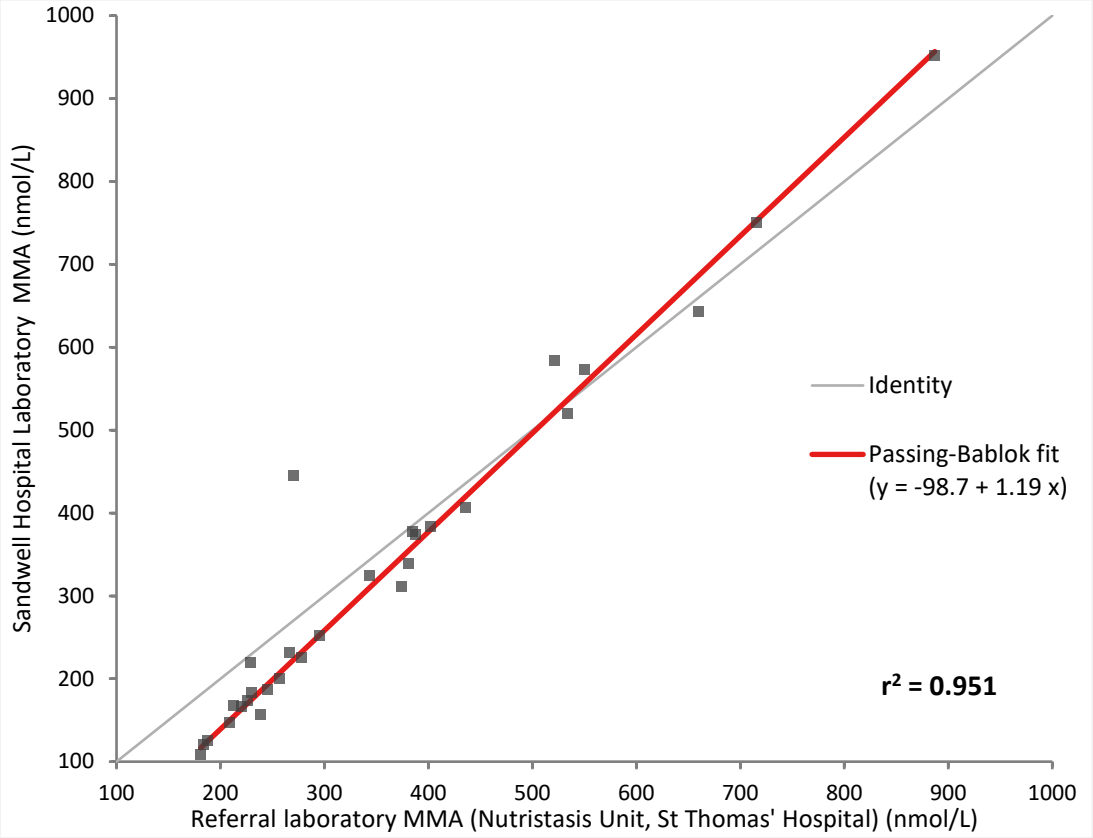
MMA	IQC Level 1	IQC Level 2
Mean (nmol/L)	236.8	548.9
SD	8.8	22.6
%CV	3.7	4.1
n	17	17

Using the data in Table 4.2 the Measurement Uncertainty (MU) of the MMA assay was also calculated, using MU = 2SD (95% confidence limits). The MMA IQC ranges were Level 1 IQC of 219.2-254.4 nmol/L and Level 2 IQC 503.8-594.0 nmol/L

4.4.2 MMA Masschrom assay bias derived from a method comparison study using patient samples, analysis of pure certified reference material (CRM) and a review of EQA performance

Patient samples with MMA results in the range 181-887 nmol/L were analysed at Sandwell Hospital using the MassChrom assay and compared to the results from an accredited referral site (Nutristasis Unit, St Thomas' Hospital) using an in-house LCMS/MS method. Figure 4.1 shows the resulting Passing Bablok method comparison with an r^2 value of 0.951 (acceptance criterion $r^2 \geq 0.950$) however the 95% CI for slope does not include 1 and 95% CI of intercept does not include 0 meaning these acceptance criteria were not met. The comparison

showed a significant proportional bias with the Masschrom assay under investigation when % difference results were calculated having up to a 40 % negative bias compared to the referral laboratory method at the lower end and a 7% positive bias at the upper end. There was also a potential outlier which was 445 nmol/L by the Chromsystem assay and 270 nmol/L by the referral laboratory assay.



Equation	Sandwell Hospital MMA (nmol/L) = -98.7 + 1.19 Referral laboratory MMA (Nutristasis Unit, St Thomas' Hospital) (nmol/L)

Parameter	Estimate	Bootstrap 95% CI
Intercept	-98.70	-114.1 to -83.63
Slope	1.190	1.140 to 1.254

Figure 4.1 MMA Passing-Bablok method comparison. Thirty samples analysed for MMA by the Black Country Pathology Service at Sandwell Hospital, Birmingham and *Nutristasis Unit, St Thomas' Hospital*, London. Results presented in nmol/L. r^2=0.951 however a significant proportional bias was seen.

No CV_I nor CV_G data were available for MMA on the European Federation of clinical chemistry and Laboratory Medicine (EFLM) website (2024), therefore the biological variation data for MMA, CV_I (within subject variation) of 7.2% and CV_G (between subject variation) of 21.1%, were taken from a paper by Lindberg et al (2019), giving a calculated maximum bias target of 8.4% for the MMA assay when using the equation $<0.375 \times (CV_I^2 + CV_G^2)^{½}$. From the Passing-Bablok method comparison a significant proportional bias was seen with the Masschrom assay giving a mean bias of -11.1% compared to the referral laboratory assay, not meeting the acceptance criteria of 8.4%.

Owing to the failure of the patient comparison study to meet the acceptance criteria, analysis of MMA pure certified reference material (CRM) of known values was undertaken as a further test of accuracy. The Masschrom MMA results for the pure CRM did not show the same significant proportional bias, compared to expected results, that was found in the patient comparison study, with positive biases of 13.5%, 14.7% and 17.4% for low, moderate and high concentrations of MMA respectively (Table 4.3), although still did not meet the 8.4% acceptance criteria.

Table 4.3. Assessment of pure CRM showing % bias results calculated for low, moderate and high concentrations of MMA.

CRM concentration expected value (nmol/L)	MMA measured value (nmol/L)	Bias (%)
84.7	96.1	13.5
423	485	14.7
846	993	17.4

The MMA result (312 µmol/L) submitted for the March 2024 ERNDiM EQA survey was within ±2SD of the target (378 µmol/L) meeting the acceptance criterion (Figure 4.2). It was noted that the ERNDiM reporting units are in µmol/L therefore the results were outside the reportable limits of the MassChrom assay.

Lab Name :	**ERN0735 - Royal Wolverhampton NHS Trust - Haematology**
Method Set :	LC-MS/MS
Sample :	SAS2024.01
Analyte :	Methylmalonic acid
Deadline :	29/03/2024
Unit :	µmol/L
Your Method :	Tandem-MS
Your Result :	312

Parameter	Method Results	All Labs Results
n :	74	96
Mean :	378	379
Median :	378	378
SD :	49.3	39.8

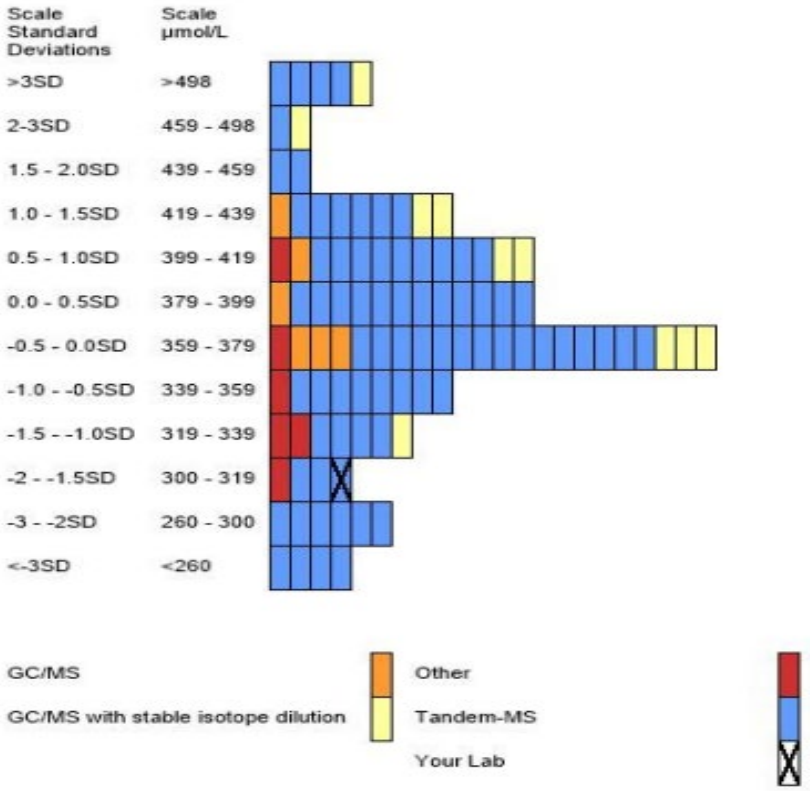


Figure 4.2. ERNDiM EQA result for survey 2024.01 (March 24) showing -2SD difference to mean, target 378 µmol/L (Acceptance criteria ± 2SD).

4.4.3 The linearity of the Masschrom MMA assay based on Chromsystems performance claims and recovery study

The Masschrom MMA assay was linear across the manufacturer's quoted linearity range of 30-5080 nmol/L with the verification showing linearity for the range 17.9-5450 nmol/L (Table 4.4 and Figure 4.3).

Table 4.4. MMA linearity of 11 pools of expected vs obtained results run in duplicate. Volumes of a high pool (created from CRM, 5450 nmol/L) and a low pool (179 nmol/L) were mixed to produce 11 different samples to test the manufacturer's quoted linearity range of 30-5080 nmol/L ensuring the highest (1670 nmol/L) and lowest calibrator (35.7 nmol/L) concentrations were covered by the range.

Sample number	% of high pool used	Replicate 1 MMA (nmol/L)	Replicate 2 MMA (nmol/L	Mean MMA result achieved (nmol/L)	Expected MMA (nmol/L)
1	0.0	17.9	17.9	17.90	17.90
2	0.625	50.1	50.4	50.25	51.85
3	1.25	83.5	78.5	81.00	85.80
4	2.5	147	152	149.50	153.70
5	5.0	249	252	250.50	289.51
6	10.0	545	556	550.50	561.11
7	20.0	1040	1040	1040.00	1104.32
8	40.0	2120	2160	2140.00	2190.74
9	60.0	3040	3060	3050.00	3277.16
10	80.0	4110	4120	4115.00	4363.58
11	100.0	5450	5450	5450.00	5450.00

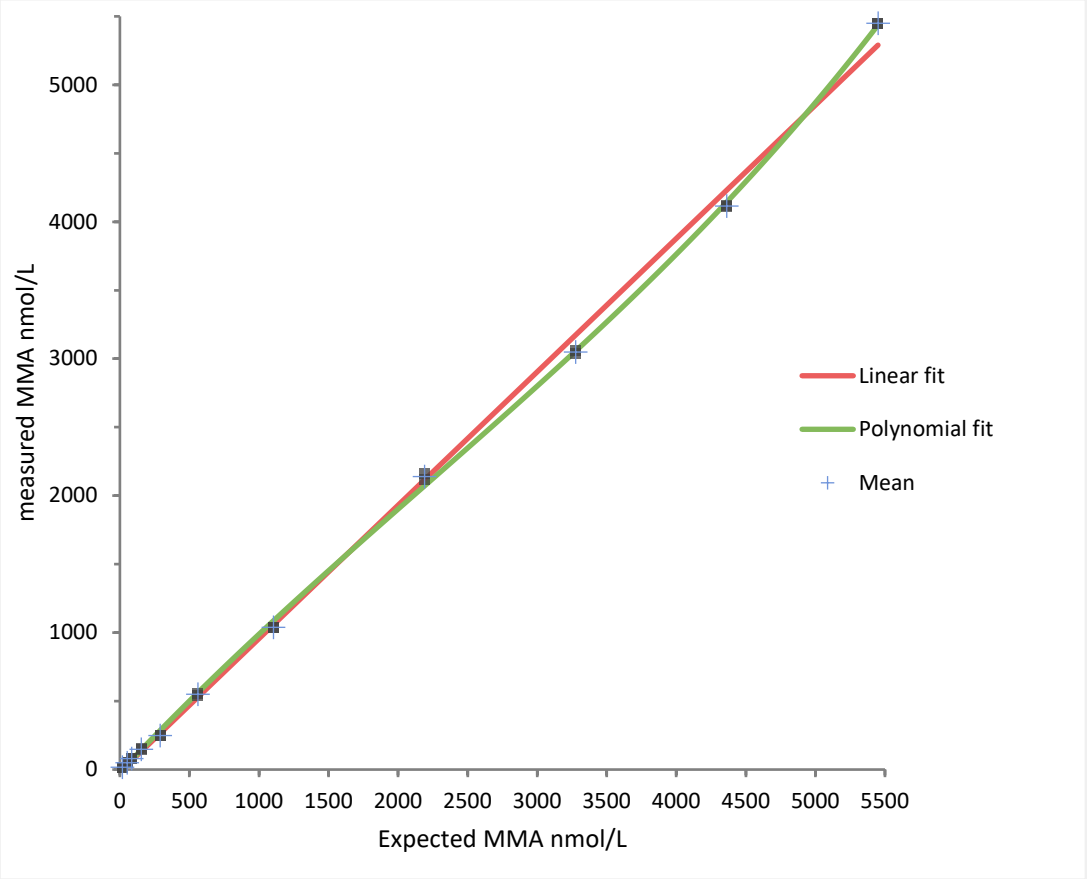


Figure 4.3. MMA linearity. Expected vs measured MMA results showing a curvilinear relationship, polynomial regression was used to improve the linear fit for the measurement range 17.9 to 5450 nmol/L.

As highlighted previously according to CLSI guideline EP6A, evaluation of the linearity of quantitative measurement procedures, the goal for linearity should be no greater than the bias (trueness) goal (CLSI, 2003). This was defined as 8.4%. Using this data allowable nonlinearity was also calculated (Table 4.5) and plotted (Figure 4.4), proving that all results during the linearity verification were within the nonlinearity acceptance criteria.

Table 4.5. MMA linearity vs nonlinear fit with allowable nonlinearity based on 8.4% bias.

Expected MMA (nmol/L)	Linear fit	Nonlinearity	Allowable nonlinearity
17.90	-0.08	-1.30	±1.50
51.85	32.98	2.21	±4.40
85.80	66.04	5.52	±7.20
153.70	132.17	11.56	±12.90
289.51	264.43	21.43	±24.30
561.11	528.94	32.93	±47.10
1104.32	1057.96	28.69	±92.80
2190.74	2116.00	-43.98	±184.00
3277.16	3174.04	-116.81	±275.30
4363.58	4232.08	-87.30	±366.50
5450.00	5290.12	147.04	±457.80

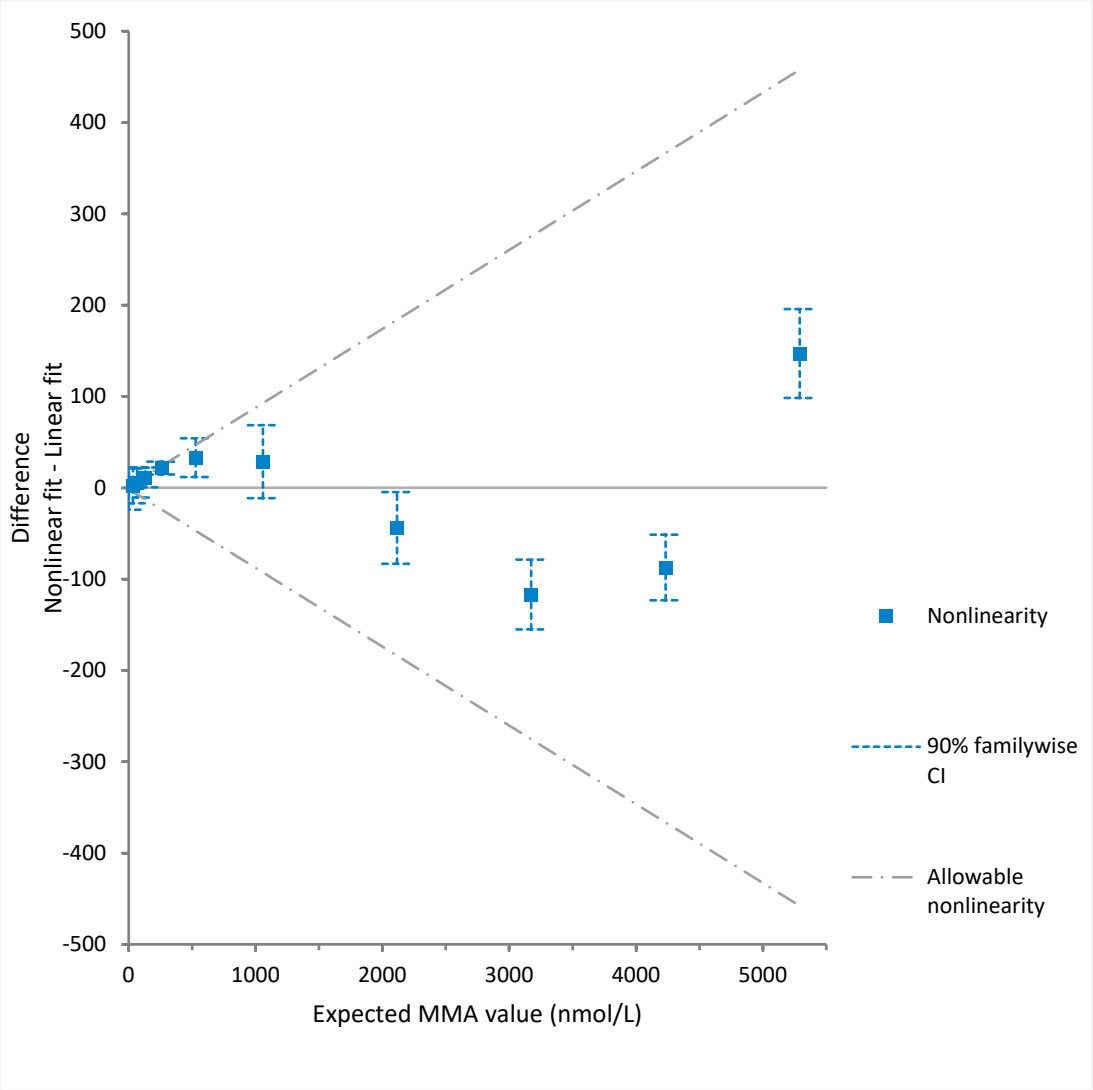


Figure 4.4 Plot of the difference between nonlinear and linear fit and expected MMA values shown in Table 4.5 showing all results are within the acceptable nonlinearity criteria.

The acceptable recovery of MMA using the MassChrom assay was 100 ±15.2%, based on bias (8.4%) and imprecision (4.1%) and the equation:

Allowable error = bias + (1.65 x CV_A) where CV_A is imprecision (CLSI, 2002).

The recovery results were between 96 and 112% for the sample pool spiked with MMA giving a mean MMA recovery of 103.3% (Table 4.6), meeting the acceptance criterion. In the Chromsystems validation of the Masschrom MMA

assay the recovery was 102-114% therefore manufacturer's acceptance criterion was also met.

Table 4.6. Recovery of MMA in serum. One pool of serum was spiked with clinically relevant concentrations of MMA. Each pool was analysed in triplicate as was an unspiked base-pool and percentage recovery was calculated for the spiked samples.

	MMA (nmol/L)					
	Replicate 1	Replicate 2	Replicate 3 (new batch)	Mean MMA (nmol/L)	Expected MMA (nmol/L)	% Recovery
serum base-pool	177	170	181	176.0		
85 nmol/L spike	252	254	248	251.3	261	96.3
423 nmol/L spike	606	627	593	608.7	599	101.6
846 nmol/L spike	1180	1190	1060	1143.3	1022	111.9
mean recovery						**103.3**

4.4.4 Effects of interfering substances on the Masschrom MMA assay including an ion suppression test and sample carryover

Many substances can interfere with mass spectrometry assays and ion suppression defined as a 10% decrease in signal at the retention time of the analytes of interest was therefore, included in the assay verification. Figures 4.5, 4.6 and 4.7 show the results of the chromatogram transitions when interfering substances were injected whilst an MMA solution was being directly infused. All three figures show no interference or ion suppression (decrease in signal) at the retention time for MMA of 1.9-2.1 minutes.

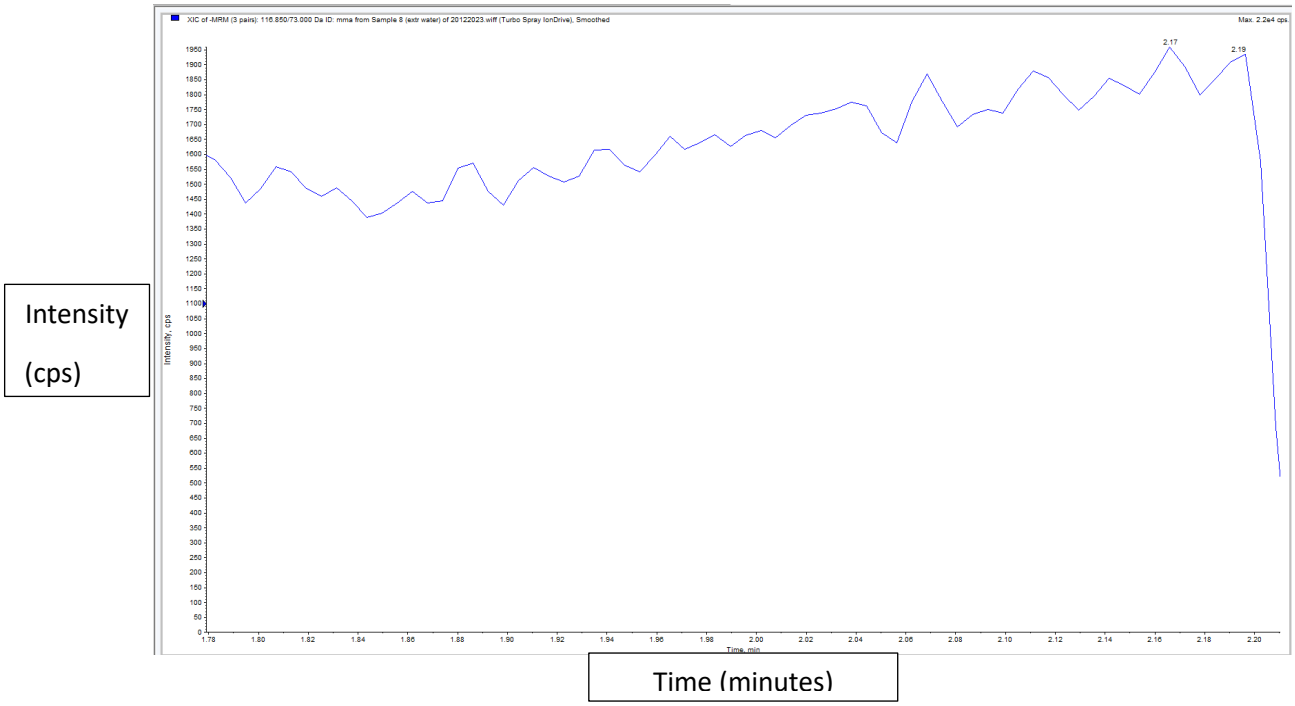


Figure 4.5. Ion suppression test of extracted water blank. MMA transition shown. No evidence of ion suppression during the retention time (RT) window for MMA of 1.9-2.1 minutes. RT shown on the x-axis (1.78 to 2.20 minutes) and intensity (counts per second) on the y-axis (screenshot taken from system data manager software).

