The impact of folate and vitamin B12 intake and biomarker status on cognitive performance in older adults on the Island of Ireland

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other University. This study utilises data from the Trinity, Ulster, Department of Agriculture (TUDA) ageing cohort study and the data collected in the first phase (Northern Ireland and Republic of Ireland) and in the second phase (Northern Ireland) was made available to me. I worked as part of the team in Dublin who were responsible for collecting data from 400 participants for the second phase of the study. I hereby certify analysis of dietary and biomarker data presented in this thesis is entirely my work.

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Acknowledgements

Summary

Suboptimal intake and biomarker status of folate, vitamin B12 and other metabolically linked B-vitamins have been associated with a range of conditions more prevalent in the ageing population such as cardiovascular disease and cognitive decline.

The Trinity, Ulster, Department of Agriculture (TUDA) ageing cohort study began in 2008 and was designed to investigate the nutritional and genetic factors in the development of chronic conditions of ageing. The TUDA study recruited 5186 participants who either had phenotypic evidence of early stages of Alzheimer disease, osteoporosis, or cardiovascular disease (CVD). A total of 987 TUDA participants aged >65 years who had a Mini Mental State Examination (MMSE) score of ≥24 at baseline returned to take part in the follow up study between 2014 and 2019.

The aim of this follow up study and thesis were to determine dietary intake of folate and vitamin B12 using a food frequency questionnaire and a four-day food diary. Determine biomarker status using red blood cell folate (RCF), serum total B12, methylmalonic acid (MMA) and homocysteine and reassess the participant's cognitive performance and investigate the relationship between folate and vitamin B12 in cognitive performance.

This follow up study found a decrease in median RCF from 940.9 nmol/L to 766nmol/L and the prevalence of B12 deficiency and elevated homocysteine rose by 7.3% and 0.7% respectively since baseline ($p<0.001$). This study found that dietary intake of folate was below the reference nutrient intake (RNI) for the 49% of the participants who were not consuming folic acid (FA) fortified foods. Whilst B12 intake exceeded the RNI.

Those who consumed FA fortified foods had a higher total folate intake than those who were non-consumers. The highest consumers had significantly lower homocysteine and MMA status (p<0.05) and higher RCF and serum B12 (P<0.05). These participants had the lowest prevalence of elevated homocysteine, MMA and low B12 status compared to non-consumers (p<0.05).

The average score in the cognitive tests decreased between baseline and the follow up study and those who were classified as having a score indicative of impairment rose by 4.6% in the RBANS, 9.8% in the FAB and 4.5% in the MMSE.

Those consuming less than 206.1µg total folate per day had significantly lower MMSE scores (p<0.05) although exceeding 357.3µg per day did not offer any benefit.

RCF concentrations above 568nmol/L were associated with a better total RBANS score (p<0.05). In the individual cognitive domains, index 3 which tests language showed that those with RCF concentration between 766.1 and 1061nmol/L had a significantly higher score than those with less than 568nmol/L (p<0.05). In index 5, which tests delayed memory, there was significant improvement in scores for those with RCF concentrations above 568nmol/L but less than 766nmol/L. Concentrations in excess of 766nmol/L did not result in significantly better cognitive scores.

MMA was also related to better cognitive scores in both the total RBANS score and in index 3, the language domain. Those with MMA concentrations less than 0.19µmol/L had significantly better total and language scores than those with concentrations greater than 0.26µmol/L which indicates lower B12 status.

This study indicates that there was a high prevalence of B12 deficiency and an increasing prevalence of elevated homocysteine which along with the decrease in RCF suggests that B vitamin status has declined in this population. The largest consumers of FA fortified foods had the lowest prevalence of elevated homocysteine and biomarkers of low B12 status as well as increased RCF concentrations ($p < 0.05$). These foods tend to have other B vitamins added which would contribute to the improvement in B12 status and homocysteine concentrations. Additionally, malabsorption in older people could be partially reduced through the consumption of fortified foods as the B12 is not bound within the food and therefore more readily absorbed.

Overall, this study has indicated that optimising RCF and MMA status which could be aided with the use of fortified foods may be beneficial in slowing the rate of cognitive decline in the older population. However, this finding requires confirmation through randomised control trials.

Personal Contribution

My role in this collaborative study was with the team in Dublin and we were responsible for recruiting participants in the Republic of Ireland (n=400). As part of the team, I conducted interviews with participants and collected data regarding their health, medication use, lifestyle, mood, dietary intake using a food frequency questionnaire and food diary and measurements including timed up and go and waist and hip circumference. Additionally, as part of the team, I reassessed the participant's cognitive performance in the frontal assessment battery (FAB), mini mental state examination (MMSE) and repeatable battery for assessment of neuropsychological status (RBANS) and taking a further blood sample which, I subsequently processed in the laboratory and at a later date these samples were sent for biomarker analysis. Our team computerised the data, I analysed the dietary data along with the University of Ulster team using Nutritics. Once all the data was collected and input to SPSS our data was merged with the data collected by our collaborators in University of Ulster.

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Abbreviations

BMI	Body Mass Index
CVD	Cardiovascular Disease
DFE	Dietary Folate Equivalents
EAR	Estimated Average Requirement
EFSA	European Food Safety Authority
EGRac	Glutathione Reductase Activity Coefficient
FA	Folic Acid
FAB	Frontal Assessment Battery
FAD	Flavin Adenine Dinucleotide
FFQ	Food Frequency Questionnaire
FMN	Flavin mononucleotide
IADL	Instrumental Activities of Daily Living
LRNI	Lower Reference Nutrient Intake
MEG	Magnetoencephalography
MMA	Methylmalonic Acid
MMSE	Mini Mental State Examination
MTHFR	Methylenetetrahydrofolate Reductase
NANS	National Adult Nutrition Survey
NDNS	National Diet and Nutrition Survey

NTD	Neural Tube Defects
PLP	Plasma pyridoxal phosphate
PSMS	Physical Self Maintenance Scale
RCF	Red Blood Cell Folate
RBANS	Repeatable Battery for Assessment of Neuropsychological Status
RDA	Recommended Daily Allowance
TIA	Transient Ischaemic Attack
TILDA	The Irish LongituDinal Study on Ageing
TUDA	Trinity, Ulster, Department of Agriculture
WHO	World Health Organisation

Chapter 1

Introduction

1.1. Background

Advances in health care have resulted in a greater life expectancy with a subsequent demographic shift towards an ageing population. In the UK, those aged over 60 years are estimated to rise from 14.9 million to 21.9 million by 2039 (Foresight, 2016). While in Ireland, by 2031 nearly 20% of the population will be aged ≥65 years (CSO, 2013). This increase will have a major impact on many economic and societal resources such as the cost, availability and provision of health care, medications, treatment and health service use and thus efforts are being made to promote health and wellbeing in this population group which is vulnerable to the chronic conditions of ageing (WHO, 2015b). The development of chronic conditions are usually accompanied by an increased risk of other comorbidities. Management of multiple comorbidities may require polypharmacy which can increase risk of falls, cognitive decline and other complications (WHO, 2015b). Having a fear of falling and/or osteoporosis can also lead to individuals limiting their activity levels (Hübscher *et al.*, 2010; Jefferis *et al.*, 2012) which can reduce cardiovascular fitness, increase the risk of weight gain and promote the development of cardiovascular health conditions (Lavie *et al.*, 2016). Preventative health care measures can have an abundance of benefits to the individual, society, and the economy. For example, taking regular physical activity may promote a greater healthy life expectancy for the individual by reducing their risk of dementia, depression, high blood pressure, type 2 diabetes and falls so that they can continue to enjoy a good quality of life, mobility and independence (Rechel *et al.*, 2013). This may delay the need for health care in older age thus benefitting the economy and society by contributing to cost savings and a reduction in the demand for health services so that these services will be more readily available to those in the greatest need. The individual may wish to continue to

contribute to the workforce for longer due to the additional years spent in good health which may offer social benefits as well as financial benefits (Rechel *et al.*, 2013). Therefore, the prevention of ill health is as important as the number of 'healthy life years' free of disease. Many modifiable risk factors are acknowledged in the promotion of health and wellbeing in ageing such as smoking cessation, obesity prevention and a healthy balanced diet (M., S. and D.B., 2016; Livingston *et al.*, 2017; Costanzo *et al.*, 2019). Among the dietary components of interest are B vitamins which are largely involved in metabolism and DNA and amino acid synthesis. Further to this, low blood concentrations of these vitamins, namely vitamin B2, B6, folate and B12 has been associated with chronic conditions of ageing such as hypertension, heart disease and cognitive decline (Porter *et al.*, 2016). Optimising vitamin B status may therefore be a valuable resource in reducing risk of such conditions and thus promote better quality of life during advanced years.

1.2 Dietary sources of B vitamins and the use of fortified foods

Major dietary contributors of vitamin B6 and B2 in the diet for adults over 65 years include meat, fish, milk, dairy products, fruit, vegetables and grains(EFSA, 2016a; Turck *et al.*, 2017). Vitamin B12 is found in animal products such as meat including beef and chicken, fish including shellfish such as clams and oysters, milk and dairy products (Laird and Molloy, 2014; EFSA, 2016b). Dietary sources of folate include; fruit and vegetables, of particular note are oranges and dark green leafy vegetables such as spinach and kale. Peanuts, almonds, offal such as liver and kidney, legumes, wheat bran and yeast extracts are also good sources (Laird and Molloy, 2014; EFSA, 2015). In addition to the foods that naturally contain B vitamins, some common place foods are fortified. In the UK and

Ireland, there is no legal obligation to do so, however, there is a voluntary fortification policy, the European Regulation (EC) No. 1925/2006 on the addition of vitamins and minerals and of certain other substances to food, which enables manufacturers to add folic acid if they wish to do so (Zicari, Carraro and Bonetta, 2006; Department Of Health, 2011) . Foods, such as breakfast cereals, cereal bars, milk and dairy alternatives and bread are often fortified with B vitamins making them important contributors of these nutrients (Hoey *et al.*, 2007). A 2017 study of Northern Ireland participants over 60 years old showed that 75% were consumers of fortified foods(Hughes *et al.*, 2017) in keeping with an earlier study which indicated 75% of men and 80% of women were consumers (Hoey *et al.*, 2007). Although this fortification is done on a voluntary basis, it has the potential to deliver a beneficial effect on folate biomarker status to those who choose to consume them (Hoey *et al.*, 2007). A study formulated using the data obtained through the National Adult Nutrition Survey (NANS) found 79% of people sampled in the Republic of Ireland consume folic acid fortified foods and 65% consume vitamin B12 fortified foods. Specifically, for those over 65 years, 81% and 65% of men and 74% and 60% of women were consumers of folic acid and B12 fortified foods, respectively. The average daily consumption of folic acid contained within fortified foods was 64µg for males and 47µg for females in this age group; and for synthetic B12 in fortified foods, 0.2µg and 0.1µg was consumed by males and females, respectively. However, the number of participants in this age group was small, therefore the data is not representative of the population (Hopkins *et al.*, 2015).

A recent study of Irish adults suggests there is a need for a mandatory fortification policy. The author found that 12% of the study population had vitamin B12 deficiency or low status, whilst 15% had low folate status or deficiency and those who were non-

consumers of fortified foods were more likely to fall into these categories. It was also noted that improvements in the prevalence of suboptimal status may be possible through adjusting current dietary intake to meet the recommended guidelines set by the government. However, the author highlights numerous concerns in regards to this, such as, some individuals may not have an awareness of the guidelines, lack the skills or access to other foods to make the dietary changes, may require medication that hinders vitamin absorption and may have reduced absorptive capacity in their gastrointestinal tracts and therefore fortification would be a more suitable option. The author also estimates that delays in implementing mandatory fortification could endanger more than 180,000 older Irish adults by leaving them at increased risk of health consequences arising from folate deficiency or suboptimal status and 145,000 older Irish adults at risk of the health consequences of suboptimal vitamin B12 status or deficiency (Laird *et al.*, 2018). The USA introduced mandatory folic acid fortification of grain products in 1998 to reduce the incidence of neural tube defects (NTDs). Since then, not only has the fortification policy decreased the incidence of NTDs (Mills and Signore, 2004) there has been a wider population benefit of reduced prevalence of low folate status (serum folate <6.8nmol/L) from 16% to 0.5% of the population which has been associated with a reduction in homocysteine levels (Pfeiffer *et al.*, 2005). One of the issues surrounding the implementation of mandatory folic acid fortification is the concerns regarding the masking of B12 deficiency which can lead to permanent neurological damage. Another concern is the increased risk of colon cancer (Mason *et al.*, 2007; Cole *et al.*, 2019). A Lancet article highlighted important misinterpretations associated with the research. For example, it is not unusual for cancer rates to appear to increase following the introduction of screening and the mandatory fortification was introduced after the

increase in colorectal cancer had occurred (Bayston *et al.*, 2008). The B-PROOF study intended to investigate the incidence of fractures in an elderly population in response to vitamin B12, folic acid and vitamin D supplementation and found no increase in mortality risk. They did, however, note a higher incidence of cancer diagnosis amongst those in the treatment group (Van Wijngaarden *et al.*, 2014). A Norwegian study of participants with Ischaemic Heart Disease found an increase in mortality and incidence of cancer in those who received folic acid and B12 supplementation (Ebbing and Meyer, 2019). Despite these concerns, a 2012 meta-analysis found no increase in the incidence of cancer when they examined trials supplementing folic acid in excess of the dose that would be achieved through intake of fortified foods (Vollset *et al.*, 2004). Another meta-analysis of B vitamin supplementation showed no increased risk of all-cause mortality, myocardial infarction, coronary heart disease and cardiovascular death. The same analysis indicated that supplementation could have a protective effect against stroke (Huang *et al.*, 2012). This suggests that there many studies that show benefit in preventing low folate and vitamin B12 status and there is not enough evidence to suggest fortifying foods will cause harm, however, longer term studies should be conducted to investigate this further.

In addition to fortification, some people may choose to supplement the diet with a nutritional supplement such as a multivitamin tablets or individual vitamins for preventative reasons, a perceived benefit or in order to treat a deficiency (Frey, Hoffmann and Heuer, 2017). B vitamins can be consumed through a variety of supplements prescribed or purchased over the counter individually or as part of a multivitamin. An example of those who may choose to supplement are those who are

consuming a vegan and vegetarian diets who can be vulnerable to B12 deficiency (EFSA, 2016b). An Irish study reported that folic acid or vitamin B12 containing supplements are used by 8% of older males and 14% of older women (Hopkins *et al.*, 2015). However, the population size in this study was very small and therefore likely not reflective of the Irish population. Another, more recent study of Irish adults has found that only 2.6% use injections or oral B12 supplements and 2.8% use folic acid supplements (Laird *et al.*, 2018). The latter study has a much larger study population (n=5895) consisting of adults over the age of 50 and therefore is more likely to give an indication of the true prevalence of supplement use in Ireland in older adults. Both studies found that there was a tendency towards higher supplement use among female participants (Hopkins *et al.*, 2015; Laird *et al.*, 2018).

1.3. The metabolic role of B vitamins

B vitamins have an important role in one carbon metabolism, methylation, amino acid metabolism and energy production. Their roles are often interconnected, and the collaborative relationship between folate, vitamin B2, B6 and B12 is illustrated in **Figure 1**.
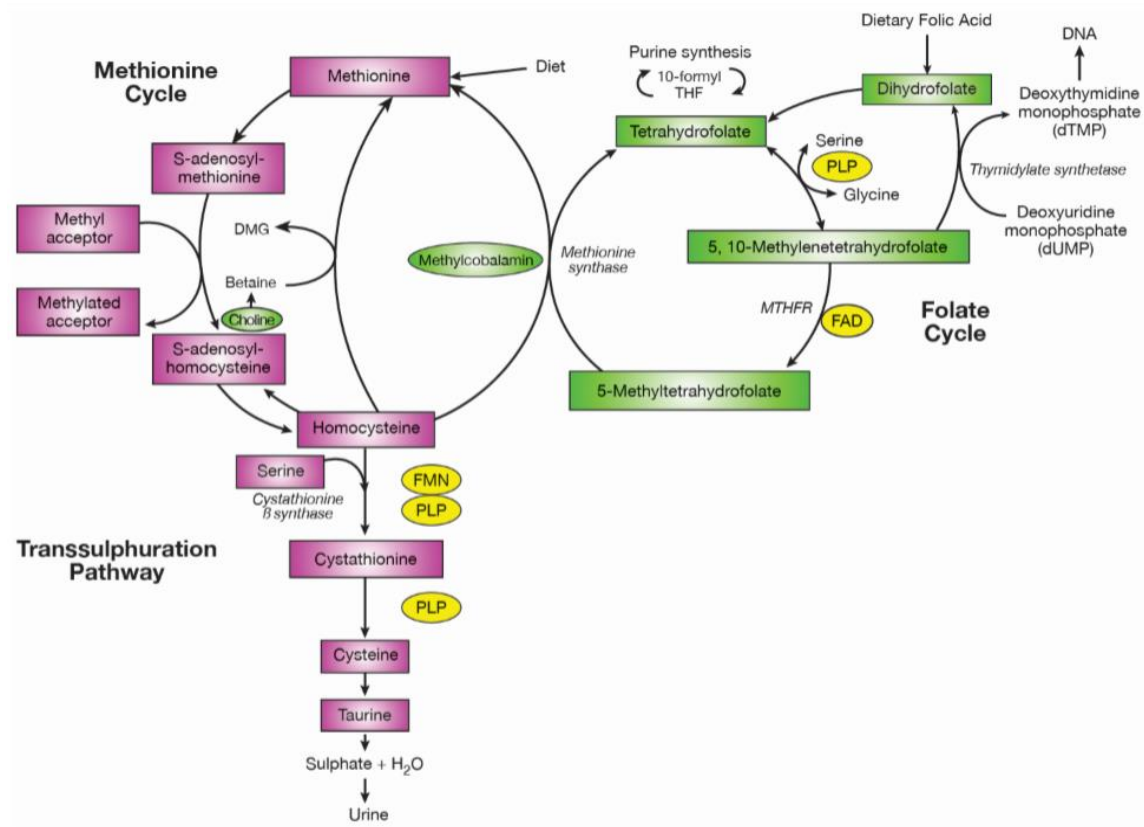
Figure 1 – One carbon metabolism. This figure illustrates the interlinking pathways that are dependent on B vitamins. Vitamin B6 is used in the form of plasma pyridoxal phosphate (PLP) and vitamin B2 is used as both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). This figure has been sourced from (Porter *et al.*, 2016).

Vitamin B12 has an important role as a cofactor for the enzymes: methylmalonyl CoA mutase and methionine synthase. Methylmalonyl CoA mutase utilises vitamin B12 in the form of adenosylcobalamin as a cofactor in the conversion of methylmalonyl CoA to succinyl CoA which is fed into the tricarboxylic acid cycle to generate energy (Hughes *et al.*, 2013). Methionine synthase is needed for the re-methylation of homocysteine. This process utilises vitamin B12 in the form of methylcobalamin to create a complex, which binds with the folate dependent 5-methyltetrahydrofolate in order to transfer a methyl group to homocysteine to produce methionine. Which is then used to create S-adenosylmethionine, a universal methyl donor, which donates methyl groups which are needed in the production of phospholipids, neurotransmitters, amines, DNA and RNA and enables modulation of gene expression (Hughes *et al.*, 2013; Porter *et al.*, 2016). The tetrahydrofolate that remains after losing the methyl group can be transformed through the folate cycle in reactions that are dependent on vitamin B6 in the form of plasma pyridoxal phosphate (PLP) and vitamin B2, in the form of flavin adenine dinucleotide (FAD) so that molecules can be produced that are required in thymidine, DNA synthesis and the methionine cycle (Porter *et al.*, 2016). Vitamin B2 is needed in the form of flavin mononucleotide (FMN) so that homocysteine can used in the transulfuration pathway which also utilises PLP to synthesise cysteine and glutathione (Laird and Molloy, 2014; Porter *et al.*, 2016). B2 also has a role in preventing oxidative damage due to its role as a cofactor in the regeneration of glutathione which serves as an antioxidant (Kennedy, 2016). When there are low levels of vitamin B2, B6, B12 or folate there are disruptions to these interlinking pathways with numerous consequences, including raised homocysteine as it cannot be remethylated to methionine and as a result there is a reduction in methylation processes. Low levels of

vitamin B12, specifically, cause increased levels of methylmalonic acid (MMA) as a result of reduced methylmalonyl CoA activity which can impact energy production and the continued cycling of folate stops due to the reduced metabolic activity of methionine synthase which traps folate so it cannot be used in DNA synthesis (Hughes *et al.*, 2013; Porter *et al.*, 2016). Low levels of either vitamin B2 or B6 can inhibit niacin production. This is because vitamin B6 is needed in the conversion of vitamin B2 to FAD which is subsequently used in the conversion of the amino acid tryptophan to niacin. Both vitamins are also needed in the transsulphuration pathway so low levels of either can also impair cysteine production (Laird and Molloy, 2014). Although, high levels of homocysteine are associated with low B vitamin status, homocysteine can also accumulate as a result of genetic conditions such as the 677C→T polymorphism in the MTHFR gene and those with this genotype will have exacerbated homocysteine concentration when accompanied by low B vitamin status (Porter *et al.*, 2016).

1.4. Nutritional biomarkers

Nutritional biomarkers are used to determine the biological response following exposure to a nutrient in terms of quantity or function of the nutrient. Given the complex nature of B vitamin's involvement in metabolic processes there are numerous biomarkers that can be used to determine B vitamin status (Raiten *et al.*, 2011). The biomarkers used in this study are listed in **Table 1** with their respective concentrations which are indicative of deficiency or low status.

Methylmalonic acid (MMA) is a metabolic product that accumulates as a consequence of low vitamin B12 availability for the conversion of methylmalonyl CoA to succinyl CoA

and it is a sensitive indicator of short to long term vitamin B12 status. However, as low levels of MMA indicate higher vitamin B12 status it is not as useful for assessing high intakes and it is recommended that other biomarkers are used in combination to improve interruptive accuracy. Serum B12 is often used but it measures the total amount of vitamin B12 regardless of metabolic activity therefore unlike MMA it is not a useful indicator of functional deficiency (Bailey *et al.*, 2015; Porter *et al.*, 2016).

Red blood cell folate is a useful biomarker for long term folate status as it correlates with liver and tissue stores and is reflective of folate status in the previous 120 days, whilst serum folate is more reflective of current intake. Homocysteine is often used in relation to folate, however, it cannot be used as a lone indicator of folate status as it can be elevated in response to other B vitamin deficiencies but it can be a useful indicator of functional B vitamin deficiency (Bailey *et al.*, 2015; Kennedy, 2016; Porter *et al.*, 2016).

Plasma Pyridoxal-Phosphate (PLP) is commonly used to measure vitamin B6 status. PLP is relatively sensitive to changes in status as PLP concentration decreases soon after a period of low intake commences and will increase again within a short time after intake improves. The Erythrocyte glutathione reductase activation (EGRac) assay is considered the gold standard for vitamin B2 status and it is a good indicator of long term status (Porter *et al.*, 2016).

When measuring biomarker status, it is therefore important to consider using more than one biomarker to give a fuller picture of status, for example, using only MMA would show those who are and who are not deficient rather than those with high vitamin B12

status therefore serum B12 could be measured as well. Using a biomarker such as homocysteine could be used but it is not specific to a particular B vitamin therefore using it in combination with RCF would give a more meaningful result in terms of folate status. Additionally, there are often other factors to consider, such as medication use, presence of health conditions such as renal impairment and infections which may influence the result (Bailey *et al.*, 2015; Porter *et al.*, 2016). Furthermore, recommended dietary allowances (RDAs) are important guidelines in the prevention of deficiency disease, however, they do not account for individual variability in requirements and suboptimal status. Therefore, oral intakes in line with the RDA do not necessarily result in optimal biomarker status (Kennedy, 2016).

Table 1 - Recommended daily intake of B vitamins, reference ranges for their respective biomarkers and deficiency symptoms

1 Reference Nutrient Intake (RNI) for those over 50 years old.

B Vitamins	RNI [1]	Nutritional Biomarker	Deficiency	Symptoms
Folate (Salmon, 1991; Laird and Molloy, 2014; EFSA, 2015; Gandy, 2019)	200µg	Homocysteine	>15 µmol/L	Symptoms of megaloblastic anaemia such as low mood, fatigue, shortness of breath, pale skin, palpitations, neuropathy and cognitive difficulties.
		Red blood cell folate (RCF)	<226.5 nmol/L – risk of macrocytic anaemia <340 nmol/L - risk of elevated homocysteine	Reduced appetite Muscle weakness Dermatitis
B12 (Salmon, 1991; Hunt, Harrington and Robinson, 2014; Laird and Molloy, 2014; EFSA, 2016b; Gandy, 2019)	1.5 µg	Methylmalonic acid (MMA)	>0.37 µmol/L	Symptoms of megaloblastic anaemia such as low mood, fatigue, shortness of breath, pale skin, palpitations, neuropathy and cognitive difficulties.
		Serum B12	<148 pmol/L	Inflammation of the tongue and ulcers in the mouth Cognitive difficulties

1.5. B vitamin intake and status

Vitamin B2

The UK population survey, National Diet and Nutrition Survey (NDNS), has found between 2008 and 2017 that the average intake of riboflavin was in excess of the RNI, but for women over the age of 65, there was an increased number not meeting the lower reference nutrient intake (LRNI) (Roberts *et al.*, 2018; Bates *et al.*, 2019). A systematic review of micronutrient intakes in older adults has found an average of 41% of males and 31% of females are failing to meet the Estimated Average Requirement (EAR), which is concerning as the EAR is only deemed sufficient for half the healthy population (Ter Borg *et al.*, 2015). This low intake of vitamin B2 corresponds with the 29% of adults over 65 years found to be deficient as per their EGRac status (>1.3) in the NDNS (Roberts *et al.*, 2018; Bates *et al.*, 2019).

Vitamin B6

A small study of older adults living in Northern Ireland found that 11% have B6 deficiency (Hughes *et al.*, 2017). A systematic review of studies conducting in countries around the western world, however, has indicated that 31% of males and 24% of women are not meeting the EAR for vitamin B6 which would be putting individuals at greater risk of deficiency (Ter Borg *et al.*, 2015). Another study found that over 50% of participants did not meet the recommended intake and 37% have PLP of <30nmol/L which suggests deficiency (Jungert, A.and Neuhäuser-Berthold, 2019).

Folate

The NDNS suggests both folate intake and biomarker status are declining for all age and gender groups across the last 9 years of data (Bates, B, 2019). On average, however, the 2014-2016 data indicates folate intake was in excess of the RNI for adults above the age of 65, except for woman over 75 years, and there was an increase in the number of women over 65 years who failed to meet the LRNI. In 2014-2016, 3% of the women in the 65-74 years group had an oral intake less than the LRNI and this increased to 8% for the over 75's and for men over 75 years, 4% were below the LRNI. Which again differs from the findings of the systematic review which found that including and beyond the UK 29% of males and 35% of females were not meeting the EAR, however, this review also analysed studies conducted outside of the UK and Ireland (Ter Borg *et al.*, 2015). In terms of biomarker status, the NDNS found 14% of men and 10% of women over 65 years had RCF values that are indicative of deficiency (Roberts *et al.*, 2018). A Northern Ireland study found 3% to be deficient in folate (Hughes *et al.*, 2017) whilst in Ireland 15% had deficiency or low levels of folate, however, the level of deficiency rose to 23.1% for those over the age of 80 years old (Laird *et al.*, 2018). Furthermore, on a global scale folate is considered one of the most common micronutrient deficiencies (Bailey, West and Black, 2015).

Vitamin B12

Vitamin B12 deficiency can be found often. Hunt et al estimate that B12 deficiency affects around 20% of the older population in the UK (Hunt, Harrington and Robinson, 2014). Specifically in Ireland, Laird et al found 12% of older people to have low levels or deficiency in vitamin B12, and those over 80 years old had highest incidence of B12

deficiency (Laird *et al.*, 2018). For vegans, in the UK, 11% are said to be deficient (Hunt, Harrington and Robinson, 2014). The common occurrence of vitamin B12 deficiency may be attributable, in part, to the finding that 16% of males and 19% of females are not meeting the EAR for vitamin B12 (Ter Borg *et al.*, 2015). The NDNS reports an improvement, overall in the UK, of the number of women over 65 who had low B12 status (Bailey *et al.*, 2015).

1.6. Causes of low status

Low biomarker status of B vitamins can occur for many reasons. In general, micronutrient deficiencies are more prevalent in lower income countries (Bailey, West and Black, 2015), but as previously discussed they are still present in the UK and Ireland and they can occur more frequently in certain at risk groups.

Poverty is a common cause of micronutrient deficiencies and this can be due to lack of food security for those with low income and it is often associated with poorer access to health care (Bailey, West and Black, 2015). A survey in Ireland indicated that 51.1% of older population have reported prioritising their heating costs during the winter months rather than spending money on food and clothing (Goodman *et al.*, 2011). Those with low income can experience greater difficulty in accessing shops (Schmitthenner, 2019).

The older population are more likely to have conditions which increase their risk of developing mobility and cognitive problems which can influence a person's ability to access and prepare food. Additionally, depression, frailty, dysphagia, poor dentition, sensory perception of food such as smell and taste, social isolation and in greater need

for support with activities of daily living can all contribute to nutritional status (Mudge *et al.*, 2011; Roberts *et al.*, 2019; Schmitthenner, 2019).

Malnutrition tends to increase with the level of care an older person requires. Hospital admissions have been shown to increase the risk of malnutrition as acute illness can present with infection, worsening cognition for example delirium, poor appetite and increased need for support with daily activities such as eating and drinking. The consequences of malnutrition are symptoms such as fatigue and muscle wasting which can further exacerbate oral intake (Mudge *et al.*, 2011).

In the older Irish population, Laird et al have indicated a low consumption of dairy products and particularly for those with the lowest intakes there has been an association with poorer vitamin B2, B6, B12 and folate biomarker status (Laird *et al.*, 2017). Additionally, vegans and those with low consumption of foods from an animal origin can be at greater risk of B12 deficiency (Hunt, Harrington and Robinson, 2014).

Low oral intake of these vitamins is also common in people who are experiencing drug or alcohol abuse or anorexia nervosa (Porter *et al.*, 2016). In addition to poor oral intake, those who chronically abuse alcohol develop impaired organ function and therefore have lower folate and vitamin B12 storage and uptake capability. Chronic alcohol abuse is associated with lower biomarker status of folate, vitamin B2 and B6 and B12 (Cravo and Camilo, 2000; Bailey *et al.*, 2015).

Low intake can also be partially attributed to the nutrient losses as a result of cooking methods and manufacturing processes as B vitamins are water soluble, heat and light sensitive therefore overcooking, boiling foods and processes such as canning reduces their vitamin content (Laird and Molloy, 2014; Porter *et al.*, 2016).