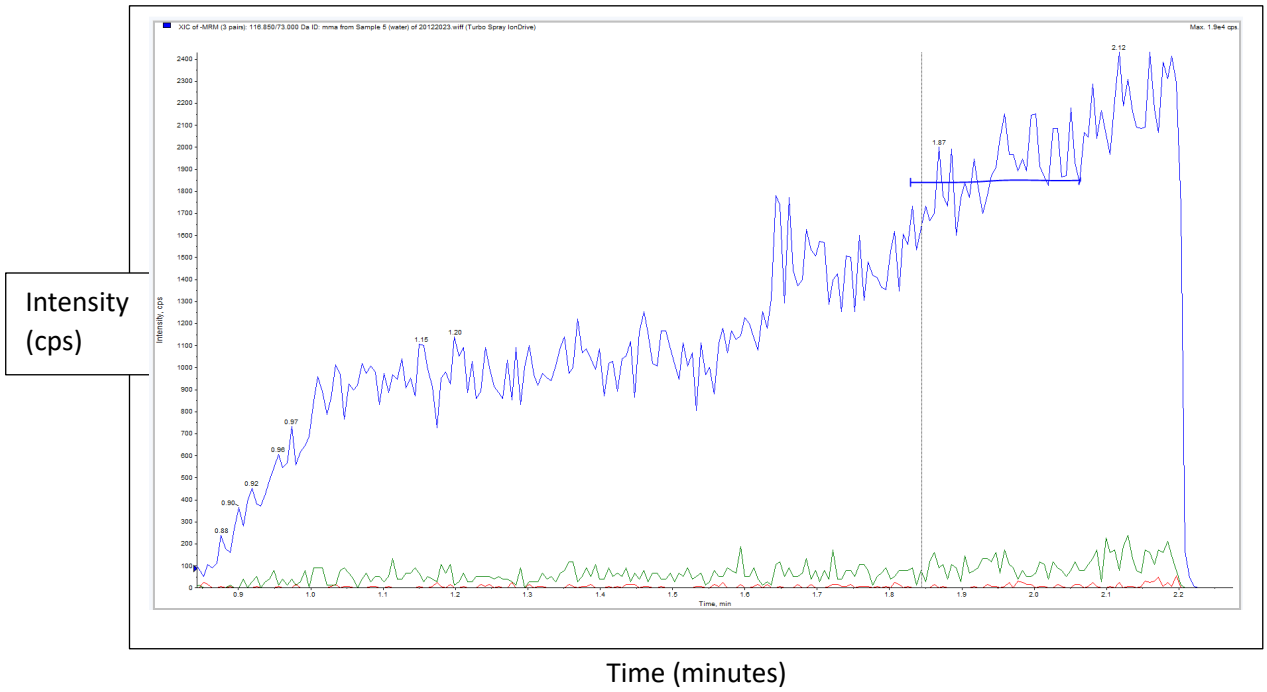


Figure 4.6. Ion suppression test, system blank. MMA transition shown in blue. No evidence of ion suppression during the RT window for MMA of 1.9-2.1 minutes. The blue horizontal line is the MMA RT period of 1.9-2.1 minutes with no loss in signal. RT shown on the x-axis (0.9 to 2.20 minutes) and intensity on the y-axis (screenshot taken from system data manager software).

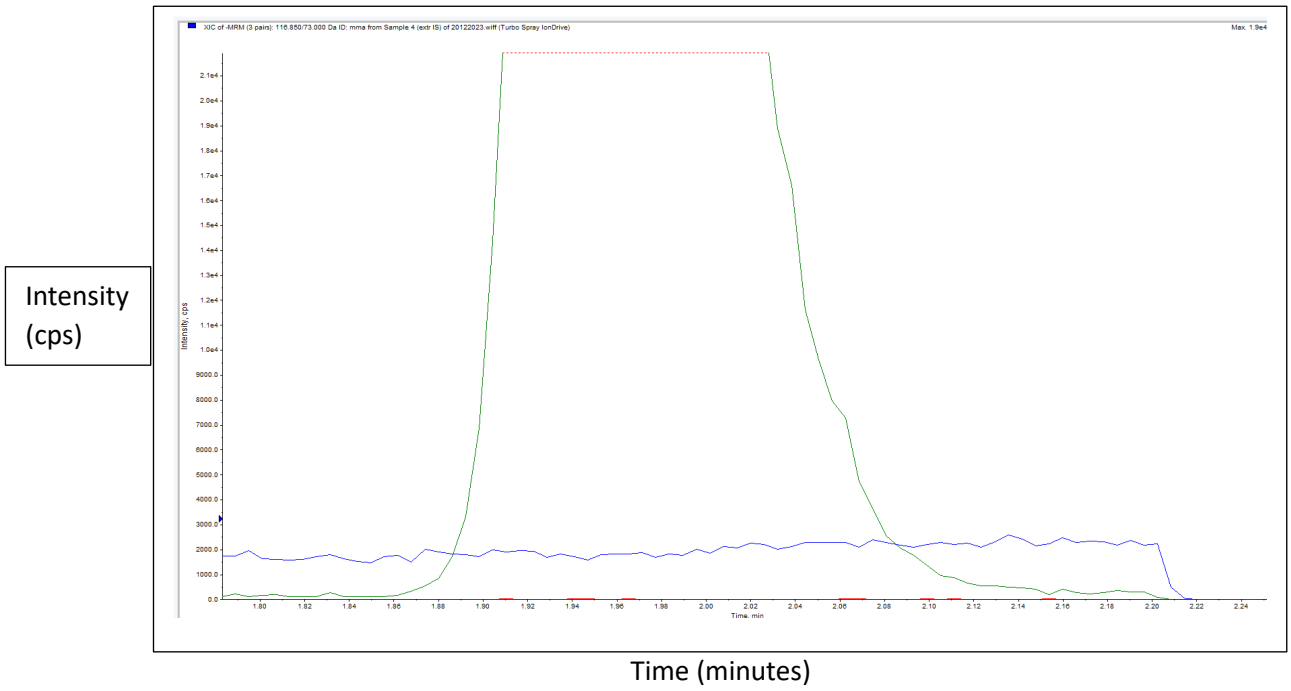


Figure 4.7. Ion suppression test extracted internal standard (IS). MMA transition shown in blue. No evidence of ion suppression during the RT window for MMA of 1.9-2.1 minutes. The green trace is the IS. RT shown on the x-axis (1.80 to 2.24 minutes) and intensity on the y-axis (screenshot taken from system data manager software).

Succinic acid is an isobar of MMA and if not separated chromatographically will interfere with MMA analysis. The results of a sample spiked with succinic acid are shown in Figure 4.8. Resolution (Rs) was calculated from the results obtained from the trace using the formula:

Rs = (RT MMA – RT Succinic) / ((peak width MMA + peak width Succinic)/2)

Rs = (1.95-1.70) / ((0.1 + 0.15)/2) = 2

A resolution of ≥1.5 indicated that full baseline separation had occurred and confirmed that succinic acid therefore would not interfere with MMA assay results.

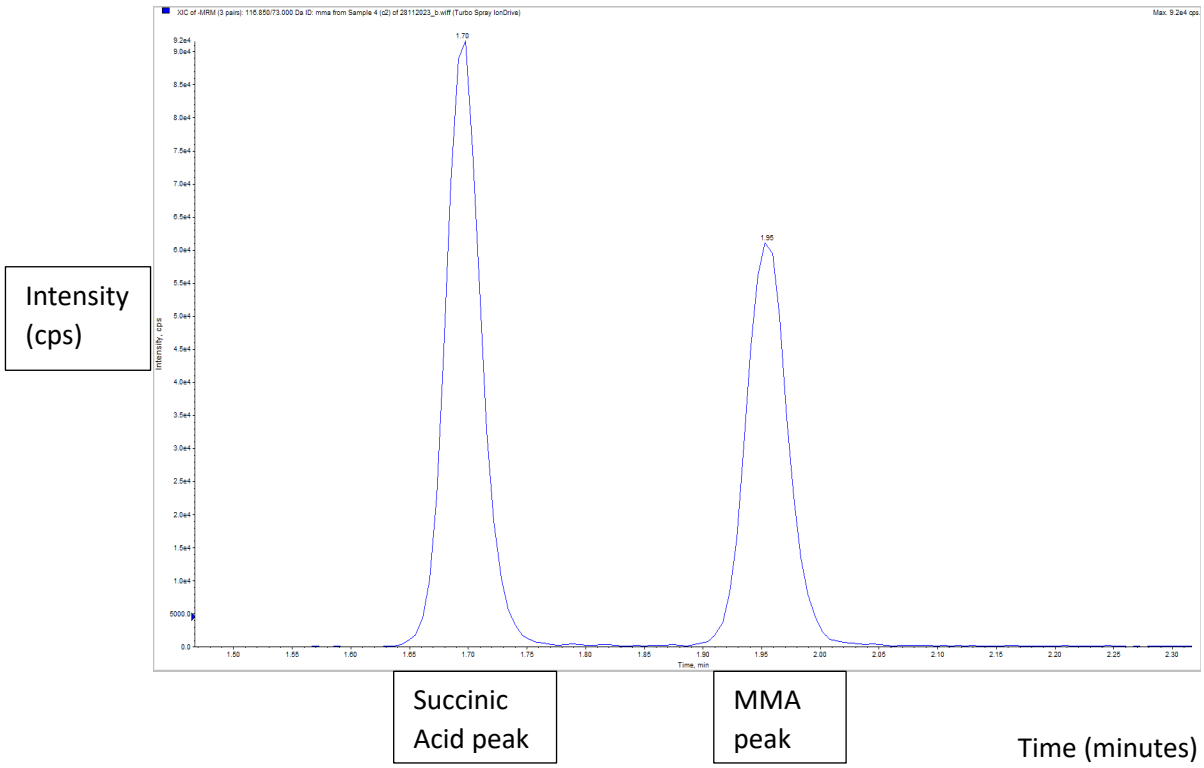


Figure 4.8. Chromatogram showing the RT of succinic acid at 1.70 minutes and MMA at 1.95 minutes with clear separation of peaks. Peak width for succinic acid was 0.15 minutes and for MMA 0.1 minute. RT shown on the x-axis (1.45 to 2.30 minutes) and intensity on the y-axis (screenshot taken from system data manager software).

The Chromsystems Masschrom MMA assay kit insert stated that no significant effects of haemolysis up to 500 mg/L, lipaemia and icterus from conjugated and unconjugated bilirubin were detected during the assay validation however these were not checked during the verification although samples with obvious signs of haemolysis, lipaemia and icterus were excluded during the sample collection process.

Carryover was assessed by analysis of the blank calibrator sample before and after a sample with a high MMA concentration (9990 nmol/L). This analysis was repeated six times and the paired t-test (p=0.3097) indicated that there was no

significant difference in the before and after high MMA samples and therefore, no carryover from samples containing MMA at concentrations approximately 25 fold higher than the anticipated upper reference limit (360 nmol/L) (Table 4.7).

Table 4.7. Pre- and post-carryover blank MMA concentration with means and SD calculated (in bold).

Sample	MMA (nmol/L) Pre-carryover	MMA (nmol/L) Post-carryover
blank 1	0.21	0.79
blank 2	0.01	0.08
blank 3	0.86	0.32
blank 4	3.42	3.40
blank 5	0.78	1.10
blank 6	0.28	1.60
mean	**0.93**	**1.22**
SD	**1.27**	**1.20**

4.4.5 The effect on MMA concentration of freeze thawing serum samples and storage of the sample extract at 4°C

The MMA concentrations in 13 serum samples, before and after one freeze thaw cycle were not significantly different (p=0.310) when analysed by paired t-test (Table 4.8).

Table 4.8. MMA concentration in 13 serum samples before and after one freeze thaw cycle. Following initial test the samples were refrozen at -80°C until thawing and re-extraction. Paired t-test result shown in bold. NS = not significant. IQR= Interquartile range.

Initial MMA concentration (nmol/L)	MMA concentration after freeze thawing (nmol/L)	Paired t-test of the MMA results
115	107	**p=0.310 (NS)**
85.8	50.6	
186	159	
112	110	
220	264	
159	152	
119	132	
317	310	
63.2	76.5	
190	218	
948	865	
148	107	
95.8	83.3	
148	132	Median (nmol/L)
112	107	25% IQR (nmol/L)
190	218	75% IQR (nmol/L)

MMA concentrations in 70 sample extracts, before and after storage at 2-8°C for 4 days, were not significantly different (p=0.194) when analysed by paired t-test (Table 4.9).

Table 4.9. MMA concentration in 70 sample extracts before and after storage at 2-8°C for 4 days. Paired t-test result shown in bold. NS = not significant. IQR= Interquartile range.

Initial MMA (nmol/L)	MMA post-storage @ 2-8°C for 4 days (nmol/L)	Initial MMA (nmol/L)	MMA post-storage @ 2-8°C for 4 days (nmol/L)	Paired t-test of the MMA results
217	255	295	301	**p= 0.194 (NS)**
163	206	83.7	124	
201	238	220	237	
58.2	93.6	312	322	
338	345	113	137	
115	159	354	387	
146	158	114	169	
1000	875	232	226	
159	163	737	645	
92.9	107	240	302	
50.5	90.3	94.5	134	
207	227	279	244	
143	173	193	199	
129	146	421	383	
205	197	77.8	115	
180	185	613	551	
158	173	148	154	
118	144	182	218	
1670	1490	840	777	
400	370	406	460	
206	190	47.5	76.7	
151	183	328	386	
48.8	75.3	206	213	
179	205	216	218	
269	248	132	152	
960	886	405	393	
131	146	369	432	
479	448	317	377	
316	282	141	175	
156	152	135	161	
126	127	118	154	
183	186	247	218	
563	564	218	222	
241	263	257	273	
155	156	163	192	
		203	209.5	Median (n=70)
		136.5	156.5	25% IQR
		315	317	75% IQR

4.4.6 MMA reference range review

MMA reference ranges were reviewed by Mineva et al (2019) (Table 4.10). The original reference range of 73-271 nmol/L (Allen et al, 1990) has limitations because it was derived from a small population of healthy adults aged <65 years (highlighted in green in Table 4.10). The suggested reference range in the kit insert for the Chromsystems Masschrom MMA assay is 50-440 nmol/L. However, it is widely recognised that MMA increases with age and therefore the ranges of Vogiatzoglou et al (2009) (≤280 for ≤65 years and ≤360 for >65 years) from assumed B12 replete, middle aged and elderly adults (TB12 ≥400 pmol/L) were used in this study (highlighted in yellow in Table 4.10).

Table 4.10. Published age specific reference intervals for serum MMA (Table reproduced from Mineva et al, 2019). Most commonly used ranges were those of Vogiatzoglou, highlighted in yellow, derived in B12 replete (TB12 ≥400 pmol/L) middle-aged and elderly patients.

Selected published reference intervals for serum or plasma methylmalonic acid

Author (year) [Reference]	Population	Central 0.95 reference interval, nmol/L	Additional information
Allen 1990 [10]	Healthy U.S. middle-aged adults	73–271	Men and women, *n*=50; 18–65 y
Rasmussen 1990 [11]	Healthy Danish middle-aged adults	50–370	Men and women, *n*=58; 40–68 y (median: 53 y)
Rasmussen 1996 [4]	Healthy Danish middle-aged adults before and after vitamin B-12 supplementation for 1 wk (in a few cases for 2 wk)	80–280	Men (*n*=109) and women (*n*=126); 20–84 y (men; median: 50 y) and 20–85 y (women; median: 49 y); all but 1 subject had plasma creatinine concentrations within the reference interval for healthy subjects
Joosten 1996 [5]	Healthy Belgian, Dutch, and German middle-aged adults Healthy Dutch elderly living at home Healthy German elderly after B-vitamin supplementation for 3 wk	62–247 72–476 55–278	Men and women, *n*=99; 19–55 y (mean: 30 y) Men and women, *n*=64; 65–88 y (mean: 76 y); no participant had creatinine clearance <30 mL/min Men and women, *n*=143; 65–96 y (mean: 75 y); no participant had creatinine clearance <30 mL/min
Lewerin 2003 [12]	Swedish elderly with and without B-vitamin supplementation Total study group at baseline Healthy elderly at baseline Healthy elderly after B-vitamin supplementation for 4 mo	 110–480 120–380 20–340	Men and women, *n*=209; 70–88 y (women) and 70–93 y (men) (overall median: 76 y) *n*=208 *n*=123 *n*=78 (vitamin B-12 replete)
Milman 2007 [13]	Healthy Danish pregnant women 18 wk gestation 32 wk gestation 39 wk gestation 8 wk post-partum	 40–290 50–340 60–360 80–350	Women (n=434) with a normal pregnancy ≥37 wk *n*=413 *n*=390 *n*=250 *n*=160
Vogiatzoglou 2009 [14]	Norwegian middle-aged adults: Unselected Vitamin B-12 ≥200 pmol/L Vitamin B-12 ≥400 pmol/L Norwegian elderly: Unselected Vitamin B-12 ≥200 pmol/L Vitamin B-12 ≥400 pmol/L	 100–320 100–300 100–280 110–490 110–410 100–360	Men and women, *n*=3,684; 47–49 y *n*=3,684 *n*=3,568 *n*=1,306 (vitamin B-12 replete) Men and women, *n*=3,262; 71–74 y *n*=3,262 *n*=3,043 *n*=1,058 (vitamin B-12 replete)
Erdogan 2010 [6]	Healthy US adults US persons tested for MMA (unknown clinical history) 0–10 y 11–20 y 21–30 y 31–40 y 41–50 y 51–60 y 61–70 y ≥71 y	60–360 0–510 30–260 50–330 50–400 50–400 50–420 50–440 50–480	Men (*n*=16) and women (*n*=24) Males and females (*n*=4,944); highest 10% of results disregarded (potentially unhealthy persons) *n*=28 *n*=39 *n*=165 *n*=287 *n*=545 *n*=813 *n*=918 *n*=2149

A summary of verification results and associated acceptance criteria is given in Table 4.11.

Table 4.11. MMA method performance achieved compared with published Chromsystems claims, EQA scheme provider acceptance criteria and biological variability data taken from literature. Acceptance criteria was achieved in all aspects except method comparison bias (in bold).

Performance Characteristic	**Parameter**	**Target**	**Achieved**	**Acceptance criteria met Yes/No**
EQA Bias		± 2SD	± 2SD	Yes
Method Comparison	PB regression	$r^2 \geq 0.950$ Bias <8.4%	$r^2 = 0.951$ -11.1%	Yes **No**
Imprecision % (inter-batch)	Level 1 IQC	6.7%	3.7%	Yes
	Level 2 IQC	4.8%	4.1%	Yes
Accuracy (% Recovery)	Allowable error %	±15.2% Chromsystem 102-114%	± 3.3%	Yes
LOQ	nmol/L	30	16.8	Yes
	CV%	<20	5.3	Yes
Linearity	Range (nmol/L) %	30-5080 <8.4	17.9-5450 <8.4	Yes Yes

4.5 Discussion

The criteria for MMA method selection were that it must be robust, have minimal sample preparation and be suitable for the routine analysis of a significant number of samples. For this reason, a derivatised MMA method, although appealing due to the short assay time (around 1.5 minutes per patient), was discounted as the preparation steps for derivatisation and extraction are laborious and complicated with harmful chemicals used in the process (Jin et al, 2022).

The Waters Corporation MMA assay, using the Waters TQ-S mass spectrometer, Waters C18 column and Ostro-plates for sample extraction, was investigated. The sample extraction, however, involved the use of a vacuum manifold which was difficult to set up and this additional manual step would have been challenging for high throughput analysis. The first mass spectrometer trialled had been used for vitamin D blood spot testing and was found to be heavily contaminated with bacteria which was unsuitable for the MMA method.

Trialling a second Waters analyser, whilst pure CRM containing MMA produced good, well resolved peaks once the patient sample matrix was introduced, peak resolution was lost. It was concluded that the Waters instruments lacked sufficient sensitivity in ESI negative mode to quantitate MMA, in patient samples, and this method development was abandoned.

The second method investigated used a simple protein crash extraction, a HILIC C18 column from Merck and a Sciex 6500 mass spectrometer but the issues with peak resolution in patient samples persisted Various combinations of columns, solvents, pH and salt concentration were tried with the HILIC based method without success and this method was also abandoned.

The Chromsystems Masschrom® CE-IVD assay on the Sciex 6500 was ultimately chosen. Sample preparation was very quick and easy and lends itself to automated preparation ideal for the laboratory's future requirements. There were also the advantages of utilising a CE marked method because a full validation was not required and gave a level of quality assurance. Owing to the lack of an international reference plasma for MMA ChromSystems established a traceability chain using certified reference material (CRM) from ISO 17025 and ISO 17034 certified suppliers to prepare a master calibrator against which each batch is assessed using ID-LC-MS/MS (isotopic dilution tandem mass spectrometry) for the MassChrom MMA 3Plus1® Multilevel Plasma calibrator, (ChromSystems, 2019).

With respect to the verification process, the majority of Chromsystems performance claims were met or surpassed as shown in Table 4.11. Analytical sensitivity, imprecision, linearity and recovery met the acceptance criteria and were clinically useful. IQC 1 and 2 gave results within the reference range and deficient range, respectively.

A serum MMA measurement range of 20-5000 nmol/L was selected for use in the laboratory (LoQ = 16.8 nmol/L and linearity 17.9-5450 nmol/L) with results >1500 nmol/L reported following a 1/5 dilution in saline (as per manufacturer's instructions), since the top calibrator value was 1670 nmol/L.

There was no significant carryover or ion suppression observed and good baseline separation of MMA from isobaric compounds such as succinic acid was found.

The Passing and Bablok regression analysis from the patient sample comparison produced an r^2 value (0.951) just within the acceptance range however % bias was exceeded (mean -11.1%). Limitations of the patient sample comparison study were the use of 30 instead of 40 samples (CLSI, 2002) and the range of MMA values since there were few results at low and high concentrations (range 181-887 nmol/L) and very few around the deficiency cut-off value, for ≤ 65 years of 280 nmol/L.

Failure to achieve acceptable bias In MMA results between the two methods was likely to result from the use of different calibrators. The referral laboratory utilised an in-house method with in-house calibrators whilst a CE marked kit with traceable calibrators were used in this study.

Two additional checks of accuracy were performed to provide further assurance of acceptability (recovery and testing CRM). Recovery was acceptable

throughout the working range of the assay with mean recovery of 96% at the lower end and 102% at the mid-range of the assay, however analysis of pure MMA CRM showed a positive bias at the three concentrations assessed. A limitation of using CRM, in this context, is that the matrix is not comparable with patient samples.

There is no international reference standard available for MMA, which could be used to verify the method, many MMA methods are only available in research setting with non-standardised systems and there is only one ISO 15189 accredited method available in the UK. On balance it was considered that the accuracy of the assay was acceptable for routine use.

The MMA result for the March 2024 EQA sample was acceptable. A second EQA sample should have been submitted in April 2024, but unfortunately the Sciex analyser was broken at the time of the submission deadline. The sample will be tested later, and the results compared to the scheme's target value. Lack of EQA data was, therefore, a limitation of this study.

The ERNDiM MMA EQA scheme has 96 participants, with 74 laboratories using tandem mass spectrometry methods, and so is a suitable EQA scheme in this respect but the reportable units are in µmol/L and therefore results are outside the reported linearity range for the assay used in this study.

‘Vitamin B12 deficiency in over 16s: diagnosis and management guidelines’ (NG239) (NICE, 2024) contain a recommendation to consider confirmation of indeterminate range TB12 or Holo-TC with MMA. Consequently, more laboratories may develop MMA assays and the UKNEQAS scheme may become suitable for use if participant numbers increase. This scheme reports in nmol/L.

The stability of MMA at room temperature, 4^{o}C and -80^{o}C was not verified in this study. Samples for MMA analysis were frozen at -80^{o}C within 2 days of receipt and MMA testing completed within five years, in keeping with published stability limits (Mineva et al, 2015). These were both limitations of the study and further sample stability studies may be required if the MMA assay were employed routinely although long-term storage would not be anticipated. The effect of freeze thawing samples and the stability of extracted samples, following manufacturer guidance, were verified and gave acceptable results.

It was not possible to establish local laboratory decision limits using the samples collected in this study because they were all collected from patients with a suspicion of B12 deficiency and therefore, this is a limitation of this study. Further work would be required to determine B12 deficiency cut-off values based on age and the MMA results measured. NICE guidance (NG239) (NICE, 2024) recommends derivation of local laboratory MMA reference ranges for use in assessment of B12 deficiency. This would require serum MMA measurement for at least 120 B12 replete patients. The Black Country Pathology Service (BCPS) network, hosted at Royal Wolverhampton NHS Trust (hub) is ideally

placed to undertake this further study once GP work from the four partner Trusts transfers to the hub site.