Malabsorption is another factor which can lead to low biomarker status. B12, in particular, has a complex absorption process involving multiple organ systems. After ingestion, B12 is bound to proteins within the food and must be released from this complex to absorbed. The release from the protein complex is aided by the actions of pepsin and the pH of gastric secretions. B12 is then free to bind with the R-protein which is found in the salvia and gastric secretions. Pancreatic enzymes then release the bound B12 so it can bind with intrinsic factor which is secreted from the stomach lining. This complex enables B12 to bind to the receptors in the ileum for absorption to the blood stream. In the blood, a new B12 complex is formed with transcobalamin to enable transportation to cells. The body has the ability to store vitamin B12 in the liver and therefore deficiency generally takes time to develop. This absorption process can therefore be inhibited by stomach problems such as H. Pylori infection, atrophic gastritis and stomach surgeries such as gastrectomy, as well as intestinal conditions such as Coeliac disease, Crohn's disease, Ulcerative Colitis and short bowel syndrome, pancreatic insufficiency, liver disease, genetic conditions affecting the production of the proteins and enzymes required for the absorption. Issues with this process, rather than inadequate oral intake, is the most prevalent cause of B12 deficiency in the older population which is thought to affect 10-30% people over 50 years old. (Stover, 2008; Laird and Molloy, 2014; Wong, 2015).

The malabsorptive conditions mentioned above effect uptake of other nutrients including other B vitamins. In the case of coeliac disease, often diagnosis is made after a patient presents with symptoms of weight loss and gastrointestinal upset such as diarrhoea. However, it is estimated that 1% of the those over 45 years old have asymptomatic coeliac disease (West *et al.*, 2003) which may go unrecognised for numerous years until the chronic malabsorption of many nutrients including B vitamins leads to development of deficiency diseases such as megaloblastic anaemia and further investigations into causes of the latter are carried out (Bailey *et al.*, 2015).

Pernicious anaemia is an autoimmune condition that causes damage to the parietal cells and therefore impairs production of intrinsic factor which is needed to facilitate B12 absorption, but this condition is only thought to be the cause of 1-2% of the cases of deficiency (Mcnulty and Scott, 2008; Laird and Molloy, 2014).

Certain medications such as proton pump inhibitors (PPIs) and H2-receptor antagonists can interfere with B vitamin status through their impact on gastric secretions. Other interacting medications include methotrexate, metformin, anti-convulsant treatments such as phenytoin and primidone, antibiotics such as trimethoprim and isoniazid, nitrous oxide, steroids and triamterene. Reduced vitamin B12 biomarker status has also been associated with smoking (Laird and Molloy, 2014; Bailey *et al.*, 2015; Porter *et al.*, 2016).

The US Food and Nutrition Board has recommended for those over 50 years old to consume most of the recommended daily intake of vitamin B12 with the use of fortified foods or supplements to combat the common problem of food-bound malabsorption in

the older population. These sources can be absorbed by intrinsic factor without the need for detachment from food proteins by gastric secretions. Furthermore, if the severity of gastric atrophy increases to the point that intrinsic factor secretion is impaired, vitamin B12 can still be passively absorbed with high dose supplementation (Stover, 2008). In the UK and Ireland there is no mandatory policy for food to be fortified with B vitamins and supplement use tends to be low (Hoey *et al.*, 2007; Laird *et al.*, 2018).

1.7. Cardiovascular health

In Ireland, ischaemic heart disease caused 4,340 deaths while strokes resulted in 1,700 deaths in 2017, making cardiovascular diseases the second largest cause of death in Ireland (Malone, 2018). Globally, heart disease remains leading cause of death, followed by stroke and both conditions increase the risk of dementia (WHO, 2018). These conditions have share modifiable risk factors such as smoking, excessive alcohol consumption, obesity, low levels of physical activity, hypertension, hyperlipidaemia (Yusuf *et al.*, 2004).

Elevated homocysteine and hypertension are both risk factors for the development of cardiovascular disease. An increase of 20mmHg in systolic and of 10mmHg in diastolic blood pressure doubles the risk of cardiovascular disease (Lewington, 2003). Methods for reducing the prevalence of hypertension by lowering homocysteine levels through B-vitamin supplementation have been investigated. One area of particular interest is individuals with the MTHFR 677TT genotype (TT) who have 25% higher homocysteine than MTHFR 677CC genotypes (CC), and are more likely to have high blood pressure and subsequently a 24% greater risk of stroke and 16% greater risk of heart disease (Wilson

et al., 2019). The TT genotype has a worldwide prevalence of around 10% (McNulty *et al.*, 2017) and these individuals can benefit from 6-13mmHg lower blood pressure in response to riboflavin supplementation and thereby reduced risk of cardiovascular disease (McNulty *et al.*, 2017). This level of reduction in blood pressure is significant giving the research carried out by Lewington et al that indicates a 2mmHg reduction in usual systolic blood pressure can bring about a 10% reduced risk of stroke mortality and 7% ischaemic heart disease mortality (Lewington, 2003). A study of the effect of B vitamins on mortality concluded that those with higher B vitamin status had a lower mortality risk. In this study, higher B vitamin status was associated with higher levels of education, greater supplement use and diet diversity as well as better exercise levels (Huang, Lee and Wahlqvist, 2012).

Research suggests that a rise of 20mmHg in systolic and of 10mmHg in diastolic blood pressure brings about a 50% increased risk of cardiovascular disease (Lewington, 2003). A 2017 meta-analysis of stroke prevention in areas with, or without folic acid fortification, found that areas that do not have mandatory fortification benefited the most from folic acid supplementation and that doses up to 0.8mg daily were sufficient in providing these benefits (Zhao *et al.*, 2017). In agreement with this, another study which was conducted in China, found an 11% decrease in stroke risk with folic acid supplementation overall, but, specifically in areas without folic acid fortification there was a 21% decreased risk of stroke when participants took a folic acid supplement in addition to their antihypertensive treatment (Xiao *et al.*, 2017).

1.8. B vitamins and cognition

The World Health Organisation (WHO) has seen a doubling of death as a result of dementia which now make it the 5th largest killer globally (WHO, 2018). Dementia is progressive and several types have been recognised including Alzheimer's disease and vascular dementia. Dementia impacts a range of cognitive abilities such as planning, communicating, comprehending and memory. Impairment in these cognitive abilities and changes in brain structure can also lead to changes in the person's usual behaviour (National Institute for Health and Care Excellence, 2018).

In absence of a cure, the importance of preventive health is crucial and numerous protective factors have been identified. These include having a healthy, balanced diet, being active, maintaining a healthy weight, avoiding smoking and excessive alcohol consumption, as well as managing hyperlipidaemia and hypertension (WHO, 2019). These behaviours also have a protective role in the prevention of type 2 diabetes and cardiovascular events which also increase the risk of dementia (Yusuf *et al.*, 2004). Furthermore, depression, social isolation and low levels of cognitively stimulating activity also increases the risk of dementia (WHO, 2019). In addition to a well-balanced diet, the World Health Organisation (WHO) acknowledged that those with normal cognition or mild cognitive impairment who closely adhere to a Mediterranean style diet may be benefit from reduced risk of cognitive impairment or Alzheimer's disease. The use of supplements, such as B vitamins, to reduce the risk of cognitive impairment are not recommended due to the inconsistent nature of the evidence (WHO, 2019).

A review of the role of B vitamins in brain health has highlighted numerous ways in which B vitamin deficiency can affect brain function such as irritability, fatigue, depression, reduced alertness, autonomic dysfunction and seizures through their role in energy production, protein, DNA, RNA, neurotransmitter synthesis and red blood cell development (Kennedy, 2016). Further to this, studies have shown the capability of B vitamins in reducing homocysteine levels (Oort *et al.*, 2003; Anderson *et al.*, 2010; Kennedy, 2016) which has been named as a predictor of cognitive impairment (Seshadri *et al.*, 2002; Kennedy, 2016). However, the impact of B vitamins on cognition remains inconclusive (WHO, 2019).

A systematic review and meta-analysis of vitamin B6, 12 and folic acid supplementation in participants who already have cognitive diseases showed no cognitive benefit from the intervention. However, the authors note that the Mini Mental State Examination (MMSE), often used, may not be sensitive enough to detect changes (Zhang, Ye and Mu, 2017). However, using the MMSE, Hughes et al have suggested that riboflavin has a protective role in cognition in older adults. The study found after 4 years those who had the greatest riboflavin intake and biomarker status were those who showed the slowest rate of cognitive decline (Hughes *et al.*, 2017).

An intervention study of Asian adults with hyperhomocysteinemia, supplemented of 10mg vitamin B6, 25μg vitamin B12 and 800μg folic acid on a daily basis and found improved homocysteine levels and cognition after 14 weeks of treatment (Cheng *et al.*, 2014). In another systematic review which investigated the effect of dietary supplements in adults without cognitive impairment also concluded that B vitamin

intervention had no significant effect on cognitive scores in the MMSE. Improved cognition was only noted in one study in which participants with low folate intake at baseline benefited (Forbes *et al.*, 2015).

Similarly, a dietary supplement intervention in participants who have mild cognitive impairment showed an improvement in verbal memory for those who had low biomarker status at baseline and received B vitamin supplementation. Furthermore, in participants with elevated homocysteine, those who received a B vitamin supplement had a superior verbal leaning test score. However, those whose homocysteine levels were in the normal range did not benefit from intervention. The review also concluded that those who scored better in MMSE had higher homocysteine levels and therefore benefited from supplementation as it corrected their suboptimal status (Mcgrattan *et al.*, 2018).

Recently, studies have incorporated the use of neuroimaging techniques to pinpoint where the role of B vitamins in brain health is. The BrainHOP trial supplemented adults over the age of 70 with daily supplementation of 10 mg B2, 10mg B6, 400µg folic acid and 10 µg B12 for 2 years. This study utilised the Repeatable Battery of the Assessment of Neuropsychological Status (RBANS), a more robust cognitive test, which revealed that supplementation offers some protection against cognitive decline specifically in the visuospatial cognitive domain. Furthermore, the author has noted that the preliminary results from Magnetoencephalography (MEG) imaging conducted on the participants indicates some improvement in neuron function (Moore *et al.*, 2018). A small intervention study of healthy adults (n=19) between 30 and 65 years who received the

high doses multivitamin for 6 months utilised neuroimaging techniques and found increased neural metabolism markers which the authors report may reduce inflammation and oxidative stress which is favourable for brain health (Ford *et al.*, 2018). Furthermore, a range of other benefits, relevant to risk of cognitive decline, have been noted in relation to B vitamin supplementation such as improved perception of quality of life and reduced rates of depression. A small study found that provided hospitalised patients with a B vitamin containing supplement drink found lower homocysteine levels and those with the lowest levels of homocysteine also had the lowest depression scale scores (Gariballa, 2011). Using the same supplements, an assessment of quality life which consisted of questions regarding perceived health, pain, functional ability, energy levels and mental health were asked before and after the 6-week intervention. Those with the lowest homocysteine concentration had the highest quality of life (Gariballa, 2013). Another study, involved participants were with diagnosed depression and treated with citalopram and either B vitamin supplements or a placebo for 52 weeks. After 12 weeks there was no significant difference in recovery, however, after one year those on supplements were more likely to be in remission and less likely to have had a relapse at the 12th week of intervention (Ford *et al.*, 2015).

These studies suggest that correcting elevated homocysteine levels and B vitamin deficiencies will impact cognition. However, the evidence to suggest supplementation will benefit everyone is not clear as some of these studies only show benefit for those who initially had undesirable biomarker status. Therefore, further studies are required, particularly large longitudinal studies with vigorous cognitive tests as changes in cognition may take years to develop and a sensitive test would be more likely to detect

slight changes. The Irish LongituDinal Study on Ageing (TILDA) is a large, representative longitudinal study of older adult which has collected data over a ten year period. Similar to the TUDA study, TILDA has tested the participant's cognition, vitamin biomarker status such as plasma folate and vitamin B12 and has gathered information regarding the participant's medication and supplement use, medical history, anthropometry and mood. However, the TUDA study also includes RCF, MMA and homocysteine measurement, and an assessment of habitual dietary intake with the use of a food diary and FFQ.

1.9. Aims and objectives

The overall aim of this study is to quantify dietary intake of folate, folic acid and vitamin B12 and their respective biomarker concentration in older Irish adults in relation to cognitive performance.

Objectives

1. Assess dietary intake of folate, folic acid and vitamin B12 in older adults using a combination of food frequency questionnaires and a food diary.

2. Determine biomarker status using red blood cell folate, methylmalonic acid, serum B12 and homocysteine.

3. Test frontal lobe cognition using the frontal assessment battery (FAB) and global cognitive function using both the Mini Mental State Examination and the Repeatable Battery for the Assessment of Neuropsychological Status in order to give a brief and detailed assessment of global cognition.

4. Investigate if there is an association with dietary intake of folate, folic acid or vitamin B12 or their respective biomarker status on cognitive outcomes with consideration of confounding variables.

Chapter 2

Methodology

Study population

Participants for this study were initially recruited between 2008 and 2012 for the Trinity, Ulster, Department of Agriculture (TUDA) ageing cohort study. The overall aim of the cross-sectional study was to investigate nutritional and genetic factors in the development of chronic conditions of ageing. Therefore, community dwelling participants from Northern Ireland and Ireland who have phenotypic evidence of early stages of Alzheimer disease, osteoporosis, or cardiovascular disease (CVD) were invited to participate. The inclusion criteria were: patients must be >60 years of age and attend (or have attended) either the memory or bone clinic or hypertensive GP clinic and have been diagnosed with mild-moderate cognitive dysfunction or osteopenia. Recruitment of 5186 participants which included a cognitively impaired sub-cohort (n=1699) and an osteoporotic sub-cohort (n=1394) from day clinics at St. James hospital. The hypertensive cohort were recruited from Altnagelvin and Causeway Hospital in Northern Ireland. Ethical approval for this study was obtained from the Research Ethics Committee of St. James's Hospital and the Adelaide and Meath Hospital, Dublin, Northern and Western Health and Social Care Trusts and the Office for Research Committees Northern Ireland (ORECNI; reference 08/NI/RO3113). All participants provided written informed consent at the time of enrolment with all blood samples and questionnaire data coded and made anonymous prior to analysis and relevant GDPR guidelines were adhered to. Further recruitment and participant details of those in the study have been reported previously (Laird *et al.*, 2014; McCarroll *et al.*, 2015).

In the TUDA follow up study, participants who were over 65 years old who had a Mini Mental State Examination (MMSE) score of ≥24 at baseline were considered eligible and

were contacted by post (appendix 1). A total of 987 participants aged >65 years were recruited between 2014 and 2019 and the average period between their first and second appointment was 6 years. Each participant provided written informed consent on enrolment to the study, the data obtained from each participant was anonymous before analysis was carried out. **Figure 2** illustrates the participant recruitment and data collection.

Assessments

Each participant attended a research appointment lasting approximately 90 minutes. During which, participants were asked to undergo various physical measurements, complete a health and lifestyle questionnaire, cognitive tests and give a blood sample. (The health and lifestyle questionnaire and the cognitive tests can be viewed in appendix 2, 3, 4 and 5).