For the purposes of this study the reference ranges of Vogiatzoglou et al (2009) (≤280 for ≤65 years and ≤360 for >65 years) were used.

4.6 Conclusion

The MMA assay was deemed fit for purpose for use in the laboratory as the verification confirmed all the manufacturer's and most of the pre-defined performance criteria using CLSI guidelines.

Chapter 5.0 RESULTS

Assessment of clinical utility of full blood count (FBC) and symptoms in B12 deficiency. Investigation of factors affecting B12 status and comparison of diagnostic accuracy of Total B12 (TB12) and Holotranscobalamin (Holo-TC)

5.1 Background

One thousand and three patient samples were evaluated for total B12 (TB12), holotranscobalamin (Holo-TC), methylmalonic acid (MMA), full blood count (FBC), urea and electrolytes (U+E), thyroid stimulating hormone (TSH) and folate. Blood films were also analysed if they met the laboratory's film making criteria.

The diagnostic accuracies (DA) of TB12 and Holo-TC for B12 deficiency were compared, using MMA as the proxy 'gold' standard test. The DA was assessed using the manufacturer's deficiency cut-offs for TB12 and Holo-TC of <187 ng/L and <25 pmol/L, respectively and at cut offs that included the manufacturer's indeterminate ranges for TB12 and Holo-TC of <300 ng/L and <50 ng/L, respectively.

Patient symptoms, FBC and blood film were assessed for their utility in aiding the diagnosis of B12 deficiency.

Many factors have been documented to affect B12 status and the patient data was analysed to investigate some of these associations including ethnicity, prescription drugs use (metformin, proton pump inhibitors (PPI), oral contraceptive pill (OCP) and hormone replacement therapy (HRT)), gastrointestinal (GI) factors, folate status, thyroid disease and renal disease (defined by an estimated glomerular filtration rate (eGFR) of <60 mL/min/1.73 m^2).

5.2 Aim and Objectives

The aim of this chapter was to compare the DA of TB12 and Holo-TC for B12 deficiency, using MMA as the proxy 'gold' standard test and to see if a new algorithm using Holo-TC and MMA better identified B12 deficient patients.

Objectives

1. To determine if the patient results for the analytes measured were normally distributed.
2. To determine the clinical utility/relationship of full blood counts parameters (Haemoglobin (Hb), Red Cell Count (RCC), Mean Cell Volume (MCV), blood film) and symptoms in the diagnosis of B12 deficiency, using MMA as the proxy 'gold' standard test.
3. To investigate relationship of B12 status with patient factors, comorbidities and treatments

4. To determine the DA of TB12 and Holo-TC for B12 deficiency, using MMA as the proxy ‘gold’ standard test.
5. To compare the new testing algorithm to current, assessing its clinical utility based on the results obtained.

See Appendices 5 and 6 for Patient Data tables.

5.3 Method

Blood samples were identified for analysis and collected as described in section 2.3.2. TB12, Holo-TC, serum folate and TSH were measured using the Abbott Architect immunoassay i2000 analyser using chemiluminescent microparticle immunoassay as described in sections 2.4.4 (TB12), 2.4.6 (Holo-TC), 2.4.5 (folate) and 2.4.3 (TSH). U+E were measured using the Abbott Architect c16000 chemistry analyser using integrated chip technology for sodium and potassium, and enzymatic urea and creatinine and a calculated eGFR as described in section 2.4.2. FBC testing was performed on Sysmex XN-9100 series tracked instruments as described in section 2.4.1. Blood film making and staining was performed on a Sysmex SP50 analyser as described in section 2.4.1. MMA was measured on AB Sciex 6500 LC/MS/MS analyser as described in section 2.4.7.

Analyse-IT® and GraphPad Prism were utilised for statistical analysis.

5.4 Results

5.4.1 Patient Demographics

One thousand and three patients were included in this study, of which 312 (31%) were male. Patients were between 18 and 100 years old, median (IQR) 58 (46-72) and 49 (35-65) years for males and females, respectively. Patients ≤65 years accounted for 73.1% of the study population (Table 5.1).

There was an even spread of patients per decade from age 30-70 years (Figure 5.1) There were fewer patients below age 30 (particularly for males) and over age 70 (particularly for females).

Table 5.1. Patient demographics of study population by sex and age.

	Total number of patients	% of patients	Age range years	Median age years	IQR years	Number patients ≤65 years (%)	Number patients ≥66 years (%)
Male	312	31.1	18-95	58	46-72	202 (64.7)	110 (35.3)
Female	691	68.9	18-100	49	35-65	531 (76.9)	160 (23.2)

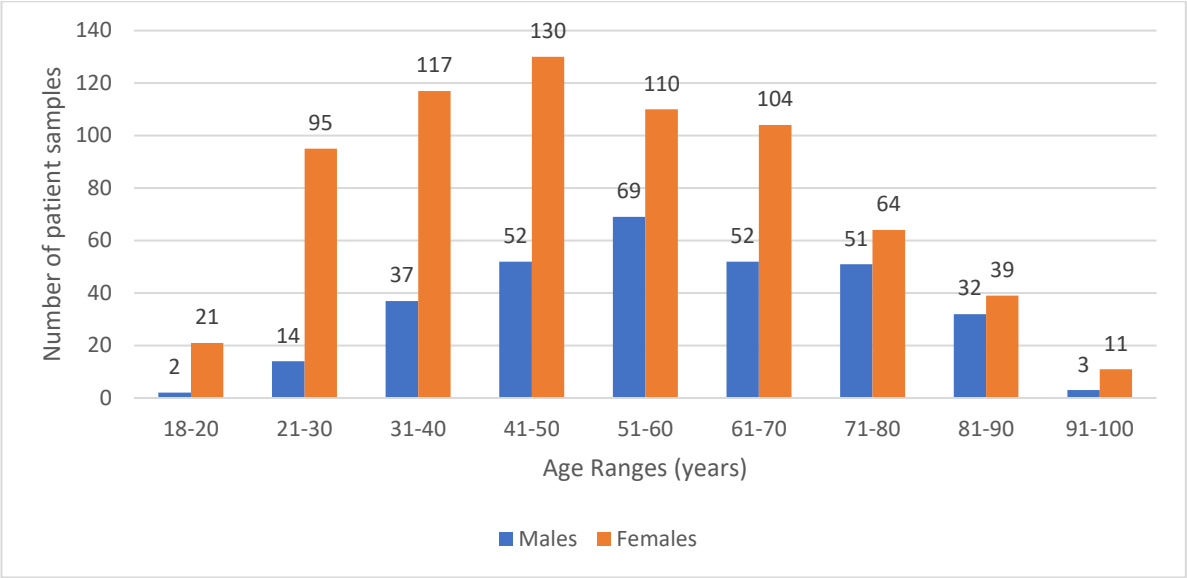


Figure 5.1 Study population shown by age in deciles for males and females. Numerical values on the columns are the numbers of patients.

Blood results (descriptive statistics) for the study population are given in Table 5.2.

5.4.2 Tests of normality

The Kolmogorov-Smirnov D (KSD) statistic showed that the data was not normally distributed for all variables ($p<0.0001$) (Table 5.3).

Table 5.2. Blood results in the study population.

	Age (years)	Na (mmol/L)	K (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)	eGFR (mL/min)	TSH (mIU/L)	Hb (g/L)	RCC ($x10^{12}$/L)	MCV (fL)	Folate (ng/mL)	Total B12 (ng/L)	Holo-TC (pmol/L)	MMA (nmol/L)
Reference ranges	-	133-146	3.5-5.3	2.5-7.8	50-98	>60	0.35-4.94	115-165 (F), 130-180 (M)	3.8-5.8	80-100	3.5-20.5	187-883	>50	<65 yr <280, >65 yr <360
Number of values	1003	1003	1003	1003	1003	1003	1000	982	982	982	981	1003	1003	1003
Minimum	18	128	2.8	1	34	19	0.004	40	2.14	8.3	1.8	83	4.9	54.3
25% Percentile	38	138	4.2	3.7	60	78	1.06	125	4.32	84.18	3.6	232	46.6	140
Median	53	140	4.5	4.6	69	90	1.575	135	4.6	87.9	5	295	67.1	196
75% Percentile	67	141	4.8	5.7	81	90	2.34	144	4.94	91.6	7.5	395	98.8	310
Maximum	100	146	6.5	26.7	684	90	90.5	186	7.02	114.1	20	2000	128	5250
Range	82	18	3.7	25.7	650	71	90.5	146	4.88	105.8	18.2	1917	123.1	5196
95% CI of median														
Actual confidence level	95.00%	95.00%	95.00%	95.00%	95.00%	95.00%	95.37%	95.57%	95.57%	95.57%	95.23%	95.00%	95.00%	95.00%
Lower confidence limit	51	139	4.5	4.5	68	90	1.52	133	4.56	87.6	4.8	288	63.8	186
Upper confidence limit	54	140	4.5	4.7	70	90	1.66	136	4.64	88.3	5.2	307	70.7	204
Mean	53.01	139.4	4.511	4.928	73.26	82.02	2.16	134.2	4.625	87.59	6.224	353	72.62	293.7
Std. Deviation	18.53	2.405	0.4328	1.861	27.15	13.36	3.996	16.56	0.5412	7.237	3.949	231.1	32.89	355.9
Std. Error of Mean	0.585	0.07594	0.01367	0.05876	0.8574	0.4218	0.1264	0.5284	0.01727	0.2309	0.1261	7.298	1.039	11.24
Lower 95% CI of mean	51.86	139.3	4.484	4.813	71.57	81.2	1.912	133.1	4.591	87.13	5.977	338.7	70.58	271.6
Upper 95% CI of mean	54.16	139.6	4.538	5.044	74.94	82.85	2.408	135.2	4.658	88.04	6.471	367.4	74.66	315.7

F = female, M = male, Yr = years of age

Table 5.3 Summary Kolmogorov-Smirnov D statistic testing for normality of all patient data. p=assumption of null hypothesis (data is normally distributed).

	Age (years)	Na (mmol/L)	K (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)	eGFR (mL/min)	TSH (mIU/L)	Hb (g/L)	RCC ($x10^{12}$/L)	MCV (fL)	Folate (ng/mL)	Total B12 (ng/L)	Holo-TC (pmol/L)	MMA (nmol/L)
Reference ranges	-	133-146	3.5-5.3	2.5-7.8	50-98	>60	0.35-4.94	115-165 (F), 130-180 (M)	3.8-5.8	80-100	3.5-20.5	187-883	>50	<65 yr <280, >65 yr <360
Data available	1003	1003	1003	1003	1003	1003	1000	982	982	982	981	1003	1003	1003
Median	53	140	4.5	4.6	69	90	1.58	135	4.60	87.9	5.0	295	67.1	196
Range	18-100	128-146	2.8-6.5	1.0-26.7	34-200	19->90	<0.004-90.5	40-186	2.14-7.02	60.8-114.1	<1.8->20.0	<83->2000	5->128	54.3-5250
1st Quartile	38	138	4.2	3.7	60	78	1.06	125	4.32	84.2	3.6	232	46.6	140
3rd Quartile	67	141	4.8	5.7	81	>90	2.34	144	4.94	91.6	7.5	395	98.7	309.7
Mean	53	139	4.5	4.9	73	82	2.16	134	4.63	87.7	6.2	353	72.6	293.7
Mean SE	0.59	0.08	0.014	0.059	0.60	0.42	0.126	0.53	0.017	0.216	0.126	7.30	1.039	11.239
SD	18.5	2.4	0.43	1.86	19.1	13.4	4.00	16.6	0.54	6.78	3.95	231.1	32.89	355.93
Skewness	0.1	-1.0	0.2	2.7	1.9	-1.9	15.6	-0.5	0.0	-0.5	1.7	4.0	0.3	7.0
Kurtosis	-0.81	3.03	0.71	20.99	7.42	2.83	299.82	1.99	1.24	2.25	2.55	22.16	-0.99	71.01
KSD statistic (D)	0.05	0.14	0.07	0.10	0.11	0.30	0.30	0.05	0.05	0.06	0.16	0.18	0.08	0.26
P value of test	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Accept or Reject null hypothesis	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject	Reject

F = female, M = male, Yr = years of age

5.4.3 Correlations of variables

Spearman Rank correlation was performed for all non-parametric variables and Figure 5.2 highlights the strongest associations (deepest colours) and whether the association is negatively or positively associated. All variables correlated with MMA using Spearman Rank correlation (for non-parametric variables) except for sodium and haemoglobin. The most statistically significant correlations, with MMA, were for age, eGFR, TB12 and Holo-TC ($p<0.0001$). The statistical significance (p values) of the correlations are shown in Table 5.4.

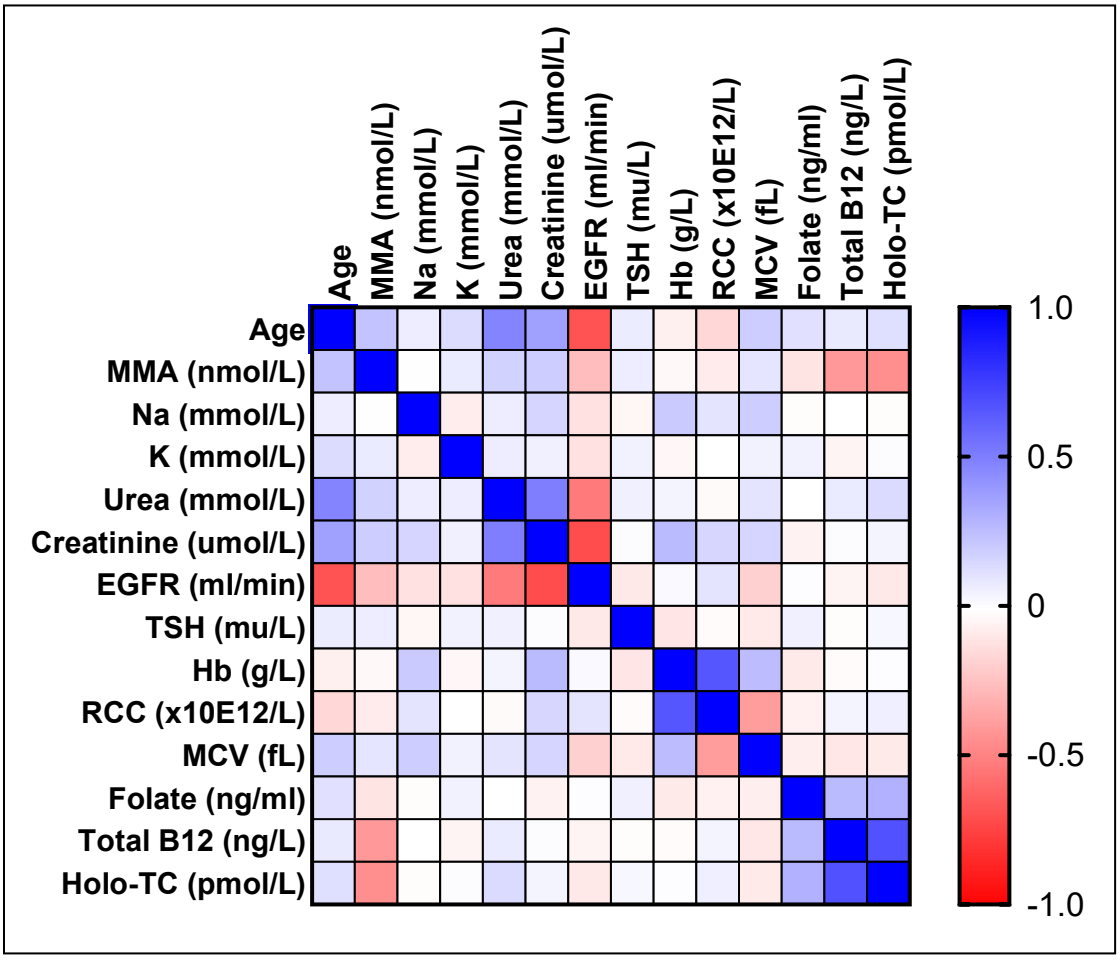


Figure 5.2. Spearman rank correlations of all non-parametric variables (deepest colours strongest corelation, red negative associations, blue positive associations). For MMA, the strongest associations were seen with age, eGFR, TB12 and Holo-TC.

Table 5.4. The statistical significance (p values) of the Spearman rank correlations. Statistical significance = p <0.05 (highlighted in yellow).

p value	Age	MMA	Na	K	Urea	Creatinine	EGFR	TSH	Hb	RCC	MCV	Folate	Total B12	Holo-TC
Age		<0.0001	0.0341	<0.0001	<0.0001	<0.0001	<0.0001	0.0185	0.0580	<0.0001	<0.0001	0.0003	0.0094	0.0001
MMA	<0.0001		0.8691	0.0130	<0.0001	<0.0001	<0.0001	0.0282	0.3392	0.0121	0.0015	0.0006	<0.0001	<0.0001
Na	0.0341	0.8691		0.0221	0.0262	<0.0001	0.0001	0.2254	<0.0001	0.0014	<0.0001	0.6604	0.9297	0.6564
K	<0.0001	0.0130	0.0221		0.0297	0.0646	0.0002	0.1166	0.2724	0.9676	0.1199	0.1250	0.1696	0.7285
Urea	<0.0001	<0.0001	0.0262	0.0297		<0.0001	<0.0001	0.0702	0.1903	0.5198	0.0013	0.9721	0.0182	<0.0001
Creatinine	<0.0001	<0.0001	<0.0001	0.0646	<0.0001		<0.0001	0.7265	<0.0001	<0.0001	<0.0001	0.0885	0.7366	0.1991
EGFR	<0.0001	<0.0001	0.0001	0.0002	<0.0001	<0.0001		0.0032	0.4736	0.0011	<0.0001	0.8236	0.1715	0.0042
TSH	0.0185	0.0282	0.2254	0.1166	0.0702	0.7265	0.0032		0.0010	0.6115	0.0051	0.0820	0.6366	0.3639
Hb	0.0580	0.3392	<0.0001	0.2724	0.1903	<0.0001	0.4736	0.0010		<0.0001	<0.0001	0.0088	0.5588	0.8895
RCC	<0.0001	0.0121	0.0014	0.9676	0.5198	<0.0001	0.0011	0.6115	<0.0001		<0.0001	0.0872	0.1895	0.0576
MCV	<0.0001	0.0015	<0.0001	0.1199	0.0013	<0.0001	<0.0001	0.0051	<0.0001	<0.0001		0.0301	0.0026	0.0092
Folate	0.0003	0.0006	0.6604	0.1250	0.9721	0.0885	0.8236	0.0820	0.0088	0.0872	0.0301		<0.0001	<0.0001
Total B12	0.0094	<0.0001	0.9297	0.1696	0.0182	0.7366	0.1715	0.6366	0.5588	0.1895	0.0026	<0.0001		<0.0001
Holo-TC	0.0001	<0.0001	0.6564	0.7285	<0.0001	0.1991	0.0042	0.3639	0.8895	0.0576	0.0092	<0.0001	<0.0001	

5.4.4 Clinical utility of FBC and blood film in the assessment of B12 deficiency

Nine hundred and eighty two patients had an FBC result, in line with the British Society Haematology guideline for the diagnosis and treatment of cobalamin and folate disorders (Devalia, Hamilton and Molloy, 2014). Twenty-one patients did not have FBC analysed as the samples were either clotted or insufficient. FBC parameters that may be associated with B12 deficiency are low Hb (<130 g/L and <120 g/L, in males and females, respectively), low RCC (<4.0 $x10^{12}$/L and <3.8 $x10^{12}$/L, in males and females, respectively) and raised MCV (>100 fL). The Spearman rank p values for these three parameters when compared to MMA, TB12 and Holo-TC are shown in Table 5.5. There was no relationship between Hb and any of tests used to identify B12 deficiency, a positive association of RCC with MMA only and strong positive associations with MCV for all three assays.

Table 5.5. Spearman rank p values for Hb, RCC and MCV (n=982); p <0.05 is statistically significant for tests of B12 deficiency.

Variable	Hb (g/L)	RCC ($x10^{12}$/L)	MCV (fL)
MMA (nmol/L)	0.3392	0.0121	0.0015
TB12 (ng/L)	0.5588	0.1894	0.0026
Holo-TC (pmol/L)	0.8895	0.0576	0.0092

When receiver operating characteristic (ROC) curve analysis was performed for Hb, RCC and MCV, using MMA as the proxy 'gold' standard test (>280 nmol/L and >360 nmol/L, if ≤65 years and >65 years, respectively) for B12 deficiency, the areas under the curve (AUC)(95% CI) were 0.552 (0.509-0.594), 0.549 (0.507-0.592), 0.521

(0.478-0.563), respectively showing that FBC parameters were not good diagnostic markers for B12 deficiency (Figure 5.3).

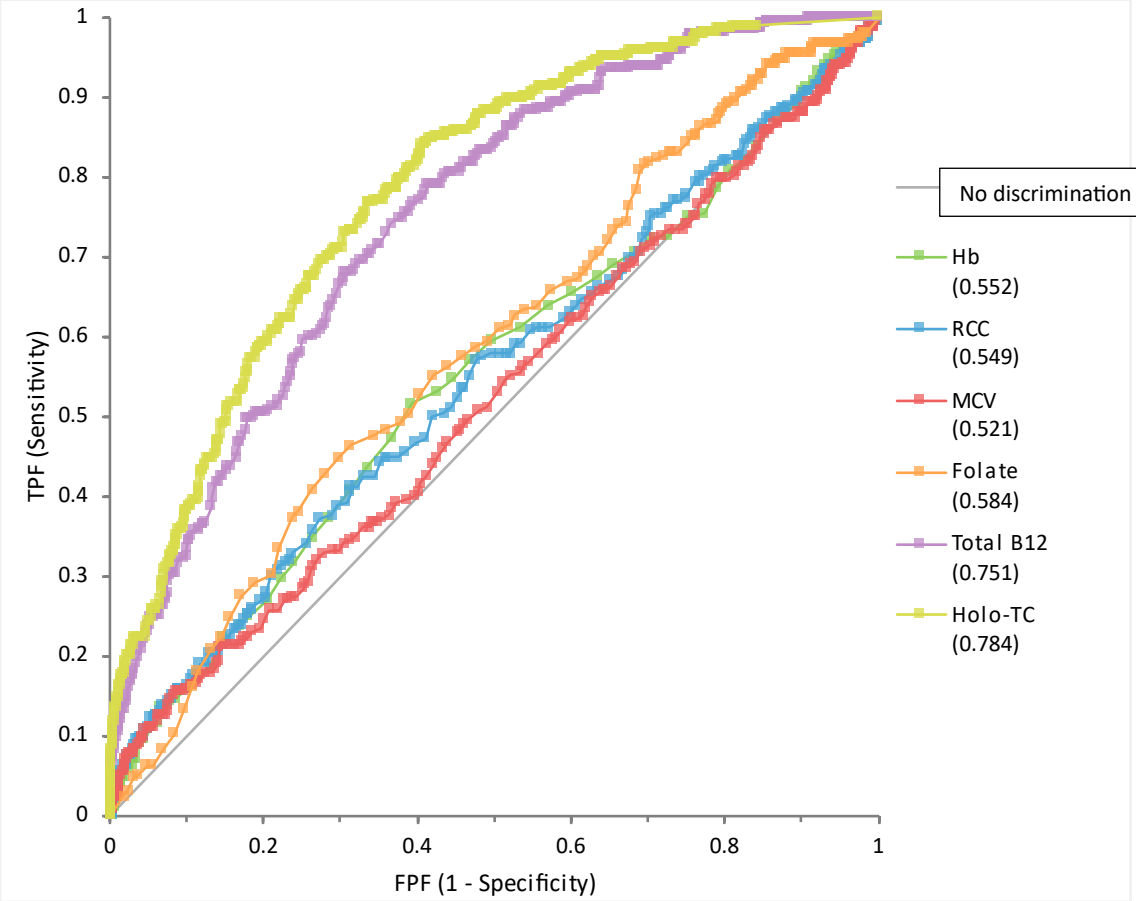


Figure 5.3. ROC curve analysis of Hb, RCC and MCV when using MMA as proxy 'gold' standard for B12 deficiency where results of 0.5 shows poor performance and 1.0 best performance. TPF = true positive rate, FPF = false positive rate.