Measurements

Height was measured using SECA 213 Stadiometer and weight using SECA scales (model 888) which were regularly calibrated. Each participant was asked to remove shoes and step onto the stadiometer with their back against the measuring stick, keeping their arms by their side and looking straight ahead. The participant was then asked to step off the stadiometer whilst bending their knees and the researcher recorded the height in centimetres. Participants were asked to keep their shoes off and to remove any heavy, loose clothing and to step onto the scale, looking straight ahead with their arms by their

sides and the researcher recorded their weight in kilograms on the questionnaire. Body mass index (BMI) was calculated using these values by dividing the participants weight in kilograms by their height in metres squared. A measurement of waist and hip circumference was taken with a SECA 200 measuring tape (accurate to 1mm) and used to calculate their waist hip ratio by dividing their waist circumference by the hip circumference. The waist measurement was obtained by asking the participant to stand and lift their top until their belly button was exposed and the researcher wrapped the measuring tape around this point. The researcher then took a measurement of the widest part of the participant's hips and recorded this for the hip measurement. All researchers were trained in conducting these measurements in University of Ulster with the wider TUDA team and each researcher followed the standard operating procedure to limit variability. Furthermore, the technique used by each researcher was checked at multiple points during the study to ensure the correct procedure was followed (the standard operating procedure can be viewed in appendix 7). Participants were also asked to complete a 'timed up and go' whereby they were timed as they rose from their seat, walked 3 metres and returned to their seat. Participants were asked to walk at their usual walking pace and if required use their usual walking aid and this was used to give an indication of functional mobility (Richardson and Podsiadlo, 1991).

Health and lifestyle questionnaire

Participants were asked to report their medical history including history of transient ischaemic attack (TIA), stroke and gastrointestinal disorders. They also reported current medication use including statins, metformin and medications which reduce gastric

secretions such as proton pump inhibitors (e.g. omeprazole, lansoprazole) and H2 receptor blockers (e.g. ranitidine). Participants were asked to report any exercise they had completed in the previous two weeks, whether they have or currently consume alcohol and if they are a current smoker. The Ability to undertake self-care was assessed using Instrumental Activities of Daily Living (IADL) and Physical Self-Maintenance Scale (PSMS) (Lawton and Brody, 1969). The Centre for Epidemiological Studies Depression Scale was used with scores of 16 or above indicating depression (Radloff, 1977). Furthermore, participants were asked to bring a detailed list of prescribed and non-prescribed supplement and to bring the product to the appointment if possible so that full details of the products could be recorded such as name, producer, contents and dosage. If sufficient detail to enable verification of the product was not provided at the research appointment, a telephone call was made to retrieve further details. The products of interest included multivitamins, vitamin B complex, single vitamins (e.g. folic acid or vitamin D), specialist products such as oral nutrition support and formulas with special claims e.g. for hair, skin and nails or joint health.

Dietary Assessment

Dietary intake data was compiled using a validated food frequency questionnaire (FFQ) and a four-day food diary (Hoey *et al.*, 2007). The FFQ consisted of B vitamin rich foods such as meat, poultry, fish, dairy and eggs. Participants were also asked to report use of B vitamin fortified foods such as breakfast cereals and milk and use of nutritional supplements. This information was collected during the research appointment and was used to support and verify the data which the participant recorded in the food diary ,

which they were asked to complete over 4 consecutive days including Saturday and Sunday and then return to the researchers. Participants were asked to record weekdays and the weekend to capture the variability which can occur in the diet across the week. Although including 2 weekdays and 2 weekend days is not representative of a typical week, analysis of the NDNS has indicated that is benefit to recording both Saturday and Sunday as there can be considerable difference in dietary intake on each of these days (Thane and Stephen, 2006). A guide was provided at the start of the food diary to indicate how the participant was required to complete it and contact details were provided if the participant wished to contact the researcher for more information. Participants were asked to give an indication of portion size (a copy of the food diary can be viewed in appendix 6).

Nutrition analysis of the 879 diaries returned was carried out in Nutritics. Nutritics is an Irish nutrition software which has a database of foods and drinks which is updated regularly. For this study, food diaries were entered into Nutritics manually as described by the participants. The researcher checked the contents of the food diary with the FFQ to verify any missing information such as brand names of fortified foods and frequency of use. In the event that the specific brand was not present on Nutritics, the researcher identified the closest match which had a similar level of fortification. Participants were asked to include portion sizes and information was provided as a guide for this, for example, how many tablespoons, slices, proportion of the packet usedif no details regarding portion size were supplied, the researcher attempted to contact the participant by telephone within one week of receiving the food diary to complete any

missing information and if this was not possible a medium portion was assigned as a standardised protocol to reduce variability within the research team. Once entered, a nutritional analysis was carried out which provided an average daily intake of natural folate, folic acid (FA), total folate and B12. To account for the greater bioavailability of FA, dietary folate equivalents (DFE) were calculated by multiplying FA (μg) intake by 1.7.

Cognitive Assessments

The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) was used to assess a range of cognitive domains including immediate and delayed memory, attention, visuospatial and language(Randolph *et al.*, 1998). The total score from RBANS and Mini Mental State Examination (MMSE) were used to assess global cognition and a score of <80 in the former and <24 in the latter was used to identify possible cognitive impairment (Folstein, Folstein and McHugh, 1975; Randolph *et al.*, 1998). Whilst the Frontal Assessment Battery (FAB) was used to assess executive function and possible impairment was indicated for those who scored ≤12 (Dubois, 2000).

Blood Sampling and Nutritional Biomarker Analysis

A non-fasted blood sample of approximately 50ml (in 8 blood tubes) was taken from each participant by a trained phlebotomist. After collection, four of the blood tubes (two EDTA, one serum and one sodium fluoride) were sent to the respective hospital laboratory for biochemical analysis of full blood count which was used to determine presence of anaemia (<12g/dL for women and <13g/dL for men) and macrocytosis

(mean cell volume ≥99fL) (Nilsson-Ehle *et al.*, 2000; WHO, 2001) and estimated glomerular filtration rate (eGFR) using a Roche Cobas analyser with full method standardisation. The other four tubes: two EDTA, one serum gel separator and one lithium heparin (the lithium heparin sample was used in other studies conducted by the TUDA researchers), were processed in the University's research laboratories and used for nutritional biomarker analysis. One of the blood samples collected in an EDTA tube was kept chilled and the remaining three tubes were kept at room temperature. The EDTA tube which was stored at room temperature was first placed on a roller for 10 minutes to ensure the blood was well mixed with the EDTA. 50µl of this blood was removed mixed by inversion with 450µl of 1% solution of ascorbic acid and left for 60 minutes at room temperature. This sample was analysed to determine the red blood cell folate concentration using chloramphenicol-resistant strain of Lactobacillus casei, this was conducted at Bevital, Bergen (Molloy and Scott, 1997). The chilled EDTA blood tube was centrifuged at 3000rpm for 15 minutes and the plasma was aliquoted. The plasma from this sample was sent to Bevital, Bergen 0.5ml of plasma was used to measure homocysteine and 0.75ml for MMA by using gas chromatography mass spectrometry (Windelberg *et al.*, 2005). The red serum gel separator blood tube was stored at room temperature for 30 minutes before centrifuging at 3000rpm for 15 minutes. The serum obtained was aliquoted for vitamin D and serum B12 analysis. 0.5ml of this serum was used for Vitamin D, which was measured using liquid chromatography mass spectroscopy which took place at St James Hospital and a L.leichmanii (delbrueckii) microbiological assay was carried out for serum B12 analysis using 1ml of serum which was conducted at Bevital, Bergen (Kelleher and Broin, 1991). Folate status was assessed using red blood cell folate concentrations and plasma homocysteine. Vitamin B12 status

was assessed using methylmalonic acid (MMA) and serum total B12 concentration. Elevated status of homocysteine and MMA, and lowered status of serum B12 was determined if analysis indicated >15µmol/L, >0.37µmol/L and <148pmol/L, respectively (Green et al., 2017). Methylenetetrahydrofolate reductase (MTHFR) genotyping was conducted at University of Ulster using the buffy coat obtained from the centrifuged EDTA blood samples. This was used to determine if participants had either the TT, CT or CC genotype which can influence homocysteine levels (Frosst *et al.*, 1995). Prior to nutritional biomarker analysis, aliquoted samples were frozen at -80°C (the blood processing protocol can be viewed in the appendix 8).

Statistical Analysis

All statistical procedures were carried out using IBM SPSS Statistics 25. Firstly, the data was checked for normality using Q-Q plots and Kolmogorov-Smimov tests and if necessary, log transformations or square roots were used. A paired t-test was used to determine if there was significant difference between the participant's weight, blood results and cognition scores between their initial recruitment and follow up appointment. Data was expressed in tables as medians with 25th and 75th centile as the data was skewed and P values less than 0.05 were considered significant.

Partial correlation, controlling for age and gender, was carried out to determine correlation between dietary intake of folate, DFE, total folate and total vitamin B12 intake and their respective biomarkers. When considering folate related intake those who reported using folic acid were removed from the correlation analysis and for

vitamin B12 intake those who reported using vitamin B12 injection or oral supplements were removed from the analysis so that true dietary intake in relation to biomarker status could be assessed.

Those who completed a food diary (n=879) were used in analysis related to fortified food intake, recommended daily intakes of folate and vitamin B12 and biomarker status. Those who did not consume fortified foods within the 4 days were allocated to the non-consumer groups and those who did consume fortified foods were split into tertiles depending on their folic acid intake. Participants were split according to folic acid intake because natural folate was quite consistent across each consumer group and therefore folic acid consumed through fortified foods was an important contributor to the variability in total folate intake. .The four groups were analysed using MANCOVA with Bonferroni to adjust for multiple comparisons to determine if there are significant changes between the each of the four group's biomarker status or nutrient intake (p<0.05).

Performance in MMSE, FAB and RBANS and its indexes were analysed in relation to dietary intake and biomarker status using MANCOVA with Bonferroni to adjust for multiple comparisons, whilst controlling for factors that may impact cognitive performance or vitamin B12 and folate status such as age, gender, waist hip ratio, history of TIA or stroke, gastrointestinal disorders, exercise, smoking, alcohol, depression, vitamin D, folic acid, vitamin B12 supplements, PPI, statin, metformin or vitamin B12 injections. Dietary intake of total folate, DFE, vitamin B12 and concentration

of serum B12, MMA, homocysteine and red blood cell folate were split into quartiles and each quartile was compared against the other for this analysis. Quartiles were used as the differences between the scores in the cognitive tests were very slight. This allowed for greater sensitivity in detecting changes which could be attributed to B vitamin intake or biomarker status. These results were illustrated in tables which displayed the median, 25th and 75th centile and significant differences ($p<0.05$).
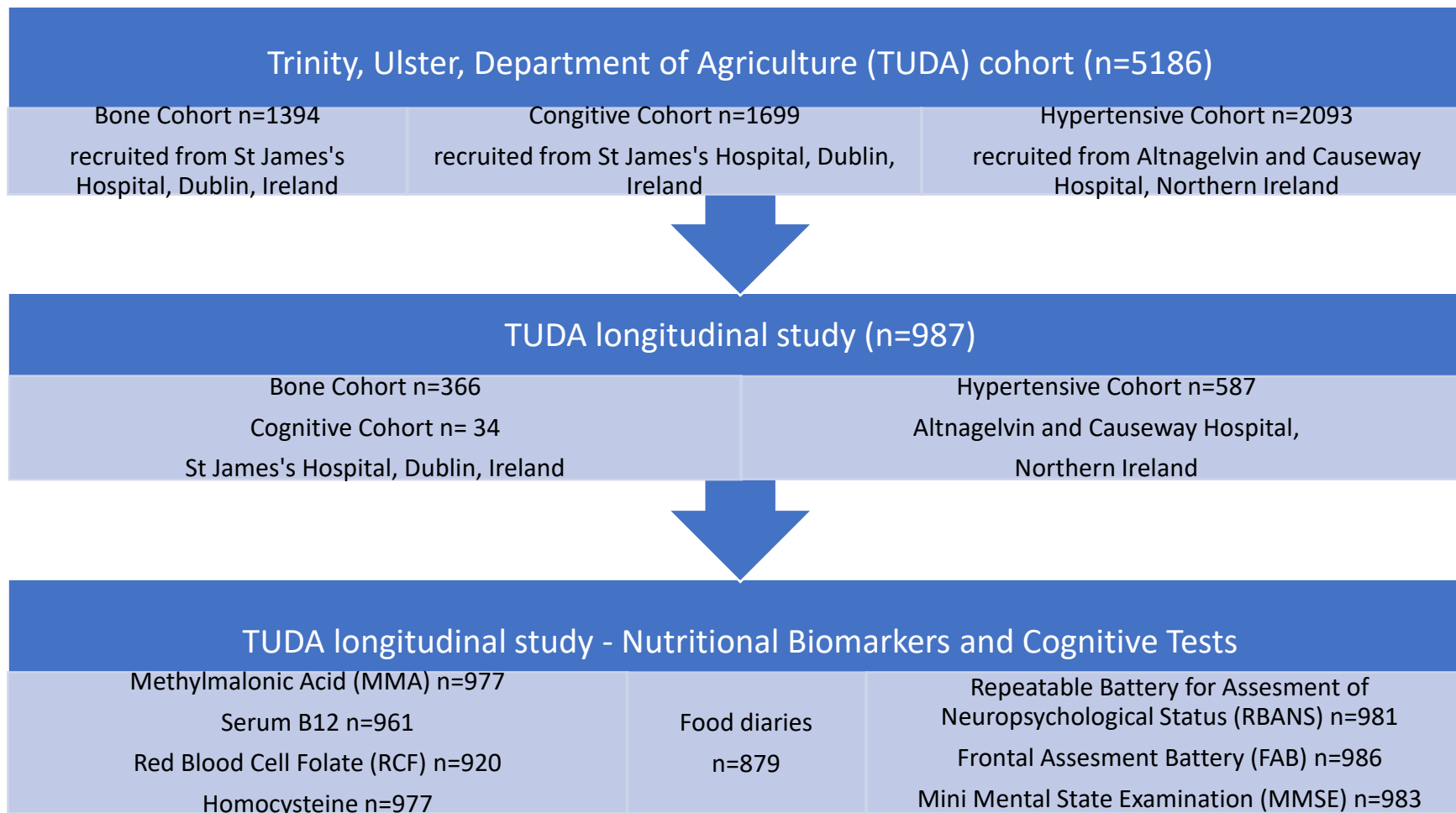
Figure 2 Participant recruitment for the Trinity Ulster Department of Agriculture (TUDA) study and relevant information gathering.

Chapter 3

Results: Demographic, lifestyle, health, and B vitamin status from the longitudinal TUDA study

It is expected that in ageing, there will be changes due to cellular damage of many years that leads to an increased risk of functional decline and the development of disease in older age. However, development of disease such as dementia is not a normal part of ageing (WHO, 2015b).

Participants underwent the same questions, measurements, and testing at baseline and in the longitudinal study which allowed comparisons to be made using a paired t-test. The health and lifestyle characteristics of the participants recruited for the TUDA study can be seen in **Table 2** and it also shows the demographic and lifestyle changes across time from the original study.

A total of 987 participants from the original 5186 participants were reassessed, on average, 6 years later. They had a median age of 75.8 years and 67.1% were female. The median body mass index (BMI) of the cohort was 27.7 kg/m^2 which, overall, did not differ from their BMI at the initial recruitment.

In terms of lifestyle factors, there was a decrease in smoking and alcohol consuming status, 9.5% to 5.6% ($p<0.001$) and 66.7% to 62.9% ($p<0.001$) respectively. The time taken to complete the timed up and go (TUG) increased from 8.0 seconds to 10.7 seconds ($p<0.001$).

The use of the medications that could impact vitamin B12 status increased from 28.2% to 37.7% (p<0.001) for proton pump inhibitors (PPI), from 5.9% to 8.2% (p<0.001) for metformin and from 51.5% to 59.0% (p<0.001) for statins. In addition to this, there also was an increase in oral folic acid (FA) and vitamin B12 supplementation, from 11.1% to 14.1% (p<0.001) and 8.0% to 10.5% (p<0.001) respectively, whilst reported use of B12 injection remained at approximately 0.4% (p>0.05).