In the study cohort only eight patients had a low Hb and a low RCC and a raised MCV and five of these had a raised MMA.

Blood films were analysed for 47 samples, the majority for a low Hb, low MCV, low platelets or lymphocytosis. Five films had features consistent with B12 deficiency, four with macrocytosis and one with hyper-segmented neutrophils. Sixteen other

FBC’s met the blood film criteria but blood films were not made, three with low Hb, seven with low MCV and six with high MCV. There were a further 64 FBC’s with various other haematological abnormalities but no film was reviewed. For the five patients with blood films suggestive of B12 deficiency, four had an MMA consistent with B12 deficiency, two samples had concurrent low TB12 and Holo-TC results. One patient had indeterminate results for both, and two had replete TB12 and Holo-TC results. Two patients had coexisting folate deficiency, and one patient had a low folate only (Table 5.6).

Table 5.6. Reported results for five patients with blood film features that were specific for B12/folate deficiency.

Hb (g/L) RR >130 (males), >120 (females)	**RCC x10^{12}/L RR >3.8**	**MCV (fL) RR 80-100**	**Blood Film comments**	**Folate (ng/mL) RR >3.5**	**TB12 (ng/L) RR 187-883**	**Holo-TC (pmol/L) RR >50**	**MMA (nmol/L) RR <280 if <65 yr, <360 if >65 yr**	**Clinical conclusion**
119	3.39	106.8	macrocytosis, target cells, platelet clumps	<1.8	130	40.2	741	True B12 deficiency, dual deficiency of B12 and folate
121	3.39	102.7	macrocytosis	4.7	<83	5.2	3930	True B12 deficiency
103	3.2	97.2	polychromasia +, macrocytosis	>20	470	109.4	386	Macrocytic comment although MCV within reference range. Raised MMA underlying B12 deficiency - missed diagnosis by TB12/Holo-TC
138	3.91	105.6	macrocytosis	2.9	208	33.3	1270	Indeterminate range for both TB12 and Holo-TC. True B12 deficiency, dual deficiency of B12 and folate
127	4.54	84.1	Hyper-segmented neutrophils (HSN), Lymphocytosis reactive lymphocytes	3.3	331	61.3	112	Replete B12 concentration, note no macrocytosis, folate is below the reference range. and early folate deficiency may explain the HSN.

RR reference range

5.4.5 Clinical utility of B12 deficiency symptoms in the assessment of B12 deficiency

For the patients where B12 deficiency symptom information was available, 119/392 patients were symptomatic at time of inclusion. Using MMA as the proxy 'gold' standard test of B12 deficiency (>280 nmol/L and >360 nmol/L, if ≤65 years and >65 years, respectively), the percentage of patients with symptoms of B12 deficiency was similar in the B12 deficient and B12 replete groups, 31% and 30%, respectively.

The common symptoms recorded are listed in Table 5.7. Tiredness was the most reported symptom (55%), and some patients had more than one symptom.

Table 5.7. Breakdown of recorded symptoms of B12 deficiency in the 119 symptomatic patients.

Symptom	n of patients
Tiredness, TATT, Lethargy	65
Pins and needles, tingling, paraesthesia, numbness	28
Aches and pains	10
Other	7
Dizziness	7
Sore mouth, glossitis, angular stomatitis	5
Memory loss, impairment? dementia	4
Anaemia	3
Hair loss/thinning	3
Total	132

5.4.6 Patient factors, comorbidities, treatments and B12 status.

5.4.6.1 B12 status and Sex

The median (IQR) concentrations for TB12 (301 (232-406) and 288 (231-372) ng/L and Holo-TC 68 (47-98) and 65 (46-99) pmol/L) were higher in females than males, respectively. The median (IQR) MMA results were lower in females than males (193 (137-289) nmol/L and 203 (146-355) nmol/L), respectively (Table 5.8). Using one-way ANOVA analysis these differences were statistically significant for TB12 (p= 0.0017) and MMA (p= 0.0005) but not for Holo-TC (p= >0.9999).

Table 5.8. Total B12, Holo-TC and MMA concentrations by sex in the study population.

B12 Assay		**Male**	**Female**	**Statistical significance**
TB12 (ng/L)	Median	288	301	p=0.0017
	IQR	231-372	232-406	
	Range	89 to 1278	<83 to >2000	
Holo-TC (pmol/L)	Median	65	68	p=>0.9999
	IQR	46-99	47-98	
	Range	5 to >128	5 to >128	
MMA (nmol/L)	Median	203	193	p= 0.0005
	IQR	146-355	137-289	
	Range	63.7 to 3625	54.3 to 5250	

IQR interquartile range. p<0.05 = statistically significant.

5.4.6.2 B12 status and Ethnicity

Ethnicity data was not available for 308/1003 patients in this study. For the 695 patients where information was available 58.3% patients were white, 32.2% were Asian and 8.3% were Black Caribbean. (Table 5.9).

Table 5.9. Ethnicity data of patient population by sex.

Ethnicity	Totals (%)	Males (%)	Females (%)
White	405 (58.3)	140 (62.5)	265 (56.3)
Asian	224 (32.2)	62 (27.7)	162 (34.4)
Black	58 (8.3)	20 (8.9)	38 (8.1)
Mixed	3 (0.4)	1 (0.5)	2 (0.4)
Other	5 (0.7)	1 (0.5)	4 (0.9)
Totals	695	224	471

In 50.0% of Black, 24.9% of White and 19.2% of Asian patients, none of the three tests of B12 deficiency were deficient or indeterminate. Using MMA as the proxy 'gold' standard test of B12 deficiency 28.4%, 40.6% and 12.1% of White, Asian and Black patients, respectively were B12 deficient. Using TB12 (< 187 ng/L) as the test of B12 deficiency, 10.1%, 22.3% and 6.9% of White, Asian and Black patients, respectively were B12 deficient. Lower levels of deficiency were seen using Holo-TC (<25 pmol/L) as the test of deficiency with deficiency in 4.9%, 7.6% and 6.9% of White, Asian and Black patients, respectively (Table 5.10).

Table 5.10. Percentage of patients with B12 deficiency in different ethnic groups

Ethnicity Measurement of B12 status	White (%)	Asian (%)	Black (%)	Mixed (%)	Other (%)
↑MMA (nmol/L)	115 (28.4)	91 (40.6)	7 (12.1)	1 (33.3)	2 (40.0)
TB12 ≤187 ng/L	41 (10.1)	50 (22.3)	4 (6.9)	1 (33.3)	3 (60.0)
TB12 188-300 ng/L	246 (60.7)	124 (55.4)	23 (39.7)	2 (66.6)	0
Holo-TC <25 pmol/L	20 (4.9)	17 (7.6)	4 (6.9)	0	0
Holo-TC 25-50 pmol/L	128 (31.6)	67 (29.9)	13 (22.4)	2 (66.6)	3 (60.0)
B12 replete by all methods	101 (24.9)	43 (19.2)	29 (50)	0	2 (40.0)

↑= Raised MMA, >280 nmol/L (≤65 years), >360 nmol/L (>65 years). B12 replete TB12 >300 ng/L, Holo-TC >50 pmol/L, MMA ≤280 ((≤65 years), ≤360 nmol/L (>65 years).

5.4.6.3 B12 status and Age

Age strongly positively correlated with MMA, as the proxy 'gold' standard marker of B12 deficiency, ($p<0.0001$) (Table 5.11).

Table 5.11. Spearman Rank correlation statistic calculated for Age versus MMA as the proxy 'gold' standard test for B12 deficiency. 95% CI = 95% Confidence intervals. p<0.05 is significant. Null hypothesis (H0) = variables are independent. n= number of patient results.

Spearman rank correlation data between age and MMA				
n	Spearman Rank rs	Fisher 95% CI	p value	Accept/reject H0
1003	0.235	0.174 to 0.295	<0.0001	Reject

5.4.6.4 B12 status and Gastrointestinal Factors

Information on gastrointestinal (GI) symptoms, disease and surgery was available for 493 patients in the study population, of these 183 had at least one of GI symptoms, GI disease or previous GI surgery. GI factors were present in 45%, 21% and 7% of B12 deficient patients using MMA, as the test of B12 deficiency (Table 5.12).

Table 5.12. Percentage of patients with and without B12 deficiency with GI symptoms, conditions and/or history of GI surgery.

Test	Patients (%) with GI factor
MMA >280 nmol/L (≤65 years), >360 nmol/L (>65 years).	63 (45.3)
B12 replete by MMA (<280 nmol/L (≤65 years), <360 nmol/L (>65 years)).	120 (33.9)

Many patients had more than one GI symptom, disease and/or GI surgery. The most common GI factor was acid reflux (35/183), followed by irritable bowel syndrome (28/183). The most common surgical procedures were cholecystectomy and bariatric surgery 18/183 and 9/183, respectively.

5.4.6.5 B12 status and folate and TSH results

Serum folate results were available for 981/1003 patients in this study (22 samples were unsuitable for analysis owing to a high haemolytic index) and of these 231(23.5%) had a folate concentration <3.5 ng/mL (indicating folate deficiency). Folate deficiency was present in 30.3% and 21.1% of patients who were B12

deficient and replete, respectively, when raised MMA was used as the marker of B12 deficiency. In the 981 patients studied 8% had both B12 (using raised MMA as the marker) and folate deficiency (Table 5.13). When ROC curve analysis was performed for folate, using MMA as the proxy 'gold' standard test of B12 deficiency, the AUC was 0.586 (95% CI 0.545-0.626) showing it was not a diagnostic marker for B12 deficiency.

TSH results were available for 1000/1003 patients in this study (three samples had insufficient serum for analysis), of which 25 (2.5%) and 45 (4.5%) were below the lower limit and above the upper limit of the reference range, respectively. Raised TSH was present in 7.8% and 3.3% of patients who were B12 deficient and replete, respectively, and low TSH was present in 3% and 2.3% of patients who were B12 deficient and replete, respectively, when raised MMA was used as the marker of B12 deficiency (Table 5.13). Seventy-five patients (7.5%) in the study population were hypothyroid on thyroxine and only four were hyperthyroid on carbimazole. For the hypothyroid patients 60 (8.1%) were B12 replete (3 patients were on oral B12 and 2 on IM injections) and 15 (5.8%) were B12 deficient, using MMA as the proxy 'gold' standard test.

Table 5.13. Relationships between B12 deficiency and serum folate deficiency (<3.5 ng/mL) and with TSH (above and below reference ranges).

	Folate<3.5 ng/mL (Reference range 3.5-20.5)	**TSH <0.350 mIU/L Hyperthyroid (Reference range 0.35-4.94)**	**TSH >4.94 mIU/L Hypothyroid (Reference range 0.35-4.94)**
Patients (%)	231 (23.5)	25 (2.5)	45 (4.5)
B12 deficient (raised MMA) (%)	78 (30.3)	8 (3)	21 (7.8)
B12 replete by MMA (%)	153 (21.1)	17(2.3)	24 (3.3)

B12 replete if MMA ≤280 nmol/L (if ≤65 years) and ≤360 nmol/L (if >65 years)

TSH (p=0.0282) and folate (p=0.0006) positively correlated and strongly negatively correlated, respectively, with MMA, as the proxy 'gold' standard marker of B12 deficiency (Table 5.14).

Table 5.14. Spearman Rank correlation statistic calculated for TSH and folate versus MMA, as the proxy 'gold' standard test for B12 deficiency. 95% CI = 95% Confidence intervals. p<0.05 is significant. Null hypothesis (H0) = variables are independent. n= number of patient results.

	Spearman rank correlation data				
Variable	n	Spearman Rank rs	Fisher 95% CI	p value	Accept/reject H0
TSH (mU/L)	1000	0.069	0.006 to 0.133	0.0282	Reject
Folate (ng/mL)	981	-0.109	-0.172 to -0.045	0.0006	Reject

5.4.6.6 B12 status and renal impairment

All patients in the study population had an estimated glomerular filtration rate (eGFR) calculated and of these 98 (9.8%) had an eGFR of <60 mL/min/1.73m^2 (indicating chronic kidney disease). An eGFR <60 mL/min/1.73m^2 was present in 15.4% and 7.7% of patients who were B12 deficient and replete, respectively, when raised MMA was used as the marker of B12 deficiency.

There was a strong negative Spearman rank correlation between eGFR and MMA (Table 5.15).

Table 5.15. Spearman Rank correlation statistic calculated for eGFR versus MMA, as the proxy 'gold' standard test for B12 deficiency. 95% CI = 95% Confidence intervals. p<0.05 is significant. Null hypothesis (H0) = variables are independent. n= number of patient results.

	Spearman rank correlation data				
Variable	n	Spearman Rank rs	Fisher 95% CI	p value	Accept/reject H0
eGFR	1003	-0.263	-0.321 to -0.202	<0.0001	Reject

To see if there were any differences in MMA, TB12 and Holo-TC between the groups with and without renal impairment a one-way ANOVA was performed which did not find any significant difference for TB12 (p=0.8182) or Holo-TC (p=0.9994) however statistically significant results were seen for MMA (p<0.0001).

5.4.6.7 B12 status and diabetes

Twenty two percent of the study population (220 patients) had diabetes (with 56% of those treated with metformin). To see if there were any differences in MMA, TB12 and Holo-TC between the groups with and without diabetes a one-way ANOVA was performed which showed no statistically significant results with TB12 (p= 0.9351), Holo-TC (p=0. 9908) or MMA (p=0.2454).

5.4.6.8 B12 status of patients on some prescription drugs

In the study cohort 124 (12%), 254 (25%), 69 (7%) and 21 (2%) patients were known to be taking metformin, PPI, OCP and HRT respectively (Table 5.16) Most of the

patients taking a PPI were doing so for gastro-protection and for these patients there were high numbers of recorded GI symptoms, conditions, or past GI surgery.

Thirty three percent and 24% of patients who were B12 deficient and B12 replete, respectively were taking metformin and 57% and 42% of patients who were B12 deficient and B12 replete, respectively were taking PPI, using MMA as the proxy 'gold' standard test. The numbers of patients taking OCP and HRT were low and therefore, further comparisons were not performed.

Table 5.16. Number of patients on prescription drugs and B12 status. n = number of patients.

Prescribed Drug	**Metformin n (%)**	**PPI n (%)**	**OCP n (%)**	**HRT n (%)**
Yes	124 (12.4)	254 (25.3)	69 (6.9)	21 (2.1)
No	31	298	247	266
Unknown	848	451	687	716
B12 deficient (raised MMA)	45 (33.1)	92 (56.8)		
B12 replete	79 (23.7)	162 (41.5)		

Raised MMA >280 nmol/L (if ≤65 years) and >360 nmol/L (if >65 years).

5.4.7 Comparison of the tests for B12 deficiency (TB12, Holo-TC and MMA)

One thousand and three patients had TB12, Holo-TC and MMA results. Figures 5.4 (TB12), 5.5 (Holo-TC), and 5.6 (MMA) summarise the number of deficient, indeterminate (where applicable) and replete results broken down by sex.

In the study population 10.4% and 4.8% of the patients were B12 deficient when assessed by TB12 (<187 ng/L) and Holo-TC (<25 pmol/L), respectively, with a further 41% and 26% potentially deficient as their results fell into the indeterminate range for TB12 (188-300 ng/L) and Holo-TC (25-50 pmol/L), respectively. Using MMA as the diagnostic test 26.3% of patients were B12 deficient.

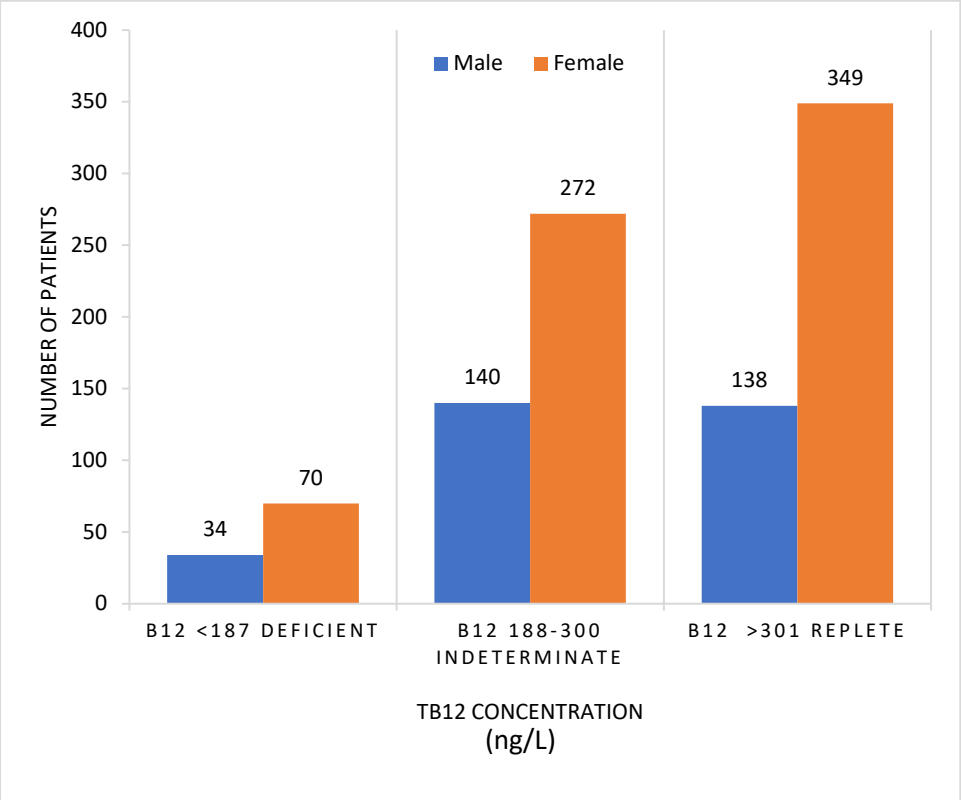


Figure 5.4. Patients' data categorised by sex and TB12 status. Figures on columns indicate actual number of patients.

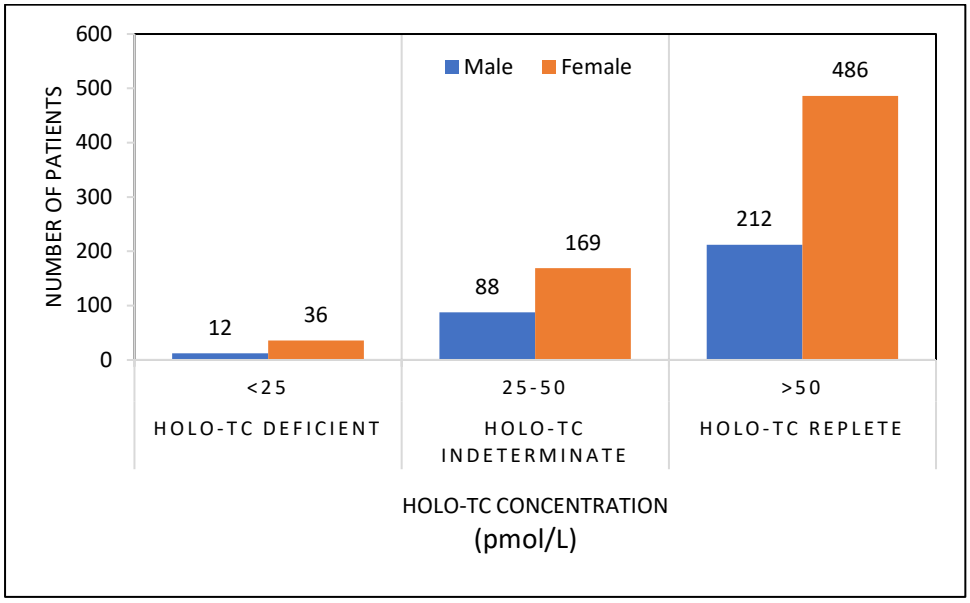


Figure 5.5. Patients' data categorised by sex and Holo-TC. Figures on columns indicate actual number of patients.

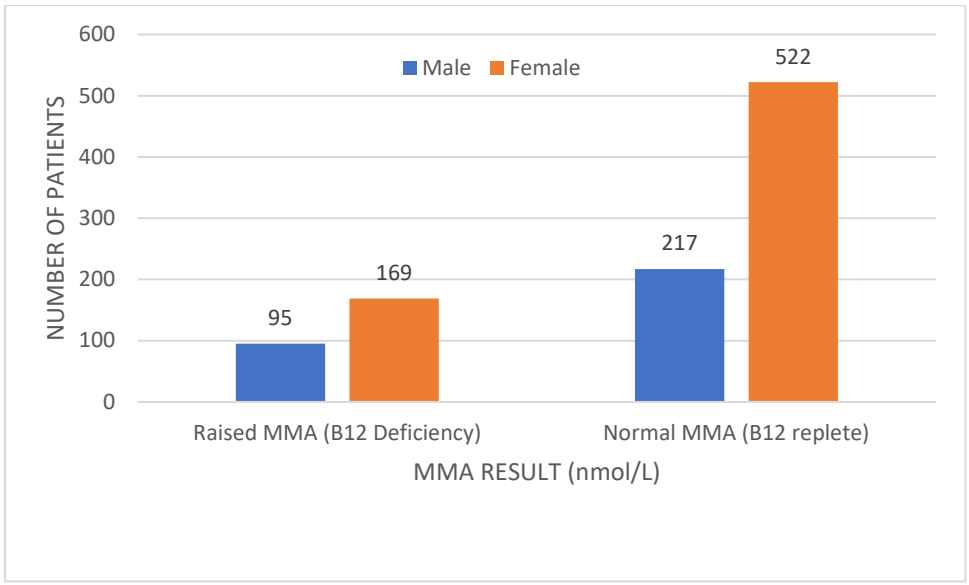


Figure 5.6. Patients' data categorised by sex and MMA status. Figures on columns indicate actual number of patients.

TB12 and Holo-TC negatively correlated strongly with MMA, as the proxy 'gold' standard marker of B12 deficiency ($p<0.0001$ in both cases) (Table 5.17).

Table 5.17. Spearman Rank correlation statistic calculated for TB12 and Holo-TC versus MMA, as the proxy 'gold' standard test for B12 deficiency. 95% CI = 95% Confidence intervals. $p<0.05$ is significant. Null hypothesis (H0) = variables are independent. n = number of patient results.

	Spearman rank correlation data				
Variable	n	Spearman Rank rs	Fisher 95% CI	p value	Accept/reject H0
TB12 ng/L	1003	-0.407	-0.459 to -0.352	<0.0001	Reject
Holo-TC pmol/L	1003	-0.443	-0.493 to -0.390	<0.0001	Reject

5.4.8 Receiver operator characteristics for diagnosis of B12 deficiency using TB12 and Holo-TC when compared to MMA as the proxy 'gold' standard test

When ROC curve analyses for B12 deficiency diagnostic accuracy were performed for TB12 and Holo-TC as individual tests using MMA as the proxy 'gold' standard test the AUC (95% CI) were 0.749 (0.717-0.782) and 0.779 (0.749 - 0.810) for TB12 and Holo-TC, respectively. Given the overlapping 95% CIs it was not possible to conclude that Holo-TC is a better test of B12 deficiency than TB12. The optimal cut-off values for diagnosing deficiency in this study were found to be 180 ng/L and 29 pmol/L for TB12 and Holo-TC, respectively.

5.4.9 Clinical utility of the proposed new algorithm for assessing B12 deficiency compared to Total B12

The DA of the test in use at the start of this study (TB12 deficient <187 ng/L) and the new proposed algorithm (Holo-TC ± MMA) (section 1.4.5) for assessing B12 deficiency, were 0.76 and 0.88, respectively, when using MMA as the proxy 'gold' standard test. For the current algorithm, all indeterminate patients were recorded as true negatives. TB12 and the new algorithm had sensitivities (95% CI) of 0.25 (0.20-0.30) and 0.60 (0.54-0.66); specificities (95% CI) of 0.95 (0.93-0.96) and 0.99 (0.98-0.99); negative predictive values (NPV) of 0.78 and 0.88 and positive predictive values (PPV) of 0.62 and 0.95, respectively for diagnosing B12 deficiency, when using MMA >280 and >360 nmol/L in ≤65 years and >65 years respectively as the proxy 'gold' standard.