Although supplement use increased, biomarker status (**Table 3**) as indicated by red blood cell folate and homocysteine did not improve. The median red cell folate decreased from 940.9 nmol/L to 766.0 nmol/L (p<0.001) and homocysteine increased from 12.5 μmol/L to 13.2 μmol/L (p<0.001) which resulted in approximately 10.0% more participants being classified as having elevated homocysteine levels (>15 μmol/L). The median serum B12 levels increased from 270.0 pmol/L to 277.5 pmol/L (p<0.001) but the proportion of participants with serum B12 levels below 148.0 pmol/L, increased from 8.5% to 16.2% (p<0.001). Median MMA levels increased from 0.33 μmol/L to 0.34 μmol/L (p<0.05) and the proportion of those with elevated MMA levels , which indicates possible deficiency, was 42.0% at baseline and 42.9% longitudinally (p=0.858). However, it is important to note that only 119 participants had MMA levels analysed at both time points.

In terms of cognitive changes (**Table 4**), three tests were used and the total scores for each test decreased significantly. For the RBANS, the median score decreased by 3.2% from 95.0 to 93.0 (p<0.001) and the proportion of participants falling below the cut off

(<80) which is for impairment increased from 12.7% to 17.3% (p<0.001). The mean total FAB score decreased by 9.1% from 16.5 to 15.0 (p<0.001) across the recruitment period and there was an increase from 2.9% to 12.7% (p<0.001) in those falling below the 12 points which indicates possible impairment. The median score for the MMSE stayed the same, the mean, however decreased by 3.2% from 28.3 to 27.4 (p<0.001) and those scoring less than 24 points increased to 5.1% (p<0.001).

The RBANS test allows for both total scores and scores for individual cognitive domains to be determined, scores for each domain decreased significantly, exception RBANS index 4 (p>0.05), which tests attention. The first domain tested immediate memory and the median score remained at 27, however the mean score decreased by 1.7% from 97.2 to 95.5 (p<0.001). The second domain tested visuospatial/ constructional cognition and the median score decreased by 4.0% from 100.0 to 96.0 (p<0.001). The third domain tested language and this decreased by 4.1% from 96.0 to 92.0 (p<0.001). And the median for the fifth domain, delayed memory, stayed at 98 but the mean score decreased by 1.5% from 95.0 to 93.6 (p<0.001).

Table 2: Health and lifestyle characteristics of the Trinity Ulster Department of Agriculuture (TUDA) cohort at baseline and longitudinally.

TUDA Cohort			
Gender	325 males (32.9%) 662 females (67.1%)		
MTHFR Genotype	CC 43.4% (n=415), CT 44.7% (n-427), TT 11.9% (n=114)		
Age left education [1]	16 (14, 18)		
	Baseline (n=987)[1]	**Longitudinal (n=987)**[1]	**P value**[2]
Age (years)	69.9 (65.6, 73.3)	75.8 (72.4, 79.1)	<0.001
BMI (kg/m^2)	27.7 (24.7, 30.8)	27.7 (24.6, 30.7)	0.149
Current smoker	9.5% (n=94)	5.6% (n=55)	<0.001
Alcohol consumer	66.7% (n=658)	62.9% (n=621)	<0.001
Gastrointestinal disorder	20.6% (n=203)	42.9% (n=423)	<0.001
TIA[3]	5.4% (n=53)	8.5% (n=84)	0.249
Stroke	2.2% (n=22)	3.4% (n=34)	0.318
PPI use	28.2% (n=278)	37.7% (n=372)	<0.001
Metformin use	5.9% (n=58)	8.2% (n=81)	<0.001
Statin use	51.5% (n=508)	59.0% (n=582)	<0.001
Folic acid use	11.1% (n=104)	14.1% (n=139)	<0.001
Oral B12 use	8.0% (n=75)	10.5% (n=104)	<0.001
B12 injection use	0.5% (n=5)	0.4% (n=4)	1.00
TUG (seconds)	8.0 (7.0, 10.0)	10.7 (9.1, 12.9)	<0.001
PSMS	24.0 (24.0, 24.0)	24.0 (23.0, 24.0)	<0.001
IADL	27.0 (26.0, 28.0)	28 (26.0, 28.0)	<0.001

1 Median followed by 25th and 75th centile in brackets.

2 Statistical differences between first and second recruitment point was calculated using a paired t-test. Differences are considered significant if <0.05.

3 TIA: Transient Ischaemic Attack, TUG: Timed up and go, PSMS: Physical self-maintenance scale, IADL: Instrumental activities of daily living.

Table 3: Changes in biomarker status of the TUDA cohort between the baseline and longitudinal study.

	Baseline (n=987) [1]	Longitudinal (n=987) [1]	P value [2]
RCF (nmol/L)	940.9 (682.2, 1326.0)	766.0 (568.0, 1061.0)	<0.001
MMA (µmol/L) [3]	0.33 (0.24, 0.54)	0.34 (0.25, 0.55)	<0.05
MMA indicating deficiency [3,4,]	42.0% (n=50)	42.9% (n=51)	0.858
Serum B12 (pmol/L)	270.0 (204.3, 356.1)	279.0 (185.0, 379.3)	<0.001
Serum B12 indicating deficiency) [4]	8.5% (n=83)	15.8% (n=152)	<0.001
Homocysteine (µmol/L)	12.5 (10.7, 14.9)	13.2 (10.8, 16.4)	<0.001
Elevated homocysteine [4]	24.5% (n=241)	34.9% (n=341)	<0.001
Anaemic [4]	10.8% (n=107)	13.5% (n=130)	<0.001
Macrocytosis [4]	1.8% (n=18)	3.0% (n=29)	<0.001
Impaired kidney function [4]	21.0% (n=203)	29.1% (n=283)	<0.001

1 Median followed by 25th and 75th centile in brackets.

2 Statistical differences between first and second recruitment point was calculated using a paired t-test. Differences are considered significant if <0.05.

3 The values and the statistical analysis displayed are for n=119 who had MMA assessed at both time points.

4 MMA deficiency >0.37µmol/L, serum B12 deficiency <148pmol/L, elevated homocysteine >15µmol/L, anaemia <12g/dL haemoglobin for females and <13g/dL for males, macrocytosis mean cell volume ≥99fL and impaired kidney function: eGFR <60ml/min/1.73m^2

Table 4: Changes in the TUDA cohort's cognitive test scores between the baseline and longitudinal study.

	Baseline (n=987) [1]	Longitudinal (n=987) [1]	P value [2]
RBANS total	95.0 (85.0, 105.0)	93.0 (83.0, 103.0)	<0.001 [2]
RBANS index 1	97.0 (87.0, 109)	97.0 (85.0, 106.0)	<0.001
RBANS index 2	100.0 (87.0, 112.0)	96.0 (84, 109)	<0.001
RBANS index 3	96.0 (90.0, 101.0)	92.0 (88.0, 96.0)	<0.001
RBANS index 4	97.0 (85.0, 109.0)	97.0 (88.0, 109.0)	0.267
RBANS index 5	98.0 (86.0, 103.0)	98.0 (84.0, 107.0)	<0.001
RBANS score indicating impairment (<80)	12.7% (n=124)	17.3% (n=170)	<0.001
FAB total score	17.0 (16.0, 18.0)	15.0 (14.0, 17.0)	<0.001
FAB score indicating impairment (<12)	2.9% (n=29)	12.7% (n=125)	<0.001
MMSE total score	28.0 (27.0, 29.0)	28.0 (27.0, 29.0)	<0.001
MMSE score indicating impairment (<24)	0.6% (n=6)	5.1% (n=50)	<0.001

1 Median followed by 25th and 75th centile in brackets.

2 Statistical differences between first and second recruitment point was calculated using a paired t-test. Differences are considered significant if <0.05.

Chapter 4

Results: Dietary analysis and biomarker status of folate and vitamin B12

A total of 879 participants returned food diaries, and these were used to assess dietary intake of folate, FA (folic acid) and B12 using Nutritics. **Table 5** details the relationship between FA fortified foods in relation to achievement of recommended nutrient intake (RNI) and biomarker status and **Figures 3 to 11** show the correlation between biomarkers and dietary intake.The results (Table 5) indicated that those who consumed the most FA fortified foods were slightly younger at a median age of 74 years rather than 76 years. The highest BMI (28.3 kg/m^2) was found in the low consumer group and this was significantly higher than both non-consumers and high consumers. Whilst the high consumers had the lowest BMI (26.3 kg/m^2) and this was significantly lower than both the non-consumers and low consumers ($p<0.05$). There were no statistical differences between level of fortified food consumption and TT genotype.

Natural folate intake is similar for each group, at approximately 200μg per day. There is a significant increase in total folate (dietary folate equivalents and natural folate intake) which increases from 200.5μg for non-consumers to 232.1μg, 287.2 μg and 438.3 μg for low, medium and high consumers respectively.

Although the median intake for folate meets the 200μg daily recommendation, 49.0% of non-consumers fail to meet this, however, those who chose to consume fortified foods were more likely to achieve this recommendation. Those who did not achieve the RNI decreases across each group to 27.3% of low consumers, 4.9% of medium consumers and 0% of high consumers.

This improvement in dietary intake is reflected in biomarker status, as high consumers of FA fortified foods have a significantly higher red blood cell folate (RCF) (948.5nmol/L) than non-consumers (648.0nmol/L). Homocysteine levels were lowest for the highest consumers (11.6 µmol/L) and this was significantly lower than non-consumers (14.3µmol/L), low consumers (14.1µmol/L) and medium consumers (13.4µmol/L) and summarily with elevated homocysteine status, there was a decrease across the groups from 45.0% in non-consumers to 19.2% in the highest consumers.

In this study, total folate intake correlated well with both RCF (**Figure 5** r^2=0.288 p<0.001) and homocysteine (**Figure 8** r^2= -0.314 p<0.001). Natural folate correlates to a lesser extent with RCF (**Figure 3** r^2= 0.117 p<0.005 and homocysteine (**Figure 6** r^2= -0.205 p<0.001. Whilst DFE shows little correlation with RCF (**Figure 4** r^2= 0.036 p=0.316) and homocysteine (**Figure 7** r^2= 0.031 p=0.377). However, this is not surprising as natural folate and DFE only represent a portion of the total intake.

FA fortified foods often contain other B vitamins, particularly vitamin B12, and the highest consumers of these foods have statistically better vitamin B12 intake and biomarker status as indicated by methylmalonyl acid (MMA) and serum B12. Dietary intakes of vitamin B12 below the RNI appeared to be very rare with only three individuals have less than 1.5µg per day. Despite this, possible deficiency was indicated for 18.1% to 24.4% of non-consumers, 18.1% to 25.0% of low consumers, 15.0% to 24.5% of medium consumers and 8.9-14.0% of high consumers based on their serum B12 and MMA status (Table 5). This can also be seen in the poor correlation between

the vitamin B12 biomarkers, serum B12 (R^2=-0.002, p=0.955) and MMA (R^2=0.01, p=0.776), and dietary intake of vitamin B12 (MMA and correlation with dietary B12 shown in **Figure 9**, serum B12 and dietary B12 shown in **Figure 10** and homocysteine and dietary B12 shown in **Figure 11**). Perhaps this disparity between vitamin B12 intake and status indicates an over estimation of intake as participants were not required to weigh their food. It also may be related to malabsorption of food-bound vitamin B12.

Table 5: Comparison of B vitamin intake and Biomarker status by intake of folic acid fortified foods [1]

Characteristic	Non-consumer (n=259) (0 µg per day)	Low consumer (n=209) (1-30 µg per day)	Medium consumer (n=205) (31-78.5 µg per day)	High consumer (n=206) (>78.76 µg per day)
Age [3]	76.6 (71.9, 79.3) [2]	76.2 (72.8, 79.6)	76.3 (72.9, 79.6)	74.3 (71.5, 76.9)
Gender- male	36.3% (n=94)	34.4% (n=72)	33.7% (n=69)	28.2% (n=58)
BMI (kg/m2) [5]	27.8 (23.9, 27.9) [a]	28.3 (25.5, 31.1) [b]	27.6 (25.0, 30.9) [abc]	26.3 (23.8, 30.0) [ac]
TT genotype	14.6% (n=37)	10.4% (n=21)	13.1% (n=26)	10.0% (n=20)
Energy (kcal) [6]	1579.6	1567.6	1589.7	1630.5
Natural folate (µg/day)	200.5 (167.7, 246.9)	202.4 (171.6, 257.7)	203.0 (170.7, 237.3)	223.5 (175.8, 265.4)

Table 5: Continued

Characteristic	Non-consumer (n=259) (0 µg per day)	Low consumer (n=209) (1-30 µg per day)	Medium consumer (n=205) (31-78.5 µg per day)	High consumer (n=206) (>78.76 µg per day)
Added folic acid (µg/day)	0.0 [a]	16.0 (8.3, 24.6) [b]	49.8 (39.1, 64.9) [c]	124.1 (95.2, 176.0) [d]
Dietary Folate Equivalents (µg/day)	0.0 [a]	27.2 (14.1, 41.8) [b]	84.7 (66.5, 110.2) [c]	210.9 (161.9, 299.3) [d]
Total folate (µg/day)	200.5 (167.7, 246.9) [a]	232.1 (196.4, 284.6) [b]	287.3 (254.6, 327.5) [c]	438.3 (374.4, 545.5) [d]
Below folate RNI [5] (200µg/day)	49.0% (n=127) [a]	27.3% (n=57) [b]	4.9% (n=10) [cd]	0.0% [d]
Dietary vitamin B12 (µg/day)	4.6 (3.4, 6.4) [a]	5.0 (3.8, 6.0) [ab]	5.0 (4.0, 6.8) [ab]	5.5 (4.1, 6.7) [b]

Table 5: Continued

Characteristic	Non-consumer (n=259) (0 µg per day)	Low consumer (n=209) (1-30 µg per day)	Medium consumer (n=205) (31-78.5 µg per day)	High consumer (n=206) (>78.76 µg per day)
Below B12 RNI [5] (1.5µg/day)	1.2% (n=3)	0.0% (n=0)	0.5% (n=1)	0.0% (n=0)
Plasma homocysteine (µmol/L)	14.3 (11.0, 18.2) [a]	14.1 (11.0, 16.8) [a]	13.4 (11.2, 16.5) [a]	11.6 (9.6, 14.4) [b]
Elevated homocysteine (>15µmol/L)	45.0% (n=116) [a]	41.2% (n=84) [a]	34.8% (n=71) [a]	19.1% (n=39) [b]
Red blood cell folate (nmol/L)	648.0 (509.0, 867.1) [a]	787.9 (568.5, 996.0) [ab]	776.0 (578.4, 1069.4) [ab]	948.5 (678.0, 1253.0) [b]
Serum B12 (pmol/L)	259.0 (166.2, 368.5) [a]	246.0 (152.0, 325.8) [a]	294.7 (192.3, 392.0) [ab]	311.5 (221.5, 431.6) [b]

Table 5: Continued

Characteristic	Non-consumer (n=259) (0 µg per day)	Low consumer (n=209) (1-30 µg per day)	Medium consumer (n=205) (31-78.5 µg per day)	High consumer (n=206) (>78.76 µg per day)
Serum B12 deficiency (<148 pmol/L)	18.1% (n=46) [ab]	24.0% (n=49) [a]	15.0% (n=30) [ab]	8.9% (n=18) [b]
MMA (µmol/L) [5]	0.26 (0.20, 0.36) [ab]	0.25 (0.20, 0.32) [a]	0.25 (0.19, 0.36) [ab]	0.22 (0.18, 0.29) [b]
MMA (>0.37µmol/L) [5]	24.4% (n=63)	18.1% (n=37)	24.5% (n=50)	14.0% (n=29)

1 Determination of consumer status was based on completion of the food diary (n=879) and those who reported using supplements were removed for this analysis. Those who consumed a folic acid fortified food in this period were splits into 3 groups according to tertiles of folic acid intake and those who did not consume a folic acid fortified food was allocated to the 'non-consumer group'.