This study utilised the original indeterminate cut-off values of 25-50 pmol/L for Holo-TC published by Abbott diagnostics for their Architect assay however when NICE published NG239 guidelines in 2024 (NICE, 2024) they recommended an indeterminate range of 25-70 pmol/L. To investigate if the DA is improved using the wider indeterminate range of Holo-TC using MMA as the proxy 'gold' standard as before, the DA was recalculated. The overall DA using the wider indeterminate range was increased to 0.93. The specificity remained the same however the sensitivity increased to 0.78 (95% CI, 0.73-0.83) from 0.60 with an increase in NPV to 0.93 (reduction of missed deficiencies of 7%) and a slight increase in PPV to 0.96. These results show that the indeterminate range for Holo-TC in the NICE guidelines

has a superior DA to the Abbott diagnostics range using the proposed diagnostic algorithm.

5.5 Discussion

Samples from twice as many females as males were included in this study, mirroring the requesting pattern for B12 deficiency in the local population. There is no evidence that B12 deficiency is more common in women and may reflect the fact that women seek medical attention from a GP more frequently than men (Wang et al, 2013). However, pernicious anaemia and autoimmune disease are seen more frequently in women (up to four-fold higher). This is thought to be due to a number of factors including hormones, X chromosomes, the environment and the microbiome (Kronzer, Bridges and Davis, 2020).

There is little in the literature about differences in B12 concentrations between males and females for TB12, Holo-TC or MMA. Margalit et al (2018), found that men had a higher prevalence of B12 deficiency (25.5% vs 18.9%), and were more severely affected. This did not appear to be related to diet or hormone levels and was postulated to be caused by genetic variations. The results of this study were largely in keeping with their findings, females had significantly higher TB12 and significantly lower MMA results, although Holo-TC results were not significantly different between the sexes.

B12 deficiency is known to increase with age due to reduced absorption of B12 caused by atrophic gastritis which leads to low production of stomach acid and intrinsic factor. Age specific cut-offs for B12 deficiency are generally not used for TB12 or Holo-TC but are in place for MMA (Mineva et al, 2019). When the Spearman Rank correlation was performed for MMA versus age there was a positive correlation.

Prevalence of B12 deficiency, in this study cohort was higher for Asians than Whites (40.6% and 23.4%, respectively) and much higher than for Black patients (12.1%), when using MMA as the test of B12 deficiency. These findings are concordant with two other large screening studies which found significantly higher B12 levels in Black compared to Asian and White participants (O'Logbon et al, 2022, Sobczyńska-Malefora et al, 2023), although these studies reported no significant differences between the Asian and White groups. Sobczyńska-Malefora et al (2023) suggested that the deficient cut-off values used for TB12 deficiency in Black people should be modified with a reference interval for Black ethnicity patients over 13 years old of 225-1091 ng/L compared to 182-692 ng/L for White and Asian ethnicity patients. The higher TB12 concentration is attributed to a combination of genetics and acquired/environmental factors in the Black ethnic group (O'Logbon et al, 2022). Higher B12 concentrations in healthy Black people was first identified in the 1980's (Kwee, Bowman and Wells, 1985, Saxena and Carmel, 1987), however, unified reference ranges for TB12 continue to be utilised with the result that B12 deficiency may have been missed, with delayed treatment leading to health inequalities. NICE

NG239 (NICE, 2024) has recognised that Black ethnicity may have a higher reference range for TB12 than White or Asian ethnicity recommending caution in patients with indeterminate results.

Earlier NICE guidance for the management of B12 deficiency (NICE, 2023) recommended that if the TB12 was in the indeterminate range then FBC results and neurological symptoms should be taken into consideration before treatment commenced. The area under the ROC curve (95% CI) for Hb 0.552 (0.509-0.594), RCC 0.549 (0.507-0.592) and MCV 0.521(0.478-0.564) indicated that they had poor diagnostic accuracy for B12 deficiency. FBC changes are known to be a late manifestation of B12 deficiency when liver stores have been totally depleted. Herrmann and Obeid, 2012 reviewed the clinical utility of the FBC in diagnosis of deficiency and concluded that the FBC cannot reliably be used as an indicator of B12 deficiency.

Only five blood films in this study had features associated with B12 deficiency which did not permit statistical analysis. Blood film features are not specific to B12 deficiency and can be mimicked by folate deficiency or both deficiencies may occur concurrently. Taken together if FBC and film findings are consistent with B12 deficiency then the likelihood of B12 or folate deficiency is high. One of the patients with blood film comments consistent with B12 deficiency had a TB12 of 470 ng/L (reference range (RR) 187-883 ng/L) and a Holo-TC of 109 pmol/L (RR >50 pmol/L) and consequently, since one of these tests are likely to be the first line test, deficiency in this patient would probably have been missed. In this patient the MMA was raised 386 nmol/L (RR <280 nmol/L for patient's age); Hb and RCC were low

(103 g/L (RR >130 g/L), 3.2 $x10^{12}$/L (RR >3.8 $x10^{12}$/L), respectively) and MCV was within the reference range at 97 fL (RR 80-100 fL). The clinical details were falling Hb and increasing MCV therefore, clinical suspicion was present, the patient was diabetic on metformin but no information regarding B12 treatment was provided. This example highlights the difficulty in identifying patients with B12 deficiency.

Symptoms are not a good indicator of B12 deficiency. In this study the percentage of patients with symptoms of B12 deficiency was similar in the B12 deficient and B12 replete groups (31% and 30%, respectively), when using MMA as the proxy 'gold' standard test of B12 deficiency. In the BSH guideline for diagnosis and treatment of B12 and folate disorders Devalia, Hamilton and Molloy (2014) discussed the variation and the non-specific nature of symptoms seen in patients with B12 deficiency.

Many factors affect B12 status such as age, ethnicity, genetic variation, renal function, therapeutic drugs, a poor diet and malabsorption and this is why B12 deficiency is difficult to diagnose.

The data looking at the prevalence of GI conditions in patients who are B12 deficient (45%) and replete (34%) has limitations as some of those that are B12 replete will be on treatment for B12 deficiency, and this information was not collected in this study. Acid reflux was the most common GI symptom observed. This is often treated with PPIs that are known to cause B12 deficiency (Swarnakari et al, 2022). IBS was also

prevalent and may result in B12 deficiency because of inadequate amounts of stomach acid and inflamed intestines that result in reduced absorption of B12 (Bek et al, 2022). Diverticulitis is commonly associated with bacterial overgrowth, occurring due to the slow movement of food through the intestines. The bacteria directly uptake B12, preventing absorption and causing deficiency (Drude, Finkelman, Davis and Ferrante, 1980). Gastric ulcers and dyspepsia may be caused by Helicobacter pylori (H. pylori) infection (Gümürdülü et al, 2003). H. pylori infection may cause B12 deficiency because it is associated with atrophic gastritis. Atrophic gastritis is an autoimmune disease characterised by parietal cell destruction leading to reduced acid production and intrinsic factor deficiency. It has been suggested that eradication of the H. pylori improves the B12 deficiency symptoms including anaemia, it is even thought that some pernicious anaemia is the final phase of H pylori associated gastritis where the parietal cell mass has been lost (Kaptan et al, 2000). One patient in this study was known to have a family history of pernicious anaemia and had MMA, TB12 and Holo-TC results consistent with B12 deficiency. This patient should have had intrinsic factor antibodies measured.

The commonest types of GI surgery found in this patient cohort were cholecystectomy, appendectomy and various forms of bariatric surgery. Any GI surgery that affects the stomach or small intestine will affect B12 absorption (Kornerup et al, 2019). None of the patients that had bariatric surgery were B12 replete. Gastric bypass results in changes in acid secretion whilst gastric sleeve surgery results in reduced acid production and lower levels of intrinsic factor (Sala et al, 2017). Up to 18% of all patients may be B12 deficient before the surgery so

levels should be checked pre-operatively, then every three months after surgery and treated accordingly, gastric bypass patients with intramuscular B12 injection and gastric sleeve/banding patients with oral B12 (Majumder et al, 2013, Lupoli et al, 2017). No patients with a history of cholecystectomy were B12 replete. After cholecystectomy bile flows directly into the intestines however, less is available. A study showed when bile was removed by ligation of the bile duct there was a 50-60% reduction in renal and hepatic uptake of B12 from the intestinal lumen which was restored once bile was replaced, therefore, suggesting that bile plays a part in the normal absorption of B12 (Teo, Scott, Neale and Weir, 1980). The Pernicious Anaemia Society webpage (PAS, 2024) has anecdotal patient reports that B12 deficiency is found in patients that have undergone appendectomy, although the mechanism is unclear, and no published evidence was found.

Serum folate deficiency has the same clinical features, FBC results and blood film findings as B12 deficiency and the two deficiencies not infrequently co-exist. For the study cohort 8% of patients were both folate and B12 deficient using the serum folate deficiency cut-off of <3.5 ng/mL in use at the author's laboratory at the time of the study (RR 3.5-20.5 ng/mL). The Spearman Rank test confirmed a negative correlation between folate and MMA concentrations. Many causes of folate deficiency are due to dietary factors however, the methyl trap hypothesis can explain an interaction in which B12 deficiency leads to lower levels of methionine synthase which results in a functional folate deficiency by trapping an increased amount of folate as the 5-methyl form, but this in itself is not sufficient to cause folate deficiency (Shane and Stokstad, 1985). It is essential that both B12 and folate concentrations

are measured before B12 treatment is commenced as treatment of folate deficiency in patients with co-existing B12 deficiency can cause irreversible neurological damage such as subacute degeneration of the cord (Langan and Goodbred, 2017).

Spearman rank correlation, in this study, showed a positive association between MMA and TSH. B12 deficiency (pernicious anaemia) has been reported to be associated with thyroid disease especially autoimmune thyroiditis (Hashimoto's). Benites-Zapata et al (2023) undertook a systematic review and meta-analysis of B12 levels in thyroid disorders and found that hypothyroid patients had lower levels of B12 than healthy individuals however this was not seen in hyperthyroid patients. Collins and Pawlak (2016) reported the prevalence of B12 deficiency in hypothyroidism as 10, 18.6 and 40.5% in 3 separate studies. There have been no reported studies of prevalence of thyroid disease in B12 deficiency. Elevated levels of anti-thyroid peroxidase antibodies have been seen in patients with B12 deficiency (Gupta, Choudhary and Chatterjee, 2023). There appears to be an association between hypothyroidism and B12 deficiency which has multifactorial causes such as alterations in the gut microbiota, bacterial overgrowth and slow intestinal motility but has been largely attributed to the autoimmune nature of thyroid disease (AITD), in which other autoimmune disorders are common such as autoimmune atrophic gastritis with increased levels of anti-parietal cell antibodies (APCA) reducing B12 absorption. The frequency of APCA in AITD has been reported to be as high as 27% (Benites-Zapata et al, 2023).

Spearman Rank correlation indicated a strong negative association between MMA and eGFR, and the MMA concentrations were significantly different, when assessed by one-way ANOVA, between the group with an eGFR <60 mL/min/1.73m^2 and the group with an eGFR ≥60 mL/min/1.73m^2, although TB12 and Holo-TC were not. Although the data was not normally distributed one-way ANOVA can be used as the test of statistical significance in this case because the sample size is ≥ 80 (Sainani, 2012). This is advantageous as ANOVA is more sensitive to changes than non-parametric tests of significance.

An association between MMA and renal impairment has previously been reported however the mechanisms are not clear, some authors report that raised MMA itself damages the kidney, or the kidney damage increases the MMA concentration due to accumulation (Ganji and Kafai, 2018). MMA accumulation influences mitochondrial dysfunction with reactive oxygen species generated causing the development and progression of various kidney diseases (Wu et al, 2023).When investigating the effect of MMA concentration, TB12 levels and renal function on mortality, Riphagen et al (2020) concluded that only some of the variation in MMA levels was explained by eGFR and speculated that the disparity was attributed to catabolism, diet or bacterial overgrowth. A limitation of the study was using an eGFR cut-off of 60 mL/min as a surrogate for chronic renal impairment as patients with an eGFR in the range of 60-89 mL/min are classified as mild chronic kidney disease (with potential increase in MMA) and the guidelines suggest using the albumin to creatinine ratio in addition to identify patients with albuminuria (Levey et al, 2009). Creatinine results were collected however albumin was not.

There was no statistical difference in TB12, Holo-TC and MMA between the groups with and without diabetes. A limitation of this analysis is that patients on metformin were not excluded. There is no published literature on diabetes and B12 deficiency when metformin has been excluded however there is evidence that B12 deficiency can promote the development of diabetes (Neal et al, 2023).

A strong link between B12 deficiency and the use of metformin has been extensively reported in the literature (Kim et al, 2019). A high percentage (33%) of diabetics on metformin in their study population were B12 deficient which is consistent with the prevalence of B12 deficiency in type 2 diabetes reported as 6-33% (Kibirige and Mwebaze, 2013). Metformin blocks B12 absorption, the mechanisms are not fully understood but interference of the calcium dependent binding of intrinsic factor/B12 complex to the cubam receptor in the terminal ileum has been proposed (Bell, 2022). The cause may be multifactorial, including altered intestinal motility, bacterial overgrown and reduced uptake of B12 in the small intestine (MHRA, 2022). The MHRA recommends that patients who are on long term or high dose metformin should be screened periodically if they present with B12 deficiency symptoms or are at higher risk of B12 deficiency and treated accordingly. Identifying B12 deficiency symptoms can be difficult for clinicians as neuropathy is a known feature of diabetes and most patients are therefore, screened annually.

The use of PPI's was high in the study cohort (25%) with 57% of patients with B12 deficiency on PPI's as compared to 42% B12 replete patients. PPIs cause B12 deficiency by inhibiting the ATPase K^+/H^+ proton pump, reducing the number of hydrogen ions released into the stomach and decreasing the production of gastric acid. Since gastric acid is required to free protein bound B12 for absorption this is a likely mechanism for B12 deficiency however, bacterial overgrowth in the digestive tract has also been suggested (Heidelbaugh, 2013, Mumtaz et al, 2022). Some patients were on PPI's following GI surgery or for conditions that may in themselves have resulted in B12 deficiency.

The numbers of patients on OCP and HRT were too low, in this study, to draw any conclusions about the association of these drugs with B12 deficiency. Berenson and Rahman (2012) found decreased B12 levels in those using OCP or HRT, but they did not cause deficiency.

When using MMA as the proxy 'gold' standard test there was very little difference in the AUC (95% CI) from the ROC analysis when using TB12 and Holo-TC as tests of B12 deficiency 0.749 (0.717-0.782) and 0.779 (0.749- 0.810), respectively. These findings were corroborated by other studies (Clarke et al, 2007a, Al Alsari et al, 2010, Carmel, 2011, Golding, 2016b) but not by others (Herrmann et al, 2003, 2005, Hvas and Nexø, 2005, Čabarkapa et al, 2007, Obeid and Herrmann, 2007, Nexø and Hoffmann-Lucke, 2011, Heil et al, 2012, Bondu et al 2020) who found Holo-TC to have superior DA to TB12. The differences seen between studies in the diagnostic accuracy of Holo-TC and TB12 for B12 deficiency is likely to be multifactorial and

dependant on which biomarker was used as the ‘gold’ standard test to confirm B12 deficiency; the size and demographics of the patient cohort including age, sex and ethnicity; the inclusion or exclusion of patients with known renal dysfunction or on medications affecting B12 metabolism; inclusion or exclusion of patients on B12 supplements/treatment; the cut-off values used to identify deficiency and indeterminate results and the impact of assay differences on the suitability of these cut-offs. This is why it is difficult to compare studies unless the same criteria are used.

A significant limitation of the study was the lack of adjustment for eGFR which has a strong association with MMA, the proxy ‘gold’ standard test of B12 deficiency used in this study. The patients were classified solely on age related cut-off values for MMA. Further work may be required to address this when the new algorithm is implemented.

Another limitation of this study was the lack of information available on B12 replacement in B12 replete patients which may have affected the relationship between B12 status and qualitative factors such as sex, ethnicity, GI conditions and medication.

A further limitation of this study was that insufficient data were available on dietary preferences to enable meaningful conclusions. Dietary insufficiency is one of the causes for B12 deficiency, more patients are now vegetarian or vegan and a third of

the patient cohort was of Asian ethnicity and therefore, it would have been helpful to look at the association between B12 deficiency and diet in the patient cohort. There were more than three million vegetarians and vegans in the UK (4.5% of the population) according to the National Diet and Nutrition survey in 2018/2019 (Stewart et al, 2021). However, according to the YouGov market research website, in January 2025 the number of adult vegetarians in the UK is 6% and vegans 3%. The results for the Midlands, specifically are similar (6% and 2%, respectively). There are no sex differences for vegans but more vegetarians are female (8% versus 4%) (YouGov, 2025). Wolverhampton is a socioeconomically deprived area and ranks in the top 20 for highest deprivation for income and employment (Indices of Deprivation, Gov UK, 2024). There is evidence that multivitamin deficiencies are more prevalent in low socioeconomic status areas with diet quality the main cause (Zhu et al, 2020).

Reference ranges have been harmonised for many common biochemical tests (Berg and Lane, 2011) but not for TB12 or Holo-TC. Suppliers of TB12 and Holo-TC kits have their own deficient and indeterminate ranges even though standard cut-offs have been recommended by the BSH (Devalia, Hamilton and Molloy, 2014) and NICE (2023, 2024) for treatment of B12 deficiency. The BSH guideline reports that TB12 below 200 ng/L (148 pmol/L) should detect 97% of B12 deficient patients (Devalia, Hamilton and Molloy, 2014). This cut-off originated from a study undertaken by Lindenbaum et al (1990) using patients with confirmed pernicious anaemia and response to B12 treatment followed up for more than 10 years. The authors found that 90-95% of patients had B12 concentrations <200 ng/L, another 5-10% 200-300

ng/L and a small percentage had B12 results >300 ng/L. The assays used included a microbiologic lactobacillus assay and a radioimmunoassay which do not compare to the immunoassays used in today's laboratories. Furthermore, the assays were not standardised, and no ethnicity information was documented for the patients. Consequently, we are still using a cut-off value which no longer likely to be appropriate for the UK population (Lindenbaum et al, 1990, Snow, 1999).

NG239 (NICE, 2024) recommends TB12 and Holo-TC cut-offs of <180 ng/L and <25 pmol/L, respectively, be used for confirmed B12 deficiency. Holo-TC <25 pmol/L was used in this study. The Abbott Architect kit for TB12, used in this study, has a cut-off of 187 ng/L which is in between the two values of 180-200 ng/L. The patient cohort, in this study, was suspected of having B12 deficiency, therefore, it was not possible to directly establish deficient and intermediate cut-off concentrations for the tests of B12 deficiency. This would be a future area of research for the laboratory.

The B12 testing algorithm in use at the Royal Wolverhampton NHS Trust at the start of this study utilised TB12 only with deficient and indeterminate cut-off values. For this algorithm, an assumption was made that all indeterminates were true negatives which is a limitation of the study as the patients were a mixture of true positives and true negatives. The proposed new testing algorithm (section 1.4.5) uses the Holo-TC test on all patients suspected of having B12 deficiency and then for those patients in the indeterminate range use an MMA assay to confirm true deficiency.

Using the new algorithm there were improvements in DA, sensitivity, specificity, NPV and PPV. The current algorithm was generating up to 38% false positive results and 22% false negative results. Using the new algorithm will reduce the false positive rate to 5% and reduce the false negative patients to 12%. Use of the NICE Holo-TC indeterminate range (25-70 pmol/L) further reduces the false negative rate to 7%. A missed diagnosis of B12 deficiency is a concern, owing to the potential for development of neurological damage due to delayed treatment. Currently if a patient has an indeterminate TB12 result it is the clinician's decision to investigate further however if the new algorithm is implemented all indeterminate Holo-TC results would be confirmed using MMA, as the proxy 'gold' standard test. Many of the patients in this study had indeterminate Holo-TC results requiring MMA confirmation which would be more costly however would improve patient care. The health economics benefits of the new testing algorithm will be explored in chapter 6.

5.6 Conclusion

There was no difference in diagnostic accuracy between the TB12 and Holo-TC assays for B12 deficiency when used as individual screening tests at the deficient cut-off concentrations. The new algorithm (Holo-TC ± MMA) results in an improvement in the diagnostic accuracy for B12 deficiency over the regime in use at the beginning of this study. The diagnostic accuracy for B12 deficiency of the FBC parameters studied was poor and the percentage of patients with symptoms of B12 was not different between the B12 deficient and B12 replete groups, highlighting

their limitations as tests of B12 deficiency. At least some of the markers of B12 deficiency had associations with age, sex, ethnicity, TSH, eGFR and folate. The study findings on prescription drugs and other diseases supported the already published literature.

Chapter 6.0 RESULTS

Health economic analysis of the current testing algorithm using Total B12 (TB12) versus the new algorithm of Holotranscobalamin (Holo-TC) ± Methylmalonic Acid (MMA) including follow-up of patients one year after initial test

6.1 Background

Health economics in the NHS involves efficiency, effectiveness and gaining value in an environment with scarce resources. Cost-effectiveness analysis (CEA) is a fundamental part of the health technology assessment process and NICE (2013) recommend the use of cost-effectiveness analysis which measures not only the cost but also the effectiveness of any new technology, be it a new test, new drug or new procedure. This approach measures outcomes in terms of the length of life spent in good health and the quality of life known as quality adjusted life years (QALY).

Decision tree analysis was chosen for the CEA rather than Markov modelling as the decision was uncomplicated, the decisions and chance events were linked from left to right in the order in which they would occur and there was a finite ending either the patient has or does not have B12 deficiency. In Markov, the events are modelled as transitions from one health state to another over time and can be used to assess disease progression which was unnecessary for the research question. Patients

were followed up for one year following either an indeterminate or low TB12 result. Data recorded included additional testing performed and treatment received for B12 deficiency. These follow-ups were fully costed and included in the cost-effectiveness analysis.

A decision tree was designed and utilised to perform the CEA of proposed algorithm Holo-TC ± MMA compared to TB12 alone.

The model used patient health measured in terms of Quality Adjusted Life Years (QALY's). Results of the CEA were reported in terms of incremental cost per QALY expressed as the incremental cost-effectiveness ratio (ICER). EuroQol-5D (EQ-5D) is a generic but descriptive preference-based questionnaire in which patients are asked to rate 5 different dimensions of mobility, self-care, usual activities, pain and discomfort and anxiety and depression, these responses can be used to generate a health utility score. Other health utility systems exist however NICE (2013) recommend using EQ-5D if available to allow standardisation between studies. However, there was a lack of EQ-5D data for this area of research with only one relevant published article found in the literature.

Deterministic sensitivity analysis was performed on the model to evaluate how variation in model inputs affect the individual model outputs.

The majority of laboratories still utilise TB12 assays which are inherently much cheaper than Holo-TC assays, therefore a second CEA was undertaken and decision tree constructed with an additional branch of TB12 ± MMA as second line to see if this was more cost effective than TB12 alone and the Holo-TC ± MMA algorithm.

6.2 Aim and objectives

The aim of this work was to investigate if the new testing algorithm (Holo-TC ± MMA was cost effective compared to the existing algorithm (TB12). A secondary aim was to see if TB12 ± MMA was also a cost-effective option.

Objectives

1. To design a decision tree for the current and proposed new diagnostic testing algorithms for the identification of B12 deficiency.
2. To calculate the costs and utilities for each branch of the decision tree.
3. To perform baseline cost-effectiveness analysis.
4. To perform sensitivity analyses, by varying costs and utilities of the model.
5. To design a second decision tree to assess cost effectiveness if using a TB12 assay with confirmatory MMA assay (plus completion of objectives 2-4 for this decision tree).

6.3 Method

Methods of health economics analysis were described in section 2.6. A decision-tree model was developed using TreeAge Pro, 2024 software (TreeAge Software, Williamstown, MA, 2024) baseline and deterministic sensitivity analysis was performed for both decision trees.