2 Median, 25th and 75th percentile displayed.

3 Significant differences in age distribution between the groups was adjusted for using MANCOVA and log transformed data was used as required.

4 Values which are significantly different from others in the same row are indicated using different letters (p<0.05), rows without these letters have no values of statistically significant difference.

5 BMI: Body Mass Index, RNI: Reference Nutrient Intake, MMA: Methylmalonic Acid

6 Mean energy intake recorded in kilocalories (kcal). Based on Basal Metabolic Rate 20.6% of food diaries were under-reported.
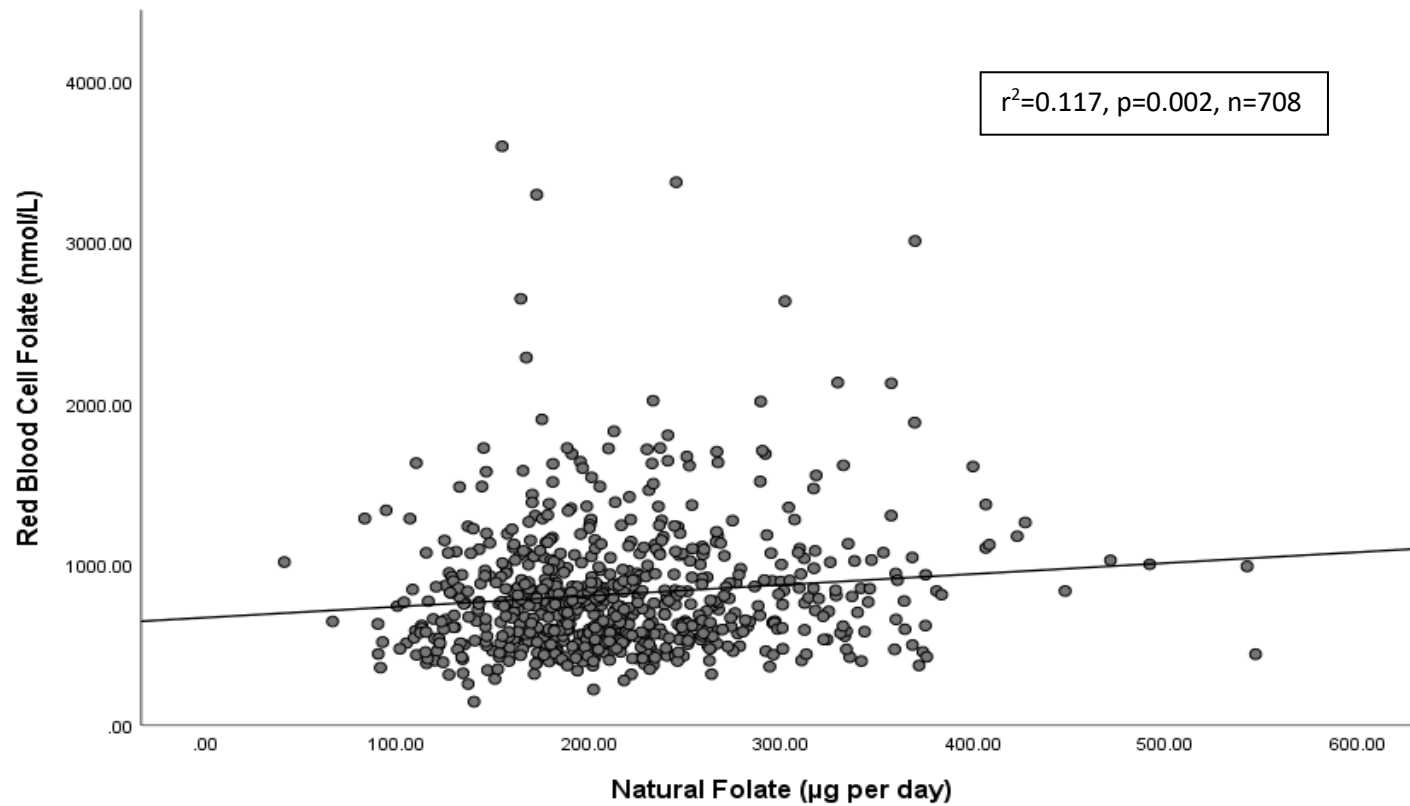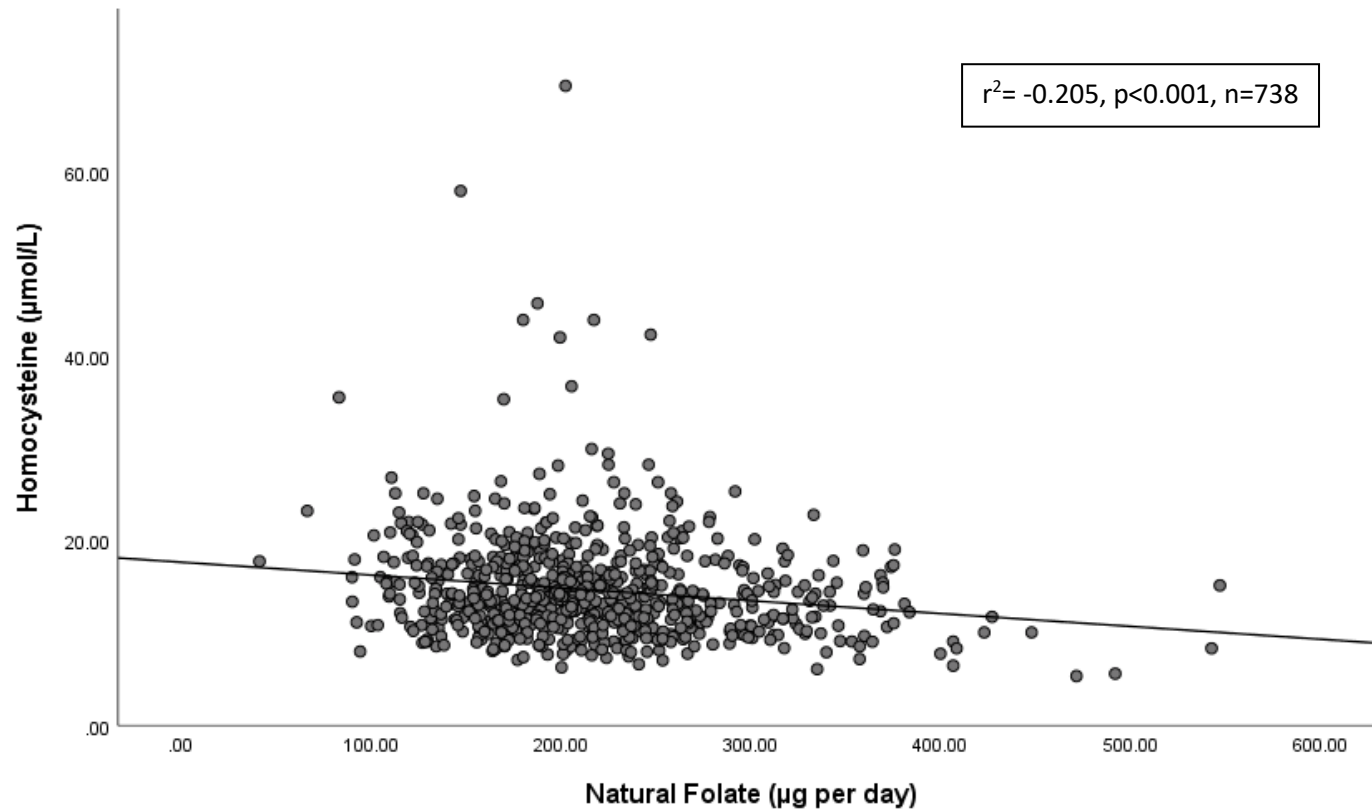
Figure 3 Correlation between natural folate intake and RCF status. Partial correlations were carried out with log transformed data as appropriate using Pearson's coefficient whilst controlling for age and gender. Those who reported using folic acid supplements were excluded.
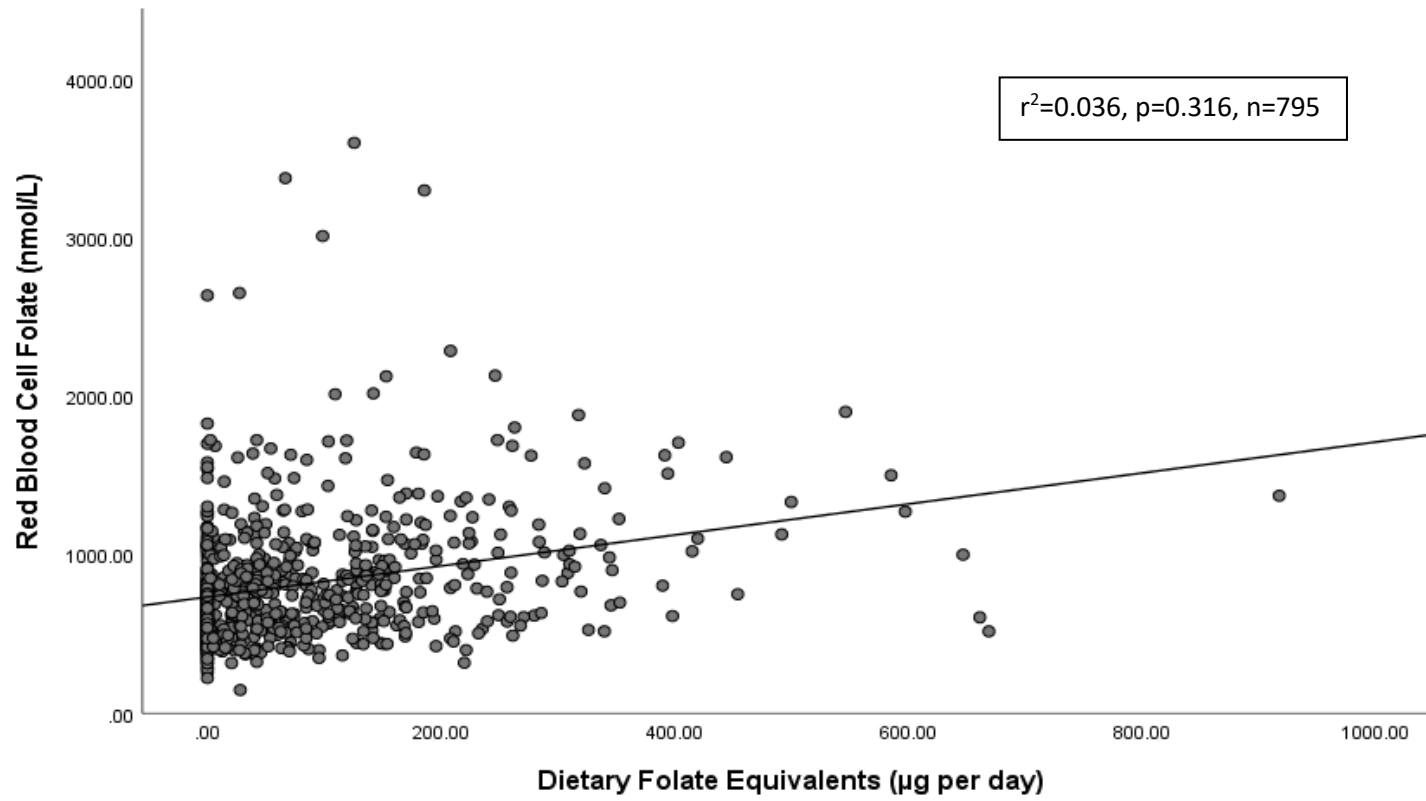
Figure 4 Correlation between DFE intake and RCF status. Partial correlations were carried out with log transformed data as appropriate using Pearson's coefficient whilst controlling for age and gender. Those who reported using folic acid supplements were excluded.

r²=0.288, p<0.001, n=708
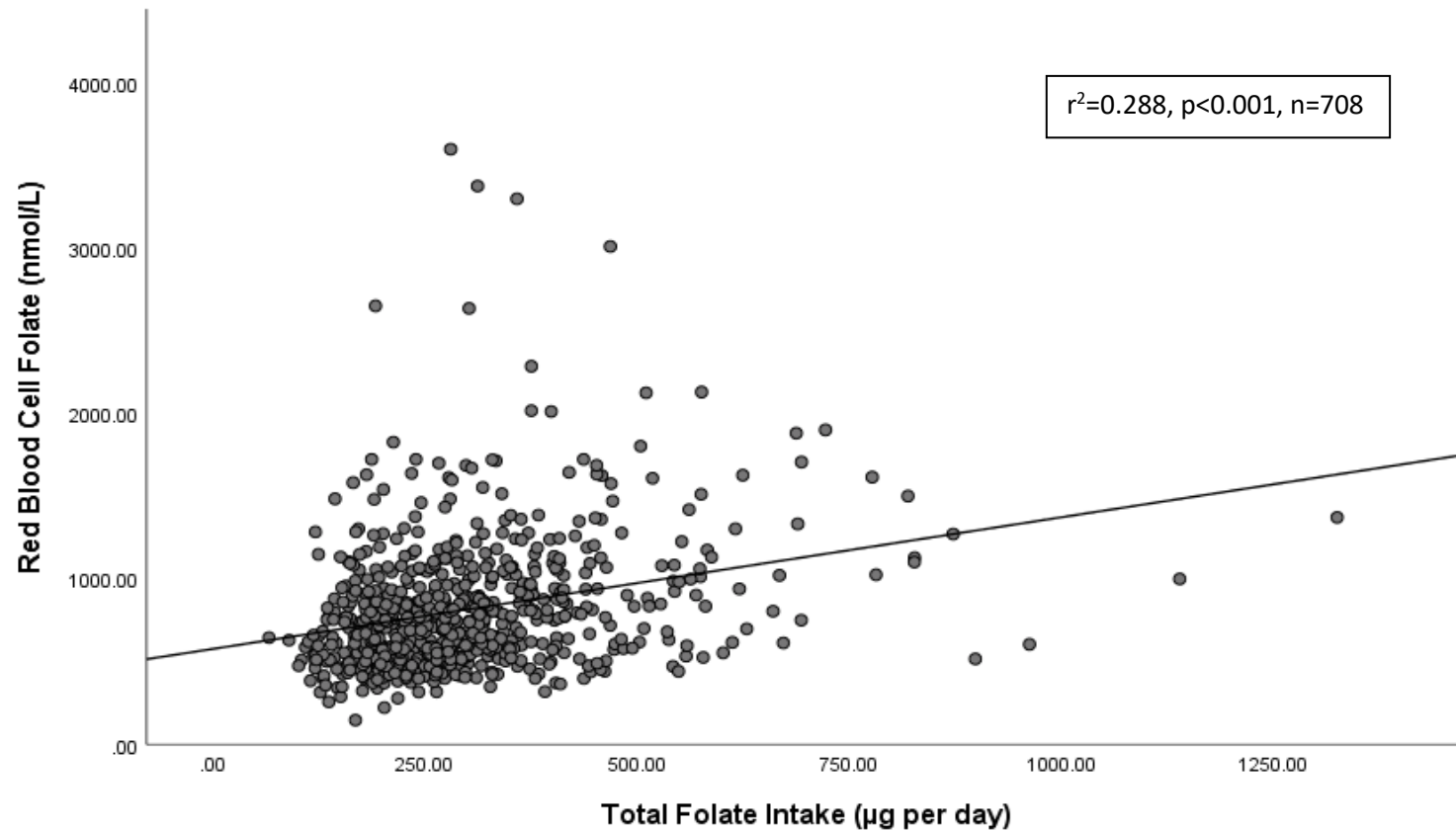
Figure 5 Correlation between total folate intake (natural folate and DFE) and RCF status. Partial correlations were carried out with log transformed data as appropriate using Pearson's coefficient whilst controlling for age and gender. Those who reported using folic acid supplements were excluded.

$r^2= -0.205$, p<0.001, n=738

Figure 6 Correlation between natural folate intake and homocysteine status. Partial correlations were carried out with log transformed data as appropriate using Pearson's coefficient whilst controlling for age and gender. Those who reported using folic acid supplements were excluded.
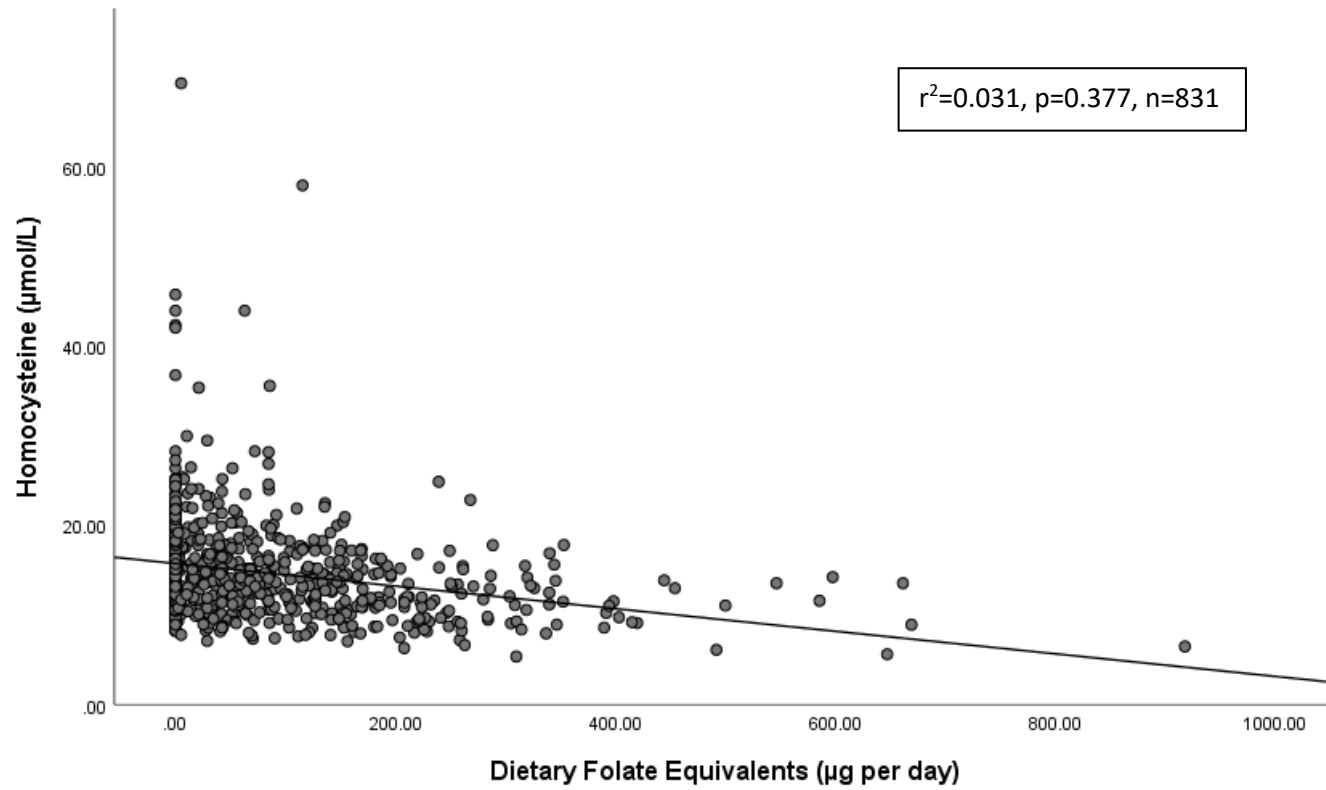
$r^2=0.031$, p=0.377, n=831

Figure 7 Correlation between DFE and homocysteine status. Partial correlations were carried out with log transformed data as appropriate using Pearson's coefficient whilst controlling for age and gender. Those who reported using folic acid supplements were excluded.
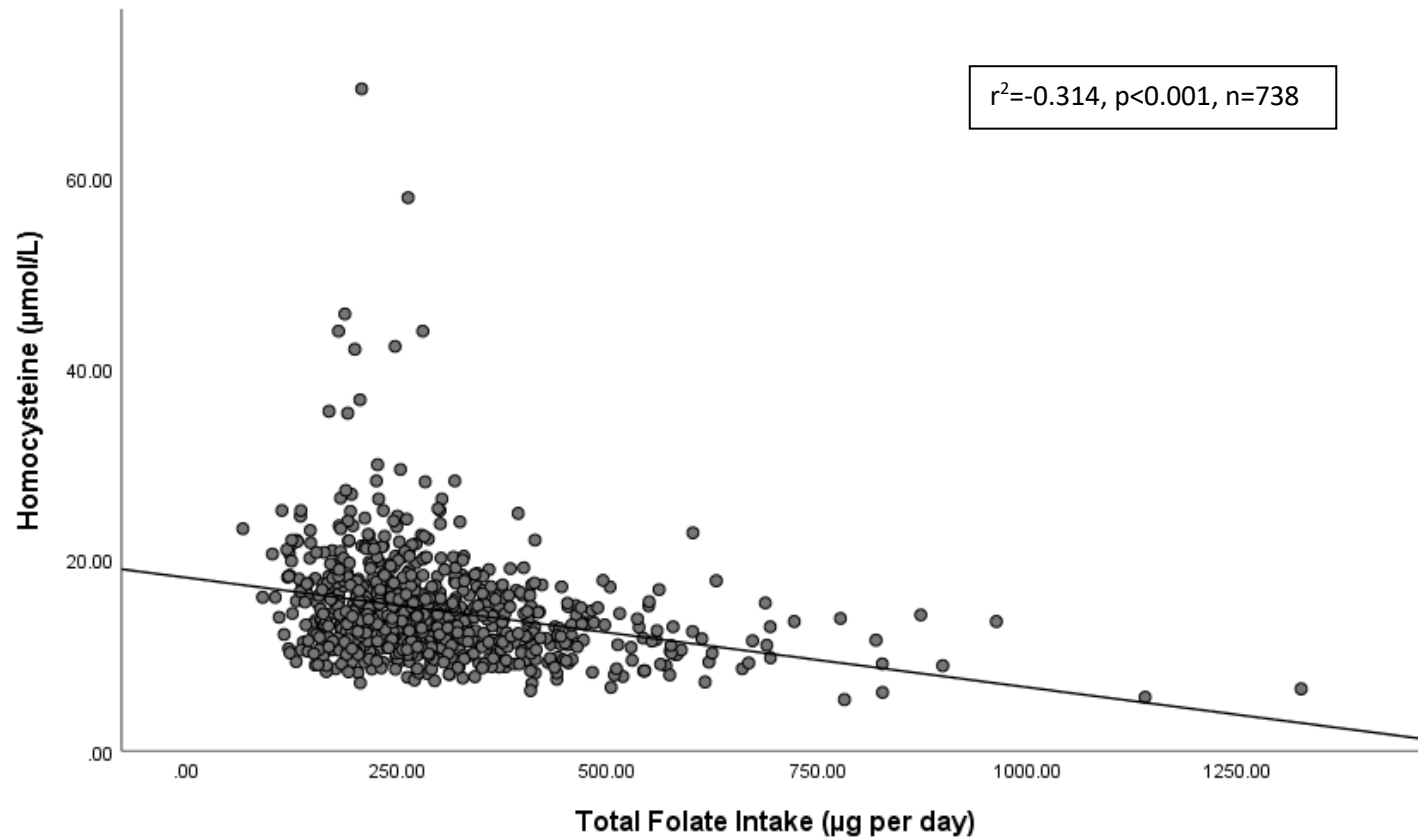
Figure 8 Correlation between total folate (natural folate and DFE) intake and homocysteine status. Partial correlations were carried out with log transformed data as appropriate using Pearson's coefficient whilst controlling for age and gender. Those who reported using folic acid supplements were excluded.
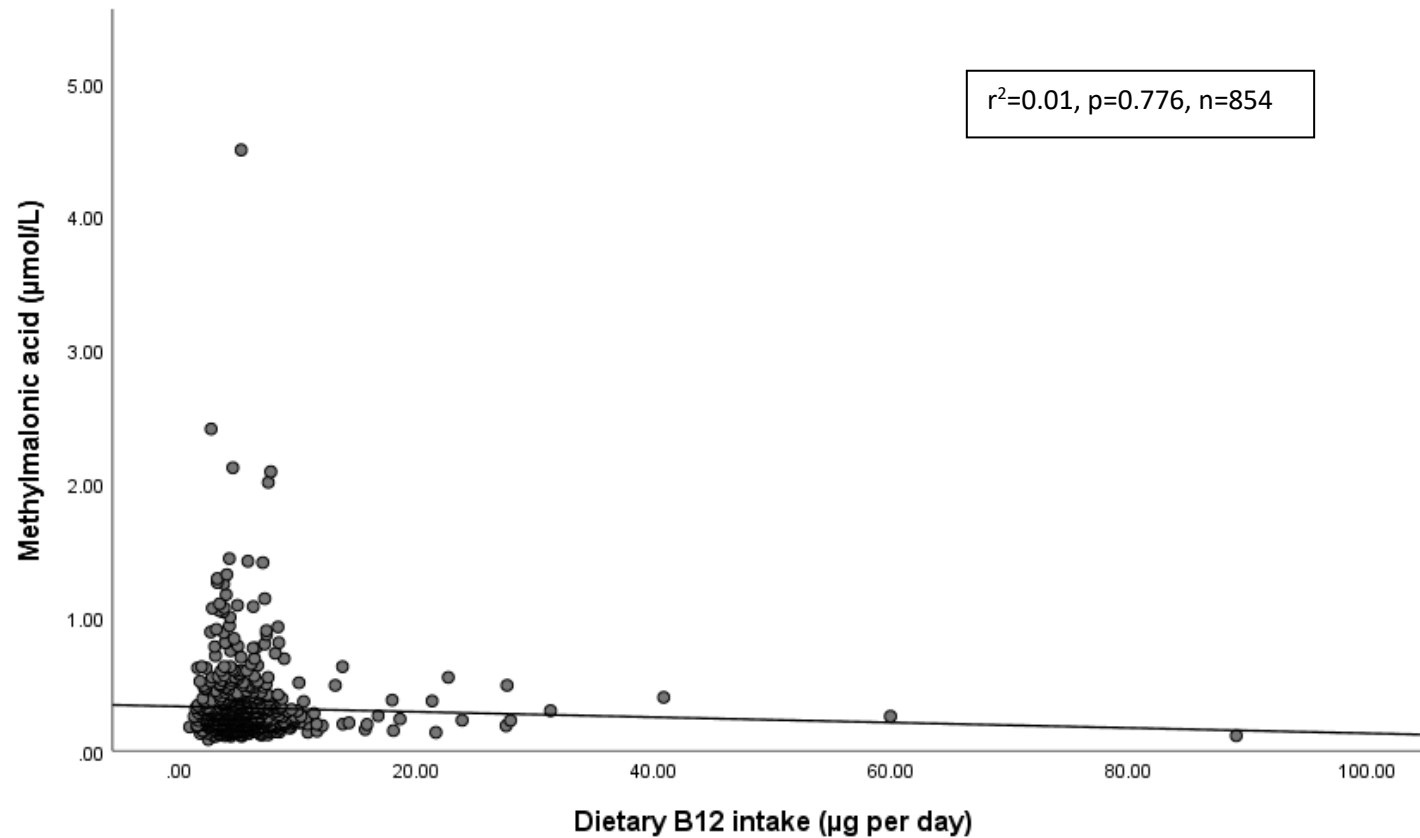
$r^2=0.01$, p=0.776, n=854

Figure 9 Correlation between dietary B12 intake and MMA status. Correlations were carried out on log transformed data as necessary using Pearson's coefficients whilst controlling for age and gender and excluding those who report using oral vitamin B12 supplements or vitamin B12 injections.
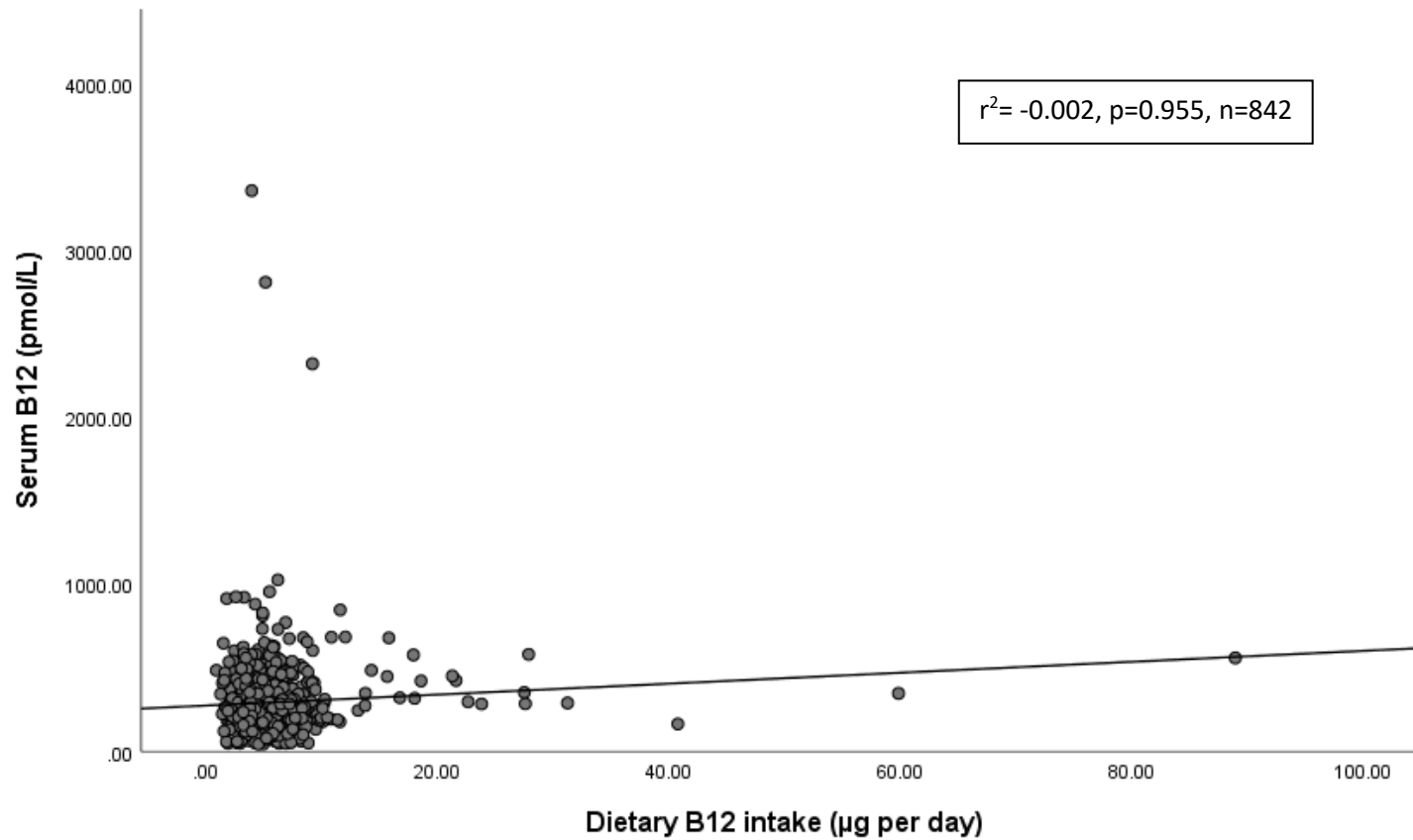
Figure 10 Correlation between dietary B12 intake and serum B12 status. Correlations were carried out on log transformed data as necessary using Pearson's coefficients whilst controlling for age and gender and excluding those who report using oral vitamin B12 supplements or vitamin B12 injections.