6.4 Results

6.4.1 Decision tree development for B12 deficiency screening algorithms (current versus new)

The first point in the tree, the decision node, represents this decision question, for example ‘suspected B12 deficiency’. The pathways that follow each option represent a series of ordered alternative events, denoted by branches stemming from chance nodes. The alternatives at each chance node are mutually exclusive and their probabilities should sum exactly to one. The end points of each pathway are denoted by terminal nodes (triangular symbols) to which values or pay-offs, e.g. costs and health utilities are assigned. Once the probabilities and pay-offs have been entered, the decision tree is rolled back, allowing the expected values of each option to be calculated (Petrou and Gray, 2011). Figure 6.1 outlines the decision tree model designed for CEA of the current B12 diagnostic testing algorithm using TB12 and the new algorithm using a combination of Holo-TC and MMA testing. With the current algorithm the indeterminate patients are not further categorised but the advantage of

the new algorithm using the combination of Holo-TC and MMA gives a definite classification of B12 deficiency (assumed 100% accuracy). The baseline model includes the laboratory (manufacturer derived) cut-off concentrations for TB12 at the deficient (<187 ng/L), indeterminate (188-300 ng/L), and replete status(>300 ng/L); for Holo-TC (chapter 3 Holo-TC verification) deficient (<25 pmol/L), indeterminate (25-50 pmol/L), replete status (>50 pmol/L) and MMA (chapter 4 MMA verification) age adjusted ranges of deficient >280 nmol/L (if ≤65 years), >360 nmol/L (if >65 years) and replete status <280 nmol/L (if ≤65 years), <360 nmol/L (if >65 years).

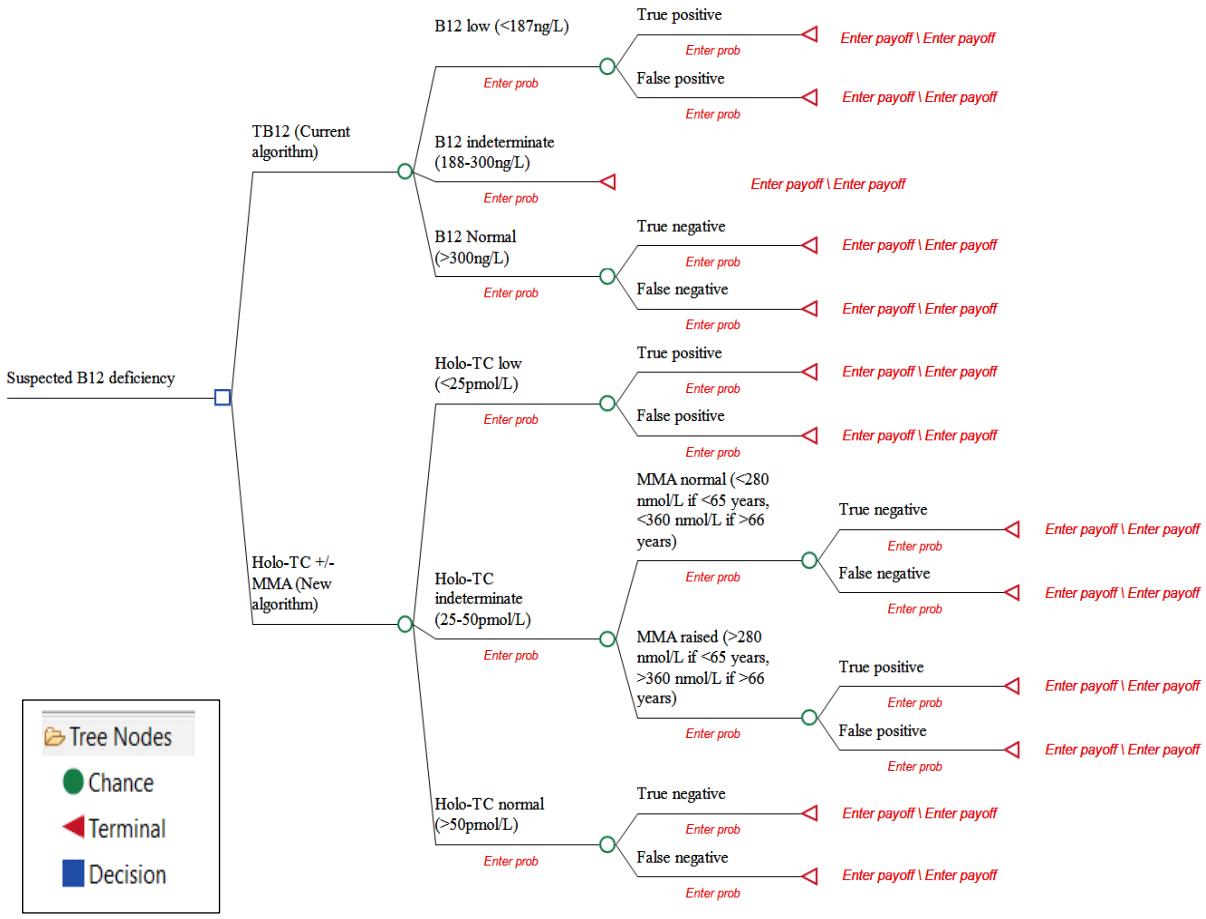


Figure 6.1. Decision tree model used for health economics analysis showing current algorithm of TB12 versus the proposed new algorithm of Holo-TC ± MMA. The tree node key details the symbols used in the tree.

6.4.2 Calculation of costs and utilities for branches of B12 algorithm decision trees

The model utilised costs from The Royal Wolverhampton NHS Trust (RWT) finance department for laboratory tests already in place and calculated test costs for the new MMA assay. Staffing costs for GP appointments, nurse-led treatment appointments and phlebotomy appointments were taken from the latest update of personal social services research unit data (PSSRU, 2023) and treatment costs from the British National Formulary (BNF, NICE, 2024) with patient health measured in terms of QALY's. The expected costs and QALY's incurred in the one year follow-up period after the initial TB12 test, based on diagnostic test accuracy, were calculated. Costs and QALY payoffs were assigned based on the decision tree pathway followed.

The health care costs included:

1) the costs of conducting the tests
    a. equipment/consumables.
    b. staff resource.
    c. overheads.
2) Follow-up for 1 year
    a. Repeat/additional test costs including phlebotomy costs and additional GP appointments.
    b. Treatment costs including therapy and nurse appointments for Intramuscular injections.

The TB12 and Holo-TC tests were performed on analytical platforms already available in the laboratory at RWT, details of cost per test, staffing and overheads were obtained from the RWT finance department for these and the follow-up tests performed.

The new MMA assay was a manual test with various grades of staff involved in the test process: an assistant practitioner (Agenda for change (AfC) Band 4) for sample preparation and test conducted by a biomedical scientist (AfC Band 6). After completion of the test, the results were authorised by a clinical scientist (AfC Band 8a) and released for review. The staff costs for the MMA assay were taken from PSSRU 2023 data, Band 4 £34 per hour, Band 6 £50 per hour, Band 8a £69 per hour. The MMA cost per test calculation was based on running a daily batch of patient samples Monday to Friday (79 samples), 20,000 samples pa (Table 6.1).

As the laboratory did not have a verified MMA assay, any requests in the follow-up period were sent to a referral laboratory with their charge for MMA test utilised (£30.12).

Table 6.1. Cost per test calculation for MMA assay based on running 1 batch per weekday with an annual workload of 20000 tests.

Costed items	**MMA**
Reagents/consumables cost per sample (£)	3.06
IQC/EQA costs per sample (£)	0.03
Mass Spectrometer depreciation cost per sample (£)	2.57
Equipment maintenance cost per sample (£)	1.55
Overhead costs per sample (£)	0.69
MMA assay verification cost per sample	0.01
Staff cost for sample preparation batch samples (B4 AP 90 minutes) per test (£)	0.57
Staff cost for setup, testing and interpreting of results (B6 BMS 90 minutes) per test (£)	0.83
Staff cost for result batch authorisation (B8a CS 30 minutes) per test (£)	0.38
TOTAL COST /TEST (£) =	**9.69**

B = band, AP = Associate Practitioner, BMS = Biomedical Scientist, CS = Clinical Scientist.

Table 6.2 depicts the mean cost per test for each algorithm, £0.71 for the current TB12 and £10.40 for the combination of Holo-TC ± MMA algorithm, used in the baseline model. The one year follow-up data of costs for further tests performed and treatment costs for both the deficient and indeterminate patients by TB12 were collected. Not all patients had further tests or treatment. Of the 104 deficient patients, 83 had further tests and 84 had treatment (45 Intramuscular (IM) B12, 31 oral B12 and 8 both forms). Of the 412 indeterminate patients, 285 had further tests and 128 treatment (33 IM B12, 88 oral B12 and 7 both forms). The mean of these costs (Table 6.3) were calculated and were used as the cost payoffs in the model

(Figure 6.2). Table 6.4 shows itemised costs used to calculate mean costs for additional testing and treatment.

Table 6.2. Baseline mean test cost for current and new algorithms.

	Current algorithm (TB12) £	New algorithm Holo-TC ± MMA £
Mean test cost per patient	0.71	10.40

Table 6.3. Baseline and upper (mean +2SD) additional costs of tests and treatment in 1 year follow-up period for both deficient and indeterminate patients by TB12.

1 year follow-up	Mean cost for deficient patients £	Mean cost for indeterminate patients £
Additional tests	71.95	61.41
Treatment (Oral and IM)	87.61	34.96
Total per patient	159.57	96.36
Upper (mean +2SD) costs for sensitivity analysis	394.21	336.78

Table 6.4. Itemised costs used to calculate mean costs for additional testing and B12 treatment in follow-up period and source of data.

Follow- up additional test and treatment items	Cost	Source
Ten minute GP appointment	£56.00	PSSRU, 2023 data
Phlebotomist (B4) £34 per hour 10-minute appointment for bloods	£6.00	PSSRU, 2023 data
Full Blood Count (FBC) full costs	£1.99	RWT finance department
Urea and Electrolytes (U+E) full costs	£1.75	RWT finance department
Total B12 (TB12) full costs	£0.71	RWT finance department
Holotranscobalamin (Holo-TC) full costs	£0.71	RWT finance department
Serum Folate	£0.69	RWT finance department
Intrinsic Factor Antibodies (IF) full costs	£7.64	RWT finance department
Gastric Parietal Cell Antibodies (GPC) full costs	£7.10	RWT finance department
Methylmalonic Acid (MMA) full costs	£30.12	Nutristasis Unit, St Thomas' Hospital price
Coeliac screen (IgA-TTG) full costs	£5.88	RWT finance department
Nurse (B5) £53 per hour for B12 IM injection 10-minute appointment	£8.83	PSSRU, 2023 data
IM B12 injection 1 mg/mL cost per ampoule (Hydroxocobalamin)	£2.07	British National Formulary (BNF. NICE), 2024
Oral B12 cost per 50 mcg tablet (Cyanocobalamin)	£0.18	British National Formulary (BNF. NICE), 2024
Oral B12 cost per 1000 mcg tablet (Cyanocobalamin- Orobalin)	£0.33	British National Formulary (BNF. NICE), 2024

Full costs = reagents, consumables, maintenance, staffing and overheads

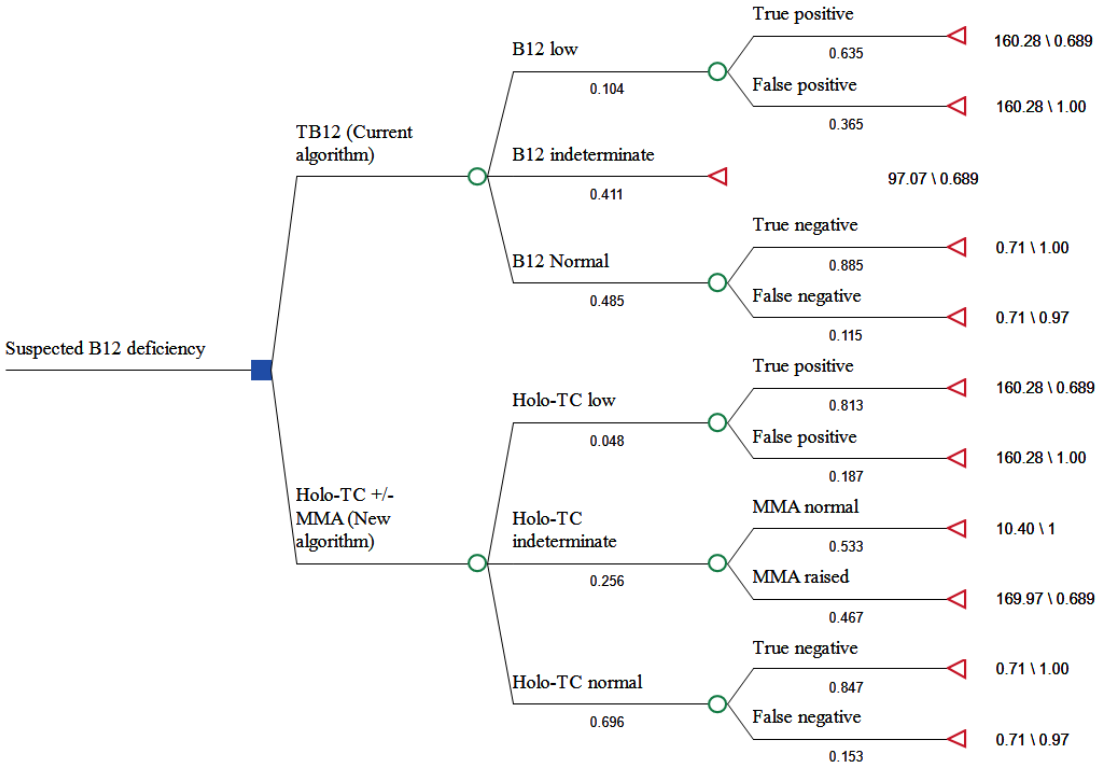


Figure 6.2. Decision tree – baseline with probabilities and pay-offs included.

The EuroQol-5 dimensions (EQ-5D) is a frequently used, preference-based tool for measuring the health utilities of patients in economic evaluations and is recommended by NICE (2013). Health utility values generated from the EQ-5D range from 0 (death) to 1 (perfect health). On review of the literature there was a paucity of data on B12 deficiency with only one relevant article available. Mnatzaganian et al (2015) published a report on patients with B12 deficiency and symptoms of fatigue that included an EQ-5D score of 11222 at baseline. Although fatigue is one of the many non-specific symptoms that occur in B12 deficiency it featured highly in the study cohort, being the top reported symptom by 55% of the patients, justifying the use of this data for the true positive (TP) patients. EQ-5D

value sets (Szende, Oppe and Devlin, 2007) was used to calculate the health utilities. In EQ-5D-3L, each of the five dimensions is scored and has three levels, no problems, some problems and severe problems (with 3 being severe problems). For good health, the score is 11111. The values sets' list the disutility for each dimension that is not scored as 1. The UK value sets score of 11222 for patients with B12 deficiency was 0.311. The utility score is calculated by subtracting the UK values sets disutility score from 1 (the score for perfect health) giving a utility of 0.689 (Table 6.5).

Table 6.5. EQ-5D calculation of utility from UK values sets for B12 deficiency. Value sets taken from Szende, Oppe and Devlin (2007). Utility score calculated by subtracting total disutility score from 1 (perfect health score).

EQ-5D (5 dimensions)	Score 1-3 (reason for score)	UK Value Sets Disutility scores	Utility score 1 minus the Total value set score
Mobility	1 (possible gait problems in some)	0	**0.689**
Self-Care	1 (not an issue)	0	
Usual Activities	2 (high levels of fatigue)	-0.036	
Pain/discomfort	2 (aches and pains common)	-0.123	
Anxiety/depression	2 (if neurological issues present)	-0.071	
	At least one 2 score	-0.081	
	Total value set score	**-0.311**	

True negative (TN) had a utility of 1.0 as they had no deficiency. Utilities for the false positive (FP) and false negative (FN) states were more difficult to calculate. Mnatzaganian in their article did not add a disutility for FP cases as they suggested

treatment with B12 was harmless. When NICE undertook their recent update to B12 guidelines NG239 (2024) they agreed that FP patients would not be affected by B12 treatment and the patients' underlying ailment would naturally resolve in 3 months. Therefore, there was no disutility associated with a FP result.

The disutility for patients with FN was more problematic. Mnatzaganian again did not add any disutility for a FN result, however included an additional charge for a further GP appointment. NICE assumed these patients were TP but not treated and so their utility would not improve over time and assigned a heath utility value of 0.689. A literature review yielded no studies identifying health utilities associated with a missed diagnosis of B12 deficiency. However, NICE DG50 reviewed missed diagnosis of non-alcoholic fatty liver disease (NAFLD) (NICE, 2023a) in which a disutility of -0.03 was used. The rationale for this was that patients would likely re-present within 6 months if symptoms persisted or worsened, with repeat tests performed and correct diagnosis made. Similarities could be drawn with B12 deficiency and therefore a disutility of -0.03 was assigned to FN patients in the baseline model.

The underlying prevalence of B12 deficiency in the study cohort by MMA was 26.3% (chapter 5) and diagnostic accuracy was calculated from the study results of the 1003 patients using functional MMA assay as proxy 'gold' standard assuming 100% accuracy generating the proportions of TP, TN, FP and FN patients.

The following were assumptions of the baseline model :-

I. Patients classed as not B12 deficient (TN) have no adverse health outcomes.
II. There are no adverse health effects of a FP result.
III. The MMA test is 100% accurate

6.4.3 Cost-effectiveness analysis results for B12 deficiency diagnosis using decision tree comparing current TB12 with Holo-TC ± MMA algorithm

The results of the model are reported as the incremental cost-effectiveness ratio (ICER) which is calculated by dividing the difference in costs between the two arms by the difference in health utilities (QALY's) between the two arms. The baseline analysis is depicted in Figure 6.3, the decision tree highlights the optimal strategy offering improved cost-effectiveness, at baseline the Holo-TC ± MMA algorithm was more cost effective than the TB12 algorithm with an incremental cost of ~£27 and incremental effect (QALY) of ~0.1 with an ICER of -276.68.

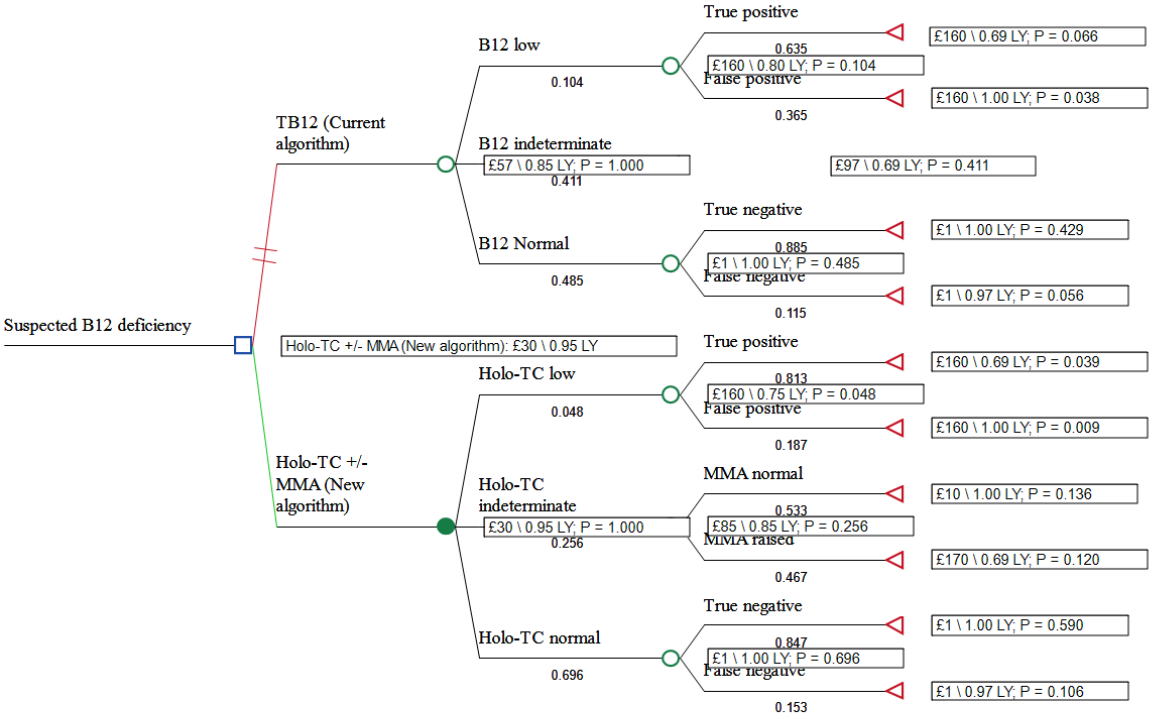


Figure 6.3. Decision tree baseline analysis exhibiting the new algorithm Holo-TC ± MMA is the optimal strategy and offers better cost-effectiveness (highlighted by green branch connector and chance node) than the alternative current algorithm TB12 (red branch connector with a double strike through marker) which is rejected.

To account for variation in model inputs different plausible ‘scenarios’ in which costs, health utilities and diagnostic accuracy were amended, were assessed (Table 6.6). The decision tree was reanalysed following input of alternative cost and QALY data.

Adjustment of the cut-off values for Holo-TC indeterminate (25-70 pmol/L) patients as recommended in the new NICE guidance (NG239) (NICE, 2024), was assessed (scenario 2). This affected the diagnostic accuracy of the algorithm. The results show that Holo-TC ± MMA was more cost-effective than TB12 with ICER -181.16 but less favourable than the baseline model.

Adjusting the costs to the upper limit +2SD (scenario 3). This scenario gave the highest ICER of -1137 between the two algorithms.

Scenario 4 changed the cost of the MMA assay to that used in NICE's cost-effectiveness analysis as most laboratories will not have their own assay and would need to send away the indeterminate patients for testing. This analysis gave an ICER of -222.3.

The increased costs of Holo-TC (NICE figure £18.50 per Holo-TC, and MMA £30.40) at the laboratories Holo-TC cut off for indeterminates of 25-50 pmol/L (scenario 5) was still more cost-effective for Holo-TC ± MMA however only just with an IC of £3.89 and IE of ~-0.1 giving an ICER of just -39.89.

Then Holo-TC cut-off of 25-70 pmol/L as per NICE (scenario 6) utilising the same costs were reanalysed. This scenario gave a positive ICER. In this scenario the Holo-TC ± MMA algorithm was more expensive (IC = £13.40) than TB12 however there was still a health benefit seen (IE = 0.079) giving an ICER of 169.76 and Holo-TC ± MMA more cost-effective than TB12.

Scenario 7 looked at adjusting the utility used (0.689) for the FN patients giving them the same utility as TP patients. Holo-TC ± MMA was again the more cost-effective algorithm with an ICER of -324. The costs for both arms were the same however the health utility was better hence the higher ICER.

The final scenario investigated changed the utilities value of the indeterminate population in the current algorithm to 1.0 i.e. treat as TN patients, until now these patients had been treated as TP (0.689) however they will be a mixture of TP and TN. This was the only scenario in which the current TB12 algorithm was more cost-effective than the proposed new algorithm with an ICER of 890.50. The cost was higher in the TB12 arm however the health utility improved more.

Table 6.6. Baseline and sensitivity analysis showing cost-effectiveness results of the current (TB12) and new B12 diagnostic testing algorithms (Holo-TC ± MMA). The most cost-effective testing algorithm is highlighted in bold.

Scenario	Test	cost (£)	incremental cost (£) IC	QALY	incremental QALY IE	ICER = cost per QALY gained IC/IE	Net Monetary Benefit (NMB) at willingness to pay (WTP) £20000/QALY (£)	Conclusion
Scenario 1: Baseline analysis: Laboratory Holo-TC cut-off (25-50 pmol/L)	TB12	56.91		0.850			16942.44	**Holo-TC ± MMA** more cost effective as cheaper and offers QALY benefit
	Holo-TC ± MMA	29.93	26.98	0.947	-0.098	-276.68	**18919.84**	
Scenario 2: Sensitivity analysis: change to Holo-TC cut-off 25-70 pmol/L NICE recommendations	TB12	56.91		0.850			16942.44	**Holo-TC ± MMA** more cost effective as cheaper and offers QALY benefit
	Holo-TC ± MMA	42.60	14.31	0.929	-0.079	-181.16	**18536.00**	
Scenario 3: Sensitivity analysis: change to Upper +2SD costs for study for additional tests and treatment	TB12	180.12		0.850			16819.22	**Holo-TC ± MMA** more cost effective as cheaper and offers QALY benefit
	Holo-TC ± MMA	69.24	110.88	0.947	-0.098	-1137.02	**18880.52**	
Scenario 4: Sensitivity analysis: change in cost of MMA assay to NICE figure £30.40	TB12	56.91		0.850			16942.44	**Holo-TC ± MMA** more cost effective as cheaper and offers QALY benefit
	Holo-TC ± MMA	35.23	21.68	0.947	-0.098	-222.32	**18914.54**	
Scenario 5: Sensitivity analysis: change in cost of Holo-TC assay to NICE figure £18.50 using laboratory Holo-TC cut-off (25-50 pmol/L), MMA cost £30.40	TB12	56.91		0.850			16942.44	**Holo-TC ± MMA** more cost effective as cheaper and offers QALY benefit
	Holo-TC ± MMA	53.02	3.89	0.947	-0.098	-39.89	**18896.75**	
Scenario 6: Sensitivity analysis: change in cost of Holo-TC assay to NICE figure £18.50 using NICE Holo-TC cut-off 25-70 pmol/L, MMA cost £30.40	TB12	56.91		0.850			16942.44	**Holo-TC ± MMA** more cost effective even though more expensive still offers QALY benefit.
	Holo-TC ± MMA	70.31	13.41	0.929	0.079	169.76	**18508.30**	
Scenario 7: Sensitivity analysis: change in utilities for FN cases to utilities used by NICE (0.689)	TB12	56.91		0.834			16628.98	**Holo-TC ± MMA** more cost effective as cheaper and offers QALY benefit
	Holo-TC ± MMA	29.93	26.98	0.918	-0.083	-324.03	18321.38	
Scenario 8: Sensitivity analysis: change in utilities for Indeterminate patients in TB12 algorithm to 1.0 TN (rather than 0.689 TP)	TB12	56.91		0.978			**19498.86**	**TB12** more cost effective though more expensive but offers QALY benefit.
	Holo-TC ± MMA	29.93	26.98	0.947	0.030	890.50	18919.84	

6.4.4 Decision tree development for a second CEA for TB12 ± MMA versus TB12 alone and Holo-TC ± MMA algorithm and results of analysis.

For this decision tree an additional branch was added to the original tree to directly compare TB12 alone, Holo-TC ± MMA and TB12 ± MMA for cost effectiveness as many laboratories do not have access to the Holo-TC assay. For the baseline model, the same costs and utilities were used as before (scenario 1). The cost for the TB12 and Holo-TC assays, at the author's laboratory, were the same (£0.71), and the cost of the MMA assay was £9.69. The proportion of the cohort with an indeterminate result was the only change between decision tree branches using TB12 and Holo-TC, respectively. The second decision tree baseline analysis (Figure 6.4) shows that the new algorithm is still the optimal strategy and offers better cost-effectiveness than the alternatives TB12 ± MMA or TB12 alone.

Holo-TC ± MMA is more cost effective as the algorithm is significantly cheaper than TB12 ± MMA (IC £14.32, IE -0.014, ICER -991.54) or B12 alone (IC £26.98, IE -0.098, ICER -276.68) and offers higher QALY benefit with an NMB of £18919.84.

As before to account for variation in model inputs, sensitivity analysis was undertaken with two different amendments made and CEA reanalysed (Table 6.7).

Scenario 2 changed the costs per test for those utilised in NG239 (Holo-TC £18.50 and MMA £30.40) with TB12 assay cost remaining the same, the indeterminate cut-

off for Holo-TC was unchanged at 25-50 pmol/L. In this scenario Holo-TC ± MMA was still more cost effective overall than the alternatives TB12 ± MMA and TB12 alone. Although in this case the Holo-TC ± MMA algorithm was more expensive (IC £8.78) giving a positive ICER of 607.92. The QALY benefit (IE 0.014) was still higher with an NMB of £18896.75. TB12 alone had an IC £3.89, IE -0.098, and ICER -39.90.

The second scenario utilised the same NICE costings however changed the Holo-TC indeterminate range to that stated in NG239 (25-70 pmol/L). In this scenario (Figure 6.5) the sensitivity analysis shows the alternative TB12 ± MMA algorithm offers better cost-effectiveness than TB12 alone or the Holo-TC ± MMA algorithm. TB12 ± MMA is more cost effective as the algorithm is significantly cheaper than Holo-TC ± MMA (IC £26.07, IE -0.004, ICER -6327.72) and B12 alone (IC £12.67, IE -0.083, ICER -152.46) and offers QALY benefit with an NMB of £18616.78.

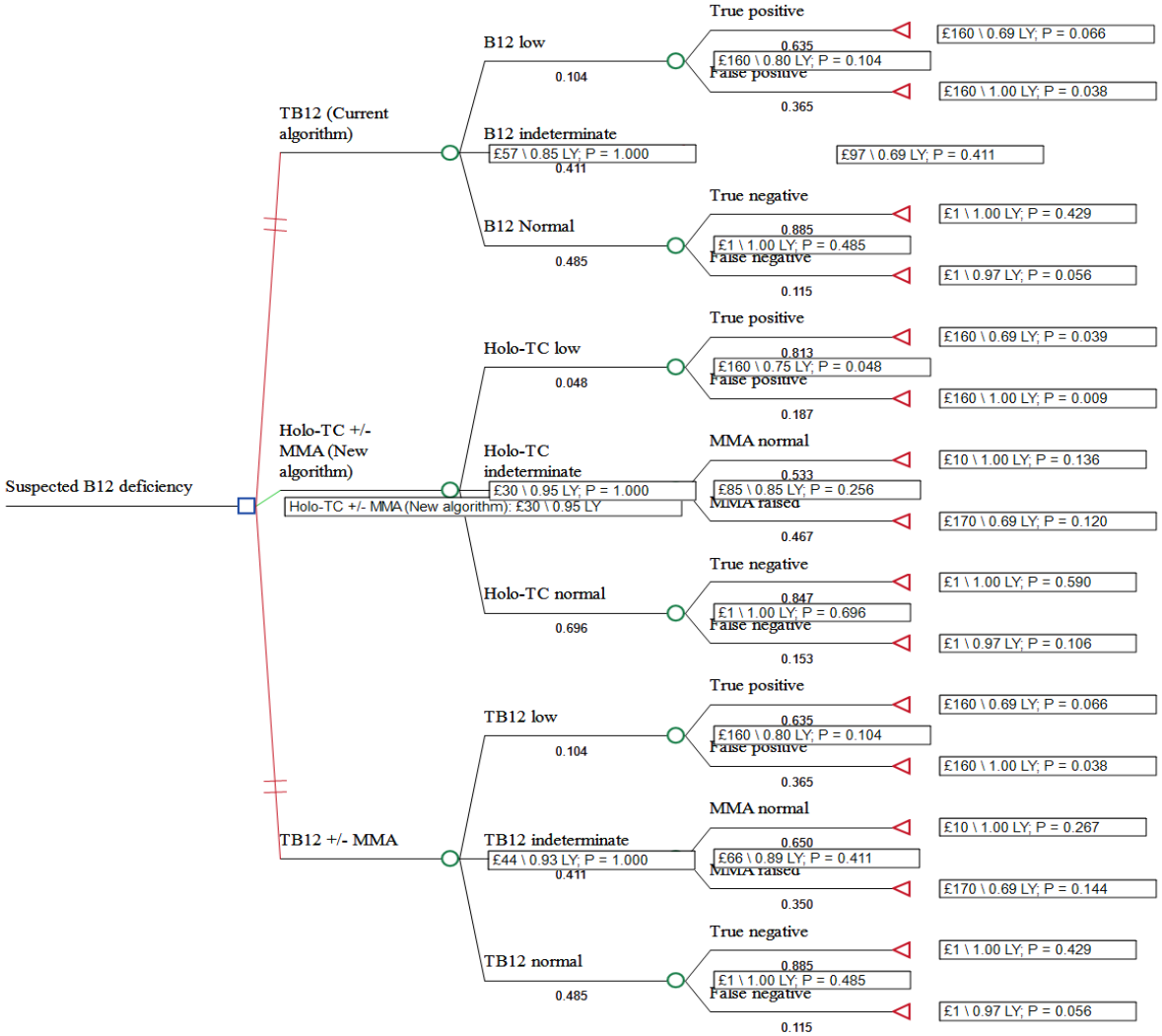


Figure 6.4. Second decision tree baseline analysis showing that the new algorithm offers better cost-effectiveness than the alternatives TB12 alone or TB12 ± MMA (red branch connector with a double strike through marker) which are rejected.

Table 6.7. Baseline and sensitivity analysis showing cost-effectiveness results of the current (TB12), new B12 diagnostic testing algorithms (Holo-TC ± MMA) and alternative TB12 ± MMA. The most cost-effective testing algorithm is highlighted in bold.

Scenario	Test	cost (£)	incremental cost (£) IC	QALY	incremental QALY IE	ICER = cost per QALY gained IC/IE	Net Monetary Benefit (NMB) at willingness to pay (WTP) £20000/QALY (£)	Conclusion
Scenario 1: **Baseline analysis: Laboratory Holo-TC cut-off (25-50 pmol/L) Using laboratory costs for Holo-TC and MMA.**	TB12	56.91	26.98	0.850	-0.098	-276.68	16942.44	**Holo-TC ± MMA** more cost effective as significantly cheaper than TB12 ± MMA and B12 alone and offers higher QALY benefit.
	Holo-TC ± MMA	29.93		0.948			**18919.84**	
	TB12 ± MMA	44.24	14.32	0.933	-0.014	-991.54	18616.78	
Scenario 2: **Sensitivity analysis: change in cost of Holo-TC assay to NICE figure £18.50 using laboratory Holo-TC cut-off (25-50 pmol/L), MMA cost £30.40**	TB12	56.91	3.89	0.850	-0.098	-39.90	16942.44	**Holo-TC ± MMA** more cost effective although more expensive than TB12 ± MMA and TB12 alone as offers more QALY benefit.
	Holo-TC ±MMA	53.02	8.78	0.948	0.014	607.92	**18896.75**	
	TB12 ±MMA	44.24		0.933			18616.78	
Scenario 3: **Sensitivity analysis: change in cost of Holo-TC assay to NICE figure £18.50 using NICE Holo-TC cut-off 25-70 pmol/L, MMA cost £30.40**	TB12	56.91	12.67	0.850	-0.083	-152.46	16942.44	**TB12 ± MMA** more cost effective as significantly cheaper than Holo-TC ± MMA and B12 alone and offers QALY benefit.
	Holo-TC ± MMA	70.31	26.07	0.929	-0.004	-6327.72	18508.30	
	TB12 ± MMA	44.24		0.933			**18616.78**	

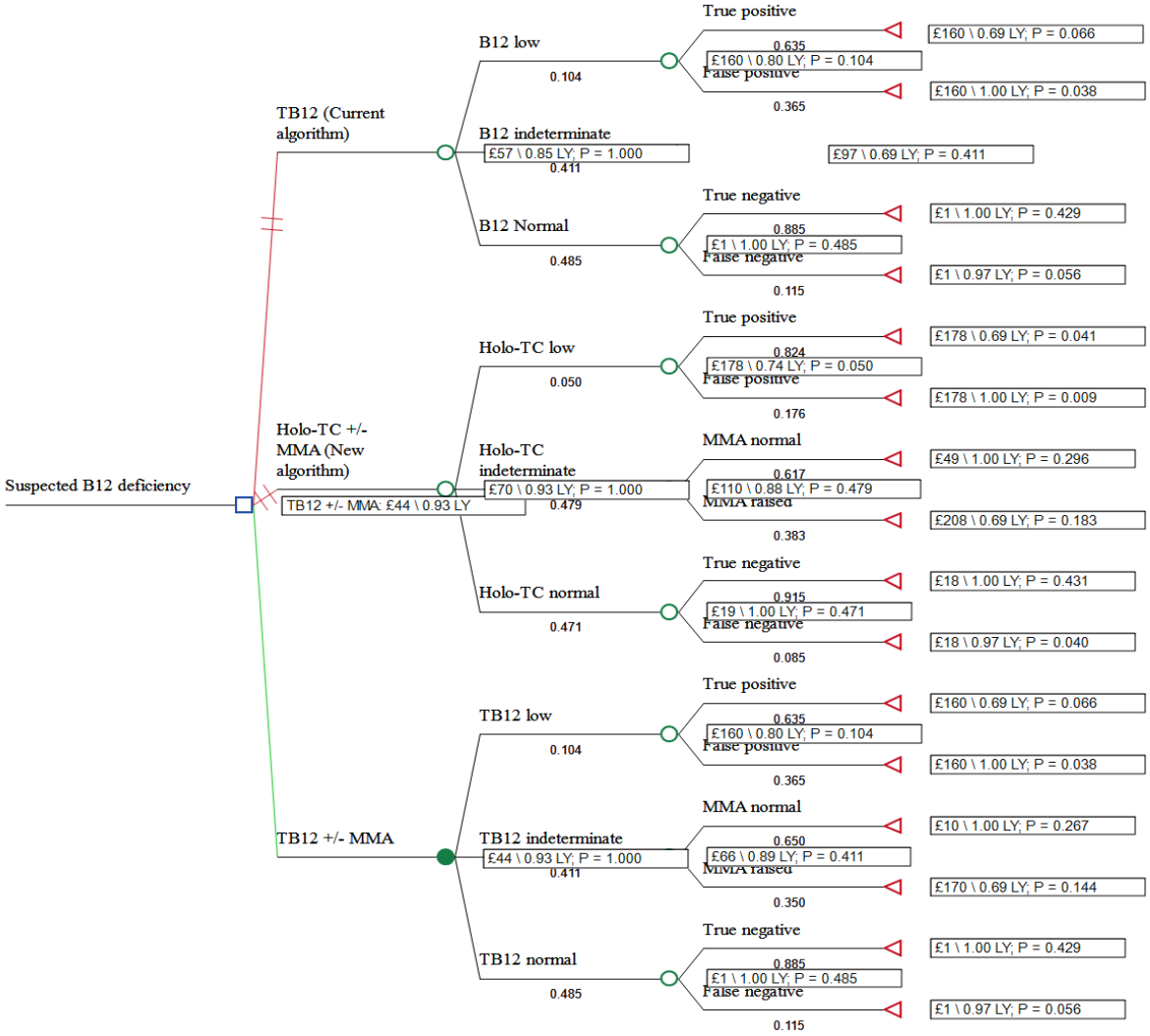


Figure 6.5. Sensitivity analysis exhibiting TB12 ± MMA (green branch connector and node) offers better cost-effectiveness than the alternative's TB12 alone or Holo-TC ± MMA (red branch connector with a double strike through marker) which are rejected.

6.5 Discussion

The baseline cost -effectiveness analysis showed the Holo-TC ± MMA algorithm was more cost effective than the TB12 algorithm with an incremental cost of ~£27 and incremental effect (QALY) of ~0.1 with an ICER of -276.68 showing cost savings per QALY gained.

When the cut-off value for Holo-TC indeterminate was adjusted to 25-70 pmol/L the new algorithm became less cost-effective, this is likely due to the higher numbers of indeterminate cases that would require MMA confirmation, and the costs associated with this algorithm. Adjusting the costs to the upper limit but using baseline data gave the highest ICER which is likely due to the high cost differences between the two arms. The standard deviation of the additional tests and treatment were high due to large variation in GP practice in the investigation and treatment of patients with B12 deficiency therefore the costs input to the model were extreme however proved to still be cost-effective. Using an increased MMA cost still proved to be cost-effective which is encouraging for laboratories that will not be able to confirm MMA in-house. Further increasing the cost per tests with the inclusion of the Holo-TC increased price, reduced the cost-effectiveness of the new algorithm considerably. There is a wide variation in prices laboratories pay for their assays and using Holo-TC may not be affordable for them. When the same cost for tests were utilised but using the recommended Holo-TC of 25-70 pmol/L, the ICER was positive showing that the new algorithm was more expensive however there was still a health benefit

meaning the new algorithm was more cost-effective than TB12. The ICER represents the additional spend required to gain an additional unit of health (QALY) in this scenario. The final two scenarios looked at adjusting the utilities rather than costs, when the FN utility was changed to that of a TP (0.689), the new algorithm was more cost effective as the costs for both arms were the same however the health utility was better hence the higher ICER. Changing the utilities of the indeterminate population in the current algorithm to treat as TN patients gave the only scenario in which the current TB12 algorithm was more cost-effective than the proposed new algorithm with a positive ICER. The cost was higher in the TB12 arm however the health utility improved more for the TB12 arm. The indeterminate group of the TB12 arm is not further classified into deficient or not deficient and it is unlikely that all indeterminates are TN therefore the benefits of this arm are likely to be overinflated.

The cost-effectiveness of Holo-TC ± MMA in the defined population was determined by whether the ICER fell above or below the willingness to pay (WTP) threshold which is currently defined by NICE as £20,000 per QALY gained (McCabe, Claxton and Culyer, 2012). The Holo-TC ± MMA algorithm was cost effective in the majority of the scenarios evaluated, with a negative ICER in most of cases meaning Holo-TC ± MMA could reduce costs when compared with the current algorithm. The net monetary benefit (NMB) was also calculated with a positive NMB indicating that Holo-TC ± MMA was cost-effective compared with current algorithm at the given WTP threshold, although all scenarios analysed gave similar NMB results.

When reviewing the literature there had been no similar cost-effectiveness analysis performed on B12 deficiency when this study began therefore a direct comparison of

results obtained was not possible. Mnatzaganian et al, (2015) looked specifically at fatigue in B12 deficiency and whether it was cheaper to test or treat all patients. Although the health utilities that they used were converted into EQ-5D information for this study these were not UK patients therefore the prevalence of B12 deficiency may not be comparable between the two study cohorts. They used 10% prevalence which was much lower than the local study population (26%). Prevalence is important in estimating the disease burden in a population and impacts both the positive and negative predictive values of diagnostic tests. The higher the prevalence the likelihood of higher costs for healthcare. In their study they concluded that treating all patients with oral B12 was more cost-effective than testing for deficiency. NICE have recently released NG239, B12 deficiency in over 16s: diagnosis and management (NICE, 2024) which also included a cost-effectiveness analysis, their CEA used MMA as second line confirmatory test for indeterminate B12 results (by either TB12 or Holo-TC) however their focus was on treatment outcomes and compared no test, no treatment against test for MMA and treat appropriately and no test, treat all patients (sensitivity analysis only). They modelled with improvements in health utilities changing every 3 months up to a year for patients treated. Their findings showed testing with MMA was cost-effective as a second line confirmatory test in B12 indeterminate patients.

NICE guideline NG239 (NICE, 2024) recommends that either TB12 or Holo-TC assays may be used as the initial screening test for B12 deficiency (in non-pregnant adults), because many laboratories only have access to TB12 assays. Furthermore, the DA of TB12 and Holo-TC for B12 deficiency, when used in isolation, was similar. Consequently, a second decision tree was constructed and analysed which included

a branch of TB12 ± MMA, as an alternative algorithm, to assess its cost effectiveness. The baseline analysis unsurprisingly found that using the author's data and laboratory costs (same price for TB12 and Holo-TC) the Holo-TC ± MMA algorithm was more cost effective than the alternatives with a negative ICER suggesting a cost saving. Similarly using the Holo-TC cut-off of 25-50 pmol/L but with the higher test costs that were used in the NICE study (Holo-TC £18.50 and MMA £30.40), the Holo-TC ± MMA algorithm was still more cost effective, with a health benefit to patients, however the ICER was positive suggesting an increase in costs would be required per QALY gained. Interestingly, when the Holo-TC cut-off was adjusted to the range recommended in NG239 (25-70 pmol/L), the CEA showed that the TB12 ± MMA algorithm was more cost effective than TB12 alone and the Holo-TC ± MMA algorithm, respectively as it was significantly cheaper whilst providing greater health benefits to patients by providing an earlier confirmed diagnosis. This is consistent with the findings of the study undertaken by NICE (NICE, 2024) and is reassuring for laboratories that are unable to afford Holo-TC testing.

The cost-effectiveness analysis had several limitations which are important to acknowledge when considering applicability of this model to other populations. The prevalence of B12 deficiency was 26.3% which was higher than historical published values. Prevalence is not well defined in the UK, Hunt, Harrington and Robinson (2014) defined B12 deficiency prevalence of between 6% in <60-year-olds and >20% in >60-year-olds however the source of this data is unclear. The evidence appears to come from studies of patients >65 years old with reported deficiency of between 5-10% (Clarke et al, 2004) and true deficiency in 6% (Clarke et al, 2007a) however the

quality of the data is questioned due to potential sample degradation before testing (frozen storage for >10 years). There appears to be no evidence for the 20% prevalence figure at this age cut-off (>20% was seen in >75-year-olds) or testing performed in subjects <60 years old or why <60, >60 years were chosen which generates uncertainty on these prevalence assumptions. NICE (2023) report 0.1% of the population has pernicious anaemia, with B12 deficiency prevalence of 1.9% in >60 year olds, 11% in vegans and many 'subclinical' patients have results in 'grey zone 200-300 ng/L' up to 15% of those aged 20-59 years and >20% if >60 years, others suggest higher prevalence figures of 26% of the population affected (Bailey et al, 2013) which is in keeping with the study findings. The reasons for the high prevalence in the local study may be due to the fact that the population was not normal with patients selected for the study on the basis of suspicion of B12 deficiency; the population potentially had a high percentage vegetarian diets due to patient ethnicity but also Wolverhampton has poor socioeconomical factors and high levels of deprivation meaning the patients diets might be poorer, so this was somewhat explained.

From the original literature review it was evident that there was no consensus definition of B12 deficiency nor harmonisation of cut-off values used which ranged from <157 ng/L (<116 pmol/L) (Bolann et al, 2000) to <310 ng/L (<229 pmol/L) (Campos et al, 2020) and assay manufacturers ranging from <145 ng/L (<107 pmol/L) (Beckman Coulter, 2023) to <232 ng/L (<171 pmol/L) (Roche Diagnostics, 2023). Similarly, many diagnostic accuracy studies to calculate sensitivity and specificity have used different B12 concentration cut-offs and different reference

standards making it exceedingly difficult to compare the studies undertaken. NICE (2024) did not undertake a meta-analysis as part of NG239 but a diagnostic review of the literature. They confirmed that, of 10 studies a variety of cut-offs were used for the index tests, different index tests were used, and no two studies used the same reference standard. Of the studies reviewed, the closest to the study cohort was by Heil et al (2012). Heil et al (2012) used a higher TB12 cut-off of <200 ng/L, lower Holo-TC cut-off of 21 pmol/L and MMA at higher cut-off of >450 nmol/L with a cohort of 360 patients however from the article it was not clear which test was used as gold standard. The cut-offs used were calculated from 100 blood donors. The prevalence of B12 deficiency was reported to be 13%, therefore significantly lower than the study prevalence. The sensitivity of TB12 was reported to be 0.53 (95% CI 0.38-0.68) and specificity 0.81 (95% CI 0.76-0.85) and for Holo-TC sensitivity was 0.64 (95% CI 0.49-0.77) and specificity 0.88 (95% CI 0.84-0.91). The sensitivity and specificity of the local study were higher for both TB12 and Holo-TC. The MMA cut-off used by Heil was also higher, if this cut-off had been used in the local study, then the prevalence would be similar (around 14%).

A limitation of the diagnostic accuracy data was that patients with renal disease were not removed from the cost-effectiveness analysis, the patients were classified using the age adjusted MMA cut-off ranges which would be how the algorithm would be utilised in the laboratory. This may have affected the FP rate. Adjusting the MMA concentration for the eGFR is an option to compensate for this limitation and could be undertaken as a future piece of work.

A further limitation associated with the cost-effectiveness analysis was related to the use of the MMA as the 'gold' standard test, as MMA may not accurately identify deficiency in patients >65 years of age with renal disease, bacterial overgrowth in the small intestines or when patients are dehydrated as falsely high levels of MMA are seen in these circumstances (NICE, 2015), however neither TB12 nor Holo-TC have been reported to be affected. Also, MMA increases with age and age adjusted reference ranges are required.

Subclinical B12 deficiency defined as B12 concentrations of between 200-300 ng/L (148-221 pmol/L) is very difficult to identify, is often asymptomatic and is approximately 10 times more common than clinical B12 deficiency and these patients would be included as FN's, however it is not possible to predict which asymptomatic patients will go on to become symptomatic (Hvas, Ellegaard and Nexø, 2001). Other than a disutility of -0.03 added to FN patients the costs associated with a missed diagnosis were not included in the model. These costs could include additional GP appointments for ongoing symptoms or for repeat blood tests, inappropriate or unnecessary investigations, secondary care referrals, delayed treatment, long-term complications of anaemia and neurological problems including dementia, sick days and loss of earnings. These costs are difficult to quantify. If the diagnostic assay used does not detect deficient patients', then the model will be more cost-effective as the patients will not be followed up for further tests or treatment so therefore cheaper. If no concurrent disutility is included the results of the model may be skewed. It is reasonable to assume that a negative result may lead to reduced monitoring. In this case the risk of FN results may be detrimental to patients with worsening outcomes however the assumption made was that patients

would re-present if symptoms had not resolved however damage may already have occurred. When the utility of these patients was adjusted to that of a true positive patient this improved the cost-effectiveness because no additional costs were associated with the result i.e. both arms the same. Analysis of the FN group would be an interesting area to focus on, in the future as there is a lack of research in this area.

Sensitivity analysis included changing the costs of the Holo-TC test and MMA test to those used in NG239 (NICE, 2024) as many laboratories would not have access to the specialist equipment and expertise and would have to send samples away for analysis. Holo-TC has previously been quoted as 4 times more expensive than TB12 (NICE, 2015) however the cost utilised in NG239 was £18.50 which is much higher than the cost reported by NICE MedTech innovation briefing. Different laboratories will pay varying amounts for their reagents with most under managed service contracts. When this study began the laboratory utilised the Abbott Architect TB12 assay however the laboratory's contract was re-tendered meaning the price of the assays were reviewed. The new contract provides Holo-TC for B12 screening. The cost therefore of the Holo-TC and TB12 used in the costings model was £0.71 each which was a lower price than would have been in place when the study started. Changes to the costs of MMA in the model to reflect variability in laboratory test costs reduced the cost-effectiveness of the Holo-TC ± MMA algorithm. When both the MMA and Holo-TC higher costs were modelled, there was only a small cost saving and potentially if further costs were added in such as postage or courier costs the algorithm may no longer be cost-effective.

The analysis confirms that the Holo-TC ± MMA algorithm is cost-effective (Figure 1.6, section 1.4.5) however the new algorithm would reflex all samples with an indeterminate Holo-TC, for an MMA which would have a significant operational impact on most laboratories. A recent local audit, in 2023 highlighted at RWT alone 28.6% of the 120000 B12 requests per annum were indeterminate (unpublished data). The NG239 (NICE, 2024) suggests 'consider' MMA testing in patients that have symptoms or signs of deficiency in addition to the indeterminate B12 result. Even though the CEA model is in favour of MMA it may not be feasible for laboratories to introduce the test due to the specialist equipment and expertise required to perform the test. The alternative is to send the samples to one of the few laboratories offering the test currently however they may not have the capacity to perform the national workload. Additional work could focus on an intelligent requesting system or risk-based system could be devised to incorporate the Holo-TC result, the FBC and the clinical details to select appropriate patients for MMA testing. Alternatively future analytical platforms that can automate mass spectrometry tests may become available.

Given that many laboratories are switching to Holo-TC as the first line test in place of TB12 the cost-effectiveness analysis should to be repeated with Holo-TC vs Holo-TC± MMA in the near future.

6.6 Conclusion

The Holo-TC ± MMA algorithm was cost effective compared to TB12 algorithm in most scenarios evaluated. The differences seen in each of the expected costs and QALY's were small. The local cut-off value for Holo-TC of 25-50 pmol/L was the most cost-effective scenario. Increasing the cut-off recommended in the recent NICE guidelines would increase the number of confirmatory MMA tests required and therefore reduce cost-effectiveness. The results suggest that a Holo-TC ± MMA based strategy leading to an increased diagnosis of true B12 deficiency is cost-effective. For patients this means earlier diagnosis, earlier treatment with improvement in symptoms such as fatigue and quality of life (QoL) and anaemia if present and reduced progression of neurological symptoms.

In addition, for laboratories unable to utilise the Holo-TC assay the second CEA also showed cost effectiveness when a TB12 ± MMA algorithm is used which was consistent with NG239 study (NICE, 2024).

Whilst QoL data on B12 deficiency is scarce, the health utilities assigned to the model were rationalised by the current evidence base and similar clinical scenarios. Further work could focus on the disutility of a missed diagnosis including the societal costs of repeat appointments, and emotional impact of delayed diagnoses. Referring to the original aim of the project, it can be concluded Holo-TC ± MMA combination was more cost-effective than TB12 for the diagnosis/exclusion of B12 deficiency in

adult patients (>18 years). Despite the favourable CEA laboratories should consider the feasibility of the introduction of an MMA based algorithm for the diagnosis of B12 deficiency given the specialist equipment and expertise currently required to analyse MMA.

Chapter 7.0 DISCUSSION SUMMARY AND CONCLUSION

7.1 Discussion

The overall aim of this project was to assess the cost-effectiveness of Total B12 (TB12) algorithm compared to an algorithm using Holotranscobalamin (Holo-TC) and for indeterminate cases Methylmalonic Acid (MMA) (Section 1.4.5). Following the recommendation in Harrington's 'Laboratory assessment of vitamin B12 status' review (2017), this project assumed a 'gold' standard testing regime of Holo-TC as the first line test, with MMA as a second line test, when Holo-TC is indeterminate, and therefore, assumed that this strategy will have greater diagnostic accuracy (DA) for B12 deficiency than using TB12, the test in use at the author's Trust at the start of this study (the Trust moved to using Holo-TC in 2023). The project assessed whether the introduction of the new testing regime would have a health economic benefit.

Health economic benefits are about improving healthcare outcomes for patients and the wider healthcare community. NICE (2011) recommend a cost-effectiveness analysis for economic evaluation of diagnostic tests. As the new testing algorithm was more expensive, this must be balanced against potential savings from a reduction in the under and over-diagnosis of B12 deficiency such as prevention of unnecessary additional testing and treatment. The Getting it Right First Time speciality report for pathology (GIRFT, 2021) highlighted that unnecessary B12

repeat testing occurs regularly with approximately 50% of the laboratories involved having no minimal retesting intervals, 20% allowing repeat testing within 1 month and a further 20% allowing 3 monthly testing. Large variations in requesting had also been documented in the 2013 Atlas of variation in which a 72-fold difference, in B12 testing, was seen across the UK and this was not correlated to prevalence of B12 deficiency (PHE, 2013).

To achieve the aim of the project both Holo-TC and MMA assays were implemented. The implementation and verification of the Abbott Diagnostics CE-IVD Holo-TC assay was successful with verification parameters meeting the acceptance criteria. The Holo-TC assay is faster than the TB12 assay but much more expensive, limiting its adoption. The indeterminate Holo-TC range (25-50 pmol/L) used in this study was taken from the Abbott Architect product insert but has also been documented in the NICE MedTech innovation briefing 40 (2015) however Sobczyńska-Malefora et al (2014) reported that using this cut-off missed a proportion of patients with elevated MMA assigning them as B12 replete rather than indeterminate which could therefore impact patient treatment. Other laboratories use a Holo-TC indeterminate range of 25-70 pmol/L which is now also recommended by NICE NG239 (2024). The sensitivity of the Holo-TC ± MMA algorithm, for B12 deficiency, increased from 60% to 78% when the NG239 Holo-TC recommended indeterminate range (25-70 pmol/L) was used instead of 25-50 pmol/L, with a reduction in falsely negative results to 7%, consistent with the findings of Sobczyńska-Malefora et al and warranting further consideration in the author's laboratory. Although the cost-effectiveness analysis, in this study, suggested better efficiency if 25-50 pmol/L is used especially with the high

local prevalence of B12 deficiency (26% using MMA) and high numbers of indeterminate results (~30% of Holo-TC requests for the Royal Wolverhampton NHS Trust (RWT) in 2023, unpublished data). The prevalence of B12 deficiency used was taken from this study and is therefore, subject to selection bias. This is a limitation of this study however, variable prevalence ranges for TB12 are reported in the literature depending on the cut-off concentrations of deficiency used of between 3-26% in the general population (Bailey et al, 2013).

Implementation of different MMA assays were attempted and ultimately the MassChrom® CE-IVD assay from Chromsystems was chosen and successfully verified against manufacturer's performance claims and was used as the proxy 'gold' standard test to calculate the DA of the TB12 and Holo-TC assays for B12 deficiency.

In this study the full blood count (FBC) parameters, haemoglobin (Hb), red cell count (RCC) and mean cell volume (MCV), individually, did not have good DA for B12 deficiency, using MMA as the proxy 'gold' standard test. It may be that if all FBC parameters are abnormal (Hb and RCC, low and MCV, raised) then the DA is improved, but there were insufficient numbers of patients with these results in this study to allow DA to be determined. Similarly, relatively few patients, in this study, met the criteria for a blood film owing to the predominance of FBC results within the reference range. This is consistent with the findings of Hermann and Obeid (2012) and Voukelatou et al (2016) that FBC changes are often a late event in deficiency.

Similarly, B12 deficiency symptoms were not reliable indicators of B12 deficiency in this study, the percentage of patients with symptoms of B12 deficiency was similar in the B12 deficient and B12 replete groups (31% and 30%, respectively), using MMA as the test of B12 deficiency. This concurs with Devalia, Hamilton and Molloy (2014) who reported that symptoms are often non-specific and do not correlate with TB12 concentrations.

Sex and age affected at least some of the markers of B12 deficiency. Males had significantly lower TB12 and significantly higher MMA results than females. Age and MMA were positively associated. It cannot be determined from this study if sex and age specific B12 marker cut-offs should be considered or if B12 deficiency is more common in females and older adults. Margalit et al (2018) reported that men are more susceptible to B12 deficiency which could not be explained by diet or oestrogen effect and suggested the involvement of genetic factors.

A large Finnish study, looking at the prevalence of B12 deficiency in older populations (65-100 years), found undiagnosed B12 deficiency in around 10% of patients but no specific clinical correlations although male gender, age ≥ 75 years and a dairy-free diet increased the likelihood of B12 deficiency (Loikas et al, 2007b).

A further study using NHANES data from 18,569 people aged ≥ 12 years found MMA concentrations were associated with age and ethnicity but not sex. The authors suggested that rising MMA concentration with age were due to the natural decline in

kidney function (Ganji and Kafai, 2018). There is evidence that TB12 decreases, and MMA increases with age due to a reduced ability to absorb B12 (Wong, 2015).

Hinds et al (2011), using data on 1770, >60 year olds from NHANES, observed that mean B12 values were higher in Blacks than in Whites and lower still for Hispanics, who were more likely to be B12 deficient and, as with other studies, males had lower concentrations of B12 than females.

Prevalence of B12 deficiency, in this study cohort was much higher for Asians than Whites, using MMA as the test of B12 deficiency. This contrasts with the findings of O'Logbon et al (2022) and Sobczyńska-Malefora et al (2023) who reported similar prevalence in Whites and Asians. Patient selection for the study, diet and deprivation are possible explanations for these differences. Similarly low levels of deficiency were seen in Black patients, when using MMA as the test of B12 deficiency, in this study and the work of O'Logbon et al (2022) and Sobczyńska-Malefora et al (2023). Sobczyńska-Malefora et al (2023) have suggested that the deficient cut-off for Black people for TB12 should be 225 ng/L as they attributed the higher TB12 concentrations seen in Black people to a combination of genetics and acquired/environmental factors. More studies are required, to whether ethnic specific cut offs, for markers of B12 deficiency should be considered or whether Black people are less susceptible to B12 deficiency. Although in the recent NICE guidance (NG239) (NICE, 2024), recommends caution when indeterminate results are obtained for these patients. The catchment area of the Black Country Pathology Service (BCPS) has a population where 4.5% are Black (Gov.UK, 2022). Therefore,

it will be important to better understand the implications of the ethnic differences in the tests for B12 deficiency with a planned review of the data again in the future.

There was a negative correlation between MMA and folate and eGFR results, respectively and a positive correlation between MMA and TSH results in this study This agrees with the findings in the literature. Benites-Zapata et al (2023) and Collins and Pawlak found lower B12 levels in hypothyroid patients. The mechanism for the association between MMA and eGFR remains unclear, however impaired clearance due to decreased eGFR is one theory. Some authors have used algorithms to correct the MMA for eGFR with a reduction in overestimation of B12 deficiency (van Loon et al, 2018) or a different cut-off for MMA may be needed in renal impairment. A limitation of this study was that MMA cut offs were not adjusted for renal impairment. For TSH and folate different cut-offs are not required as B12 deficiency is more likely in hypothyroidism and folate deficiency.

Contrary to much of the published literature (Herrmann et al, 2003, 2005, Hvas and Nexø, 2005, Obeid and Herrmann, 2007, Čabarkapa et al, 2007, Nexø and Hoffmann-Lucke, 2011, Heil et al, 2012, Bondu et al 2020), the Holo-TC test was no better at identifying B12 deficiency than the TB12 assay when compared using MMA as the proxy 'gold' standard test, with DAs of 78% (95% CI 75-81%) and 75% (95% CI 72-78%), respectively at the deficient cut-off concentrations for Holo-TC and TB12 (<25 pmol/L and <187 ng/L, respectively). Using ROC analysis, optimal cut-off values for deficiency were <180 ng/L for TB12 and < 29 pmol/L for Holo-TC so not dissimilar to the cut-offs used in this study.

In the recent NG239 NICE document (2024) they undertook a diagnostic review of literature that included DA data. The findings were that many of the publications were of poor quality with bias or imprecision with variation in index tests, the 'gold' standard test and cut-off values and they felt the studies could not be directly compared. Variable DA results were seen, and no conclusions could be drawn on the data reviewed. They recommended further research be undertaken in this area.

Using the new algorithm (Holo-TC ± MMA) there were significant improvements in DA (88% vs 76%), sensitivity (60% vs 25%), specificity (99% vs 95%), NPV (88% vs 78%) and PPV (95% to 62%) to the algorithm (TB12) in routine use at the start of this study. The 95% CI for sensitivity and specificity did not overlap indicating that the new algorithm is superior. A high sensitivity is important for a first line screening test to reduce the number of false negative (FN) patients (missed diagnoses) with high specificity in 2nd line tests to prevent false positive (FP) cases. The TB12 algorithm generated up to 38% false positive results and 22% false negative results. Using the new algorithm will reduce the false positive rate to 5% and reduce the false negative results to 12%. A further improvement in sensitivity (to 78%) was found for the Holo-TC ± MMA algorithm, when a Holo-TC equivocal range of 25-70 pmol/L was used.

The health economic analysis using TB12 alone compared to a proposed new Holo-TC ± MMA algorithm (given in Figure 1.6, section 1.4.5) to detect B12 deficiency found that the new algorithm generates cost and health benefits for patients and was therefore more cost-effective in the majority of scenarios evaluated. Scenarios were derived to demonstrate the robustness of the cost-effectiveness analysis (CEA)

when the input variables were amended to encompass plausible situations across different laboratory populations. The evidence base for the health utility input was scant and as such was derived from an article by Mnatzaganian et al (2015) who had investigated fatigue in B12 deficient patients.

The evidence base for the costs was based on the actual costs of the technology and the prices reflected actual prices paid, not just a list price. However, the Holo-TC ± MMA algorithm became almost cost neutral compared to total B12 when costs used in the recent NICE guidelines were modelled. This means that for most laboratories who do not have access to MMA locally, the algorithm may not be cost effective. To investigate this element further a second CEA was undertaken assessing an algorithm utilising TB12 ± MMA, as many laboratories do not have access to Holo-TC. Using the test prices and Holo-TC indeterminate cut-offs stated in NG239 (NICE, 2024), the model found the combination of TB12 ± MMA to be more cost effective than using TB12 alone or the Holo-TC ± MMA algorithm, being significantly cheaper whilst providing health benefits to patients. This was consistent with the findings of the study undertaken by NICE (NICE, 2024) and is reassuring for laboratories that are unable to afford Holo-TC testing in-house who may then refer samples for MMA confirmation testing.

The CEA is very much considered in isolation and does not acknowledge the feasibility of the introduction of an MMA based algorithm for the diagnosis of B12 deficiency given the specialist equipment and expertise currently required to analyse MMA. If this were to be investigated, then the cost of setting up an MMA service would need to be included and would then need to consider the time value of money

reflecting the present value of the costs and benefits accruing over a defined time period.

NHS resources in pathology are finite and if investment is made in the introduction of an MMA service, then which other new test does not get introduced, the CEA cannot account for the impact of these decisions. In addition, it could be argued that assessing the cost-effectiveness of a diagnostic test against a willingness to pay threshold of £20000 per quality adjusted life year (QALY) may be considered impractical because the NHS has a fixed budget, set by parliament (McCabe, Claxton and Culyer, 2012).

The CEA analysis did not include societal costs because of the limited availability of data. In the CEA, the repeat and additional testing and treatment costs were included however the practice across the CCG in test requesting and treatment was not standardised. The repeated GP appointments and potential psychological impact of an undiagnosed condition may have significant impacts on patient costs and productivity losses. Whilst treatment with B12 for false positive patients is considered not harmful, the missed/delayed diagnosis can have significant neurological consequences. This could be considered a key area for future research.

A limitation associated with the CEA was the lack of a true ‘gold’ standard test for B12 deficiency, MMA was used as a surrogate or proxy ‘gold’ standard due to its functional nature however MMA may not accurately identify deficiency in patients >65 years of age, with renal disease, bacterial overgrowth in the small intestines or when patients are dehydrated as falsely high levels of MMA are seen in these

circumstances (NICE, 2015). Also, MMA increases with age and age adjusted reference ranges are required with a likely requirement for eGFR adjusted ranges too. Another limitation of the CEA was related to the number of indeterminate results. Shinkins et al (2013) discuss how indeterminate results should be dealt with when performing diagnostic accuracy studies, there are three possibilities (1) exclude the tests from the analysis however this would significantly skew the data, (2) exclude the indeterminate results from the binary statistics but report an additional summary statistic such as percentage 'test yield' and (3) group valid indeterminate results with positive or negative results. For both the diagnostic accuracy and the CEA the third option was chosen. For the DA, all indeterminates were classed as true negatives (TN) and for the CEA initially all indeterminates by TB12 were classed as true positives (TP) and later sensitivity analysis classified the patients as true negatives. The numbers of TP and TN patients would fall somewhere between the two outcomes. Using the new algorithm has the advantage of confirming the indeterminate results improving diagnosis for patients.

Since the publication of NG239 (NICE, 2024) more laboratories are likely to move to Holo-TC as a first line test, especially in pregnancy. The DA of the Holo-TC test was not significantly different to TB12 in the study and only when MMA performed as confirmation as part of the algorithm were improvements seen. Future work will include repeating the CEA, using the study data gathered, for Holo-TC as a standalone test versus the Holo-TC ± MMA algorithm.

A limitation of this study was that it was not possible, in this selected population, to verify Holo-TC references ranges. Future work will involve verification of the Holo-TC reference range in patients without known B12 deficiency. The gold standard for establishing reference ranges is the direct method. At least 120 samples per range are recommended by the Clinical and Laboratory Standards Institute from healthy individuals (CLSI, 2008). Direct method studies are often very rigorous in defining health and therefore, subjects may not reflect the situation in routine practice. For this reason, indirect methods are often preferred (Ammer et al, 2022, Placzkowska, Terpińska and Piwowar, 2022). The latter use algorithms to establish reference ranges, after application of exclusion criteria to data pulled from the laboratory information system (LIMS). RWT is part of a large pathology network, with a shared LIMS and since April 2023 has routinely used Holo-TC to assess B12 status. Therefore, many patient results are available for the indirect verification of reference ranges.

Similarly, establishment of local B12 deficiency cut-off values for MMA, as recommended by NICE NG239 (NICE, 2024), are needed, prior to the introduction of the test into routine use, because MMA assays are not standardised, as illustrated by the differences seen between the referral site patient results and those using the method verified in this study.

This study has shown that sex, age and ethnicity specific reference ranges and cut-offs may be required because of the large number of genetic variations (SNPs) identified that affect various parts of B12 metabolism pathway.

The population in the BCPS is ethnically diverse and it is important that no patients suffer health inequalities as a result of unified reference ranges.

There were a number of difficulties encountered during this study. Sample collection started prior to the COVID-19 pandemic but was largely halted in 2020 and 2021. The laboratory moved to a new LIMS provider in October 2020 and collection of data from the legacy system was problematic. The LCMS/MS equipment was moved to Sandwell and West Birmingham Hospitals NHS Trust (another Trust within the BCPS) in 2021 and the COVID restriction of working on the base site only was not lifted until June 2022. Furthermore, the increase in routine workload on the LCMS/MS used for this project, impacted the MMA assay implementation and verification.

This study provides the first health economic analysis of a new testing algorithm for B12 deficiency, using Holo-TC as the first line test and MMA analysis where Holo-TC results are indeterminate using a cut-off of 25-50 pmol/L. The recent NICE NG239 guideline (NICE, 2024) included a cost-effectiveness analysis with a starting point of an indeterminate B12 test result either by TB12 or Holo-TC and then the model compared, ‘do not test, no treatment’ or ‘test with MMA’, a third branch was added as sensitivity analysis of ‘do not test, treat all patients’. NICE cut-off values for Holo-TC indeterminate result were 25-70 pmol/L with a prevalence of 24.3%. Their CEA was also cost-effective, and their recommendation is to consider MMA testing for patients with a first line indeterminate test result. When the author modelled the NICE recommended Holo-TC cut-offs it resulted in an increase in the number of

confirmatory MMA tests required and therefore reduced cost-effectiveness. When the costs per test were adjusted to those used by NICE in their CEA, the new algorithm was still cost-effective however the costs saved were very small.

Introduction of this algorithm into routine use would result in earlier diagnosis of B12 deficiency in some patients resulting in initiation of treatment, improved quality of life and prevention of potential future significant neurological complications. A B12 deficiency diagnosis before the development of debilitating disability has a financial impact on the NHS through reduced requirement for repeat GP appointments, testing and secondary care referrals to neurology and/or haematology. For society as a whole, early diagnosis is beneficial to reduce working days lost due to B12 deficiency symptoms and to reduce the impact of frailty and dementia in the elderly (Hooper et al, 2014).

Future work will include the development of a business case for the implementation of the MMA assay. Discussions will be required with the Integrated Care Board around willingness to pay for the MMA test with the knowledge that earlier B12 deficiency diagnoses would reduce the number of repeat tests and GP appointments and improve the long-term health of patients.

With the discovery that MMA is increased in various cancers and therefore could be utilised as a tumour marker in the future (Gomes et al, 2022, Li et al, 2022, Hu et al, 2023, Tejero et al, 2025), it may not be the best functional biomarker to confirm B12 deficiency, therefore further work may also be undertaken on the use of T-Hcy as a confirmatory test. T-Hcy is already acknowledged as the best biomarker for B12

deficiency in patients using N_2O (Grzych et al, 2023); the government plans to fortify wheat with folic acid thereby increasing the specificity of the test for B12 deficiency (Gov.UK, 2024) and stabilisation tubes can be procured, to counteract the inherent instability of T-Hcy in blood samples (Greiner BioOne, 2024). T-Hcy was discounted early in this study because the surplus serum used had not been subject to the strict preanalytical treatment required for T-Hcy analysis (Ueland et al, 1993).

In the recent NICE NG239 guidance (NICE, 2024) areas for future research are highlighted. Further analysis of some of the data gathered in this study may add to the evidence base in some areas, for example the follow-up data collected on treatment and additional testing undertaken.

7.2 Conclusion

In conclusion, this project has achieved the aim described in Chapter 1, to assess the cost-effectiveness of the TB12 algorithm in routine use at the start of this study with an algorithm using Holo-TC and for indeterminate cases MMA. The project had two null hypotheses, the first that there would be no difference in diagnostic accuracy between the current and new algorithm and the second was that there would be no health economics benefits seen using the new algorithm. Both null hypotheses have been rejected. The algorithm using a combination of Holo-TC and MMA is cost-effective and could be implemented for use in the BCPS improving diagnosis of B12 deficiency in primary care patients.

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