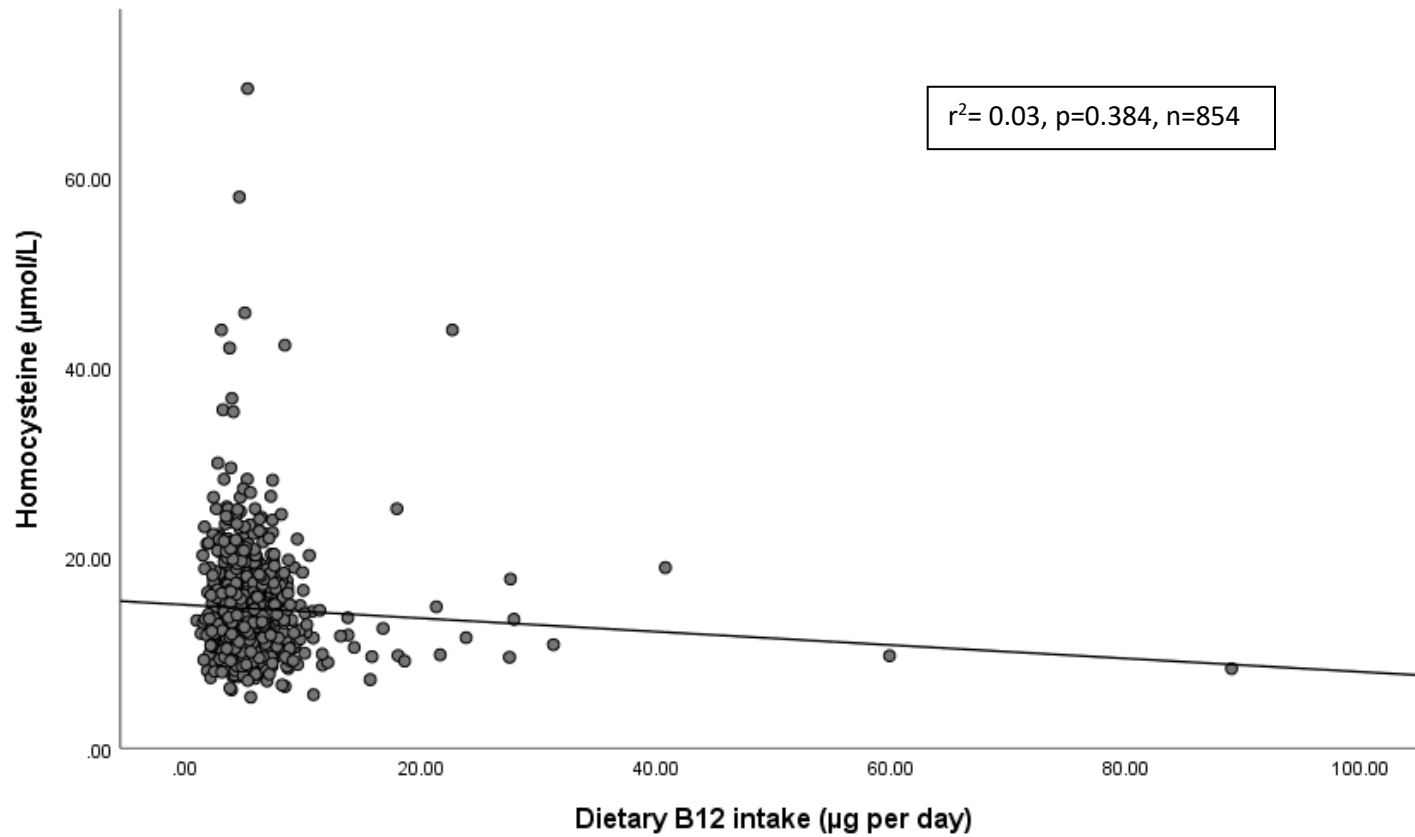**Figure 11** Correlation between dietary B12 intake and homocysteine status. Correlations were carried out on log transformed data as necessary using Pearson's coefficients whilst controlling for age and gender and excluding those who report using oral vitamin B12 supplements or vitamin B12 injections.

Chapter 5

Results: Cognitive scores in relation to folate and B12 intake and status

With the exception of RBANS's index 4, the attention domain, all cognitive scores declined significantly between the two time points in the longitudinal study **Table 4**.

Mean cognitive scores for the MMSE were significantly ($p<0.05$) higher for those in the 2nd (206.2-271.4µg) and 3rd quartile (271.5-357.3µg) of total folate intake compared to the 1st quartile (0-206.1µg), the scores obtained by those in the 4th quartile (>357.3µg) did not differ significantly. The RBANS and FAB score did not differ across these quartiles.

Cognitive scores remained constant for RCF quartiles for both the MMSE and FAB but there was improvement in the total score for RBANS and in some the individual domains. Those in the lowest quartile (0-568.0nmol/L) scored on average 89.9 while those in the 2nd (568.0-766.0nmol/L), 3rd (766.1-1061.0nmol/L) and 4th quartile (>1061.0nmol/L) had a higher median score of 93.6, 93.1 and 93.8 respectively which was significantly higher ($p<0.05$). The 1st quartile in the language domain (index 3) had the lowest score which was significantly lower ($p<0.05$) than the 3rd quartile. In the delayed memory domain (index 5), the lowest score was in the 1st quartile (90.9) and this was significantly lower ($p<0.05$) than the 2nd quartile (96.4) but there was no significant difference between the other quartiles.

Similarly, cognitive scores for MMA quartiles did not differ significantly for both the MMSE and FAB. However, the total RBANS and index 3 (language domain) score declined

as MMA levels rose. This decrease was significant ($p < 0.05$) between the 1st quartile (0-0.19µmol/L) and the 3rd (0.26-0.33µmol/L) and 4th quartile (>0.33µmol/L).

This study identified that neither natural folate intake or DFE alone had an impact of statistical significance on the MMSE, FAB, RBANS total score nor any of the indexes. This was also true of dietary B12 intake, homocysteine concentration and serum B12 status. However, this study did identify significant changes in cognition in relation to total folate intake, RCF and MMA concentration.

Table 6: Cognitive scores in relation to B vitamin intake and B vitamin biomarker status

Cognitive Score [2,3,4]	Natural Folate Intake (µg per day)[1]			
	1st quartile (0.0-171.4µg) (n=219)	2nd quartile (171.5–204.3µg) (n=221)	3rd quartile (204.4–251.2µg) (n=220)	4th quartile (>251.2 µg) (n=219)
MMSE (total score)	27.4 (27.1, 27.6)	27.5 (27.2, 27.7)	27.7 (27.5, 28.0)	27.6 (27.4, 27.9)
FAB (total score)	15.0 (14.7, 15.4)	15.1 (14.8, 15.5)	15.2 (14.9, 15.6)	15.3 (15.0, 15.7)
RBANS (total score)	92.7 (90.9, 94.5)	91.6 (89.9, 93.4)	93.7 (91.9, 95.4)	94.2 (92.4, 96.0)
RBANS INDEX 1	94.4 (90.9, 97.9)	96.7 (93.3, 100.1)	96.7 (93.3, 100.1)	96.9 (93.4, 100.4)
RBANS INDEX 2	94.8 (92.6, 97.0)	93.9 (91.7, 96.0)	95.7 (93.5, 97.9)	96.9 (94.7, 99.2)
RBANS INDEX 3	92.0 (90.6, 93.5)	91.5 (90.1, 92.9)	92.0 (90.6, 93.4)	92.0 (90.6, 93.4)
RBANS INDEX 4	96.9 (94.9, 98.8)	97.5 (95.6, 99.5)	97.7 (95.8, 99.6)	98.3 (96.4, 100.3)
RBANS INDEX 5	94.8 (92.5, 97.1)	91.8 (89.5, 94.1)	95.8 (93.6, 98.1)	94.9 (92.6, 97.2)

Table 6: continued

Cognitive Score [2,3,4]	Dietary Folate Equivalents (μg per day)[1]				
	Non-consumer (0μg) (n=259)	1st quartile (0.1-41.3μg) (n=155)	2nd quartile (41.4-84.7μg) (n=166)	3rd quartile (84.8-161.9μg) (n=144)	4th quartile (>162.0μg) (n=155)
MMSE (total score)	27.6 (27.4, 27.8)	27.5 (27.2, 27.8)	27.7 (27.4, 28.0)	27.5 (27.2, 27.8)	27.5 (27.2, 27.8)
FAB (total score)	15.4 (15.0, 15.7)	15.0 (14.6, 15.4)	15.3 (14.9, 15.7)	15.0 (14.6, 15.4)	15.2 (14.8, 15.7)
RBANS (total score)	92.9 (91.2, 94.5)	92.1 (90.0, 94.2)	94.0 (91.9, 96.0)	93.4 (91.2, 95.5)	93.0 (90.8, 95.2)
RBANS INDEX 1	95.1 (91.9, 98.2)	95.0 (90.9, 99.0)	100.9 (96.9, 104.8)	96.4 (92.2, 100.6)	94.1 (89.9, 98.2)
RBANS INDEX 2	94.9 (92.9, 96.9)	94.3 (91.7, 97.0)	96.2 (93.7,98.7)	95.0 (92.3, 97.7)	96.4 (93.7, 99.0)
RBANS INDEX 3	91.3 (90.0, 92.6)	92.5 (90.9, 94.0)	92.4 (90.7, 94.0)	91.7 (89.9, 93.4)	91.8 (90.1, 93.5)
RBANS INDEX 4	97.8 (96.0, 99.6)	95.8 (93.5, 98.1)	97.8 (95.5, 100.0)	98.0 (95.7, 100.4)	98.6 (96.2, 101.0)
RBANS INDEX 5	94.9 (92.8, 97.0)	93.1 (90.4, 95.8)	94.7 (92.1, 97.4)	95.2 (92.4, 98.0)	93.4 (90.6, 96.1)

Table 6: Continued

Cognitive Score [2,3,4]	Total Folate Intake (Natural and DFE µg per day) [1]			
	1st quartile (0.0-206.1µg) (n=220)	2nd quartile (206.2-271.4µg) (n=220)	3rd quartile (271.5-357.3µg) (n=220)	4th quartile (>357.3µg) (n=219)
MMSE (total score)	27.3 (27.0, 27.5)[a]	27.7 (27.4, 27.9)[b]	27.7 (27.4, 27.9)[b]	27.6 (27.3, 27.8)[ab]
FAB (total score)	15.1 (14.8, 15.5)	14.9 (14.6, 15.2)	15.3 (15.0, 15.6)	15.3 (15.0, 15.7)
RBANS (total score)	91.8 (90.0, 93.6)	92.4 (90.6, 94.2)	93.6 (91.9, 95.4)	94.3 (92.5, 96.1)
RBANS INDEX 1	93.0 (89.5, 96.4)	99.1 (95.7, 102.5)	96.2 (92.8, 99.6)	96.4 (93.0, 100.0)
RBANS INDEX 2	94.4 (92.2, 96.7)	94.0 (91.9, 96.2)	96.0 (93.8, 98.1)	96.9 (94.7, 99.1)
RBANS INDEX 3	91.5 (90.1, 92.9)	91.8 (90.4, 93.2)	91.9 (90.5, 93.3)	92.3 (90.8, 93.7)
RBANS INDEX 4	96.8 (94.8, 98.7)	96.7 (94.8, 98.7)	97.5 (95.6, 99.4)	99.5 (97.5, 101.4)
RBANS INDEX 5	92.7 (90.4, 95.0)	94.7 (92.5, 97.0)	95.3 (93.1, 97.6)	94.6 (92.3, 96.9)

Table 6: Continued

Cognitive Score [2,3,4]	Red Blood Cell Folate Status (nmol/L) [1]			
	1st quartile (0.0-568.0nmol/L) (n=230)	2nd quartile (568.1-766.0nmol/L) (n=231)	3rd quartile (766.1-1061.0nmol/L) (n=229)	4th quartile (>1061.0nmol/L) (n=230)
MMSE (total score)	27.2 (26.9, 27.4)	27.6 (27.4, 27.9)	27.3 (27.1, 27.6)	27.5 (27.3, 27.8)
FAB (total score)	15.2 (14.8, 15.5)	15.2 (14.8, 15.5)	15.4 (15.1, 15.7)	15.0 (15.0, 15.3)
RBANS (total score)	89.8 (88.0, 91.5)[a]	93.6 (91.8, 95.3)[b]	93.1 (91.3, 94.8)[b]	93.8 (92.0, 95.7)[b]
RBANS INDEX 1	92.1 (88.7, 95.4)	97.0 (93.7, 100.4)	97.7 (94.4, 101.0)	96.7 (93.2, 100.3)
RBANS INDEX 2	93.8 (91.6, 95.9)	94.9 (92.8, 97.1)	95.6 (93.5, 97.8)	95.8 (93.5, 98.1)
RBANS INDEX 3	90.0 (88.6, 91.3)[a]	92.5 (91.1, 93.9)[ab]	92.7 (91.3, 94.0)[b]	91.9 (90.5, 93.4)[ab]
RBANS INDEX 4	95.2 (93.2, 97.1)	96.3 (94.4, 98.2)	98.0 (96.1, 99.9)	98.3 (96.3, 100.3)
RBANS INDEX 5	90.9 (88.6, 93.2)[a]	96.4 (94.2, 98.7)[b]	93.1 (90.9, 95.4)[ab]	94.8 (92.4, 97.2)[ab]

Table 6: Continued

Cognitive Score [2,3,4]	Homocysteine Status (µmol/L) [1]			
	1st quartile (0.0-10.8µmol/L) (n=250)	2nd quartile (10.9-13.2µmol/L) (n=239)	3rd quartile (13.3-16.4µmol/L) (n=245)	4th quartile (>16.4 µmol/L) (n=243)
MMSE (total score)	27.3 (27.1, 27.6)	27.4 (27.2, 27.7)	27.5 (27.2, 27.7)	27.6 (27.3, 27.9)
FAB (total score)	15.2 (14.8, 15.6)	15.2 (14.9, 15.6)	15.2 (14.8, 15.5)	15.1 (14.8, 15.5)
RBANS (total score)	92.6 (91.8, 94.4)	93.7 (92.0, 95.4)	91.6 (89.8, 93.3)	92.2 (90.4, 94.0)
RBANS INDEX 1	94.6 (91.3, 97.9)	96.3 (93.1, 99.5)	96.9 (93.7, 100.1)	95.2 (91.9, 98.4)
RBANS INDEX 2	95.2 (93.1, 97.4)	95.4 (93.4, 97.5)	94.3 (92.2, 96.4)	94.9 (92.7, 97.0)
RBANS INDEX 3	91.9 (90.4, 93.3)	91.9 (90.6, 93.2)	91.4 (90.1, 92.8)	91.2 (89.8, 92.6)
RBANS INDEX 4	96.9 (94.9, 98.8)	97.5 (95.6, 99.3)	96.8 (94.9, 98.6)	97.0 (95.1, 99.0)
RBANS INDEX 5	93.7 (91.4, 96.0)	95.1 (92.9, 97.3)	92.6 (90.4, 94.8)	93.9 (91.7, 96.2)

Table 6: Continued

Cognitive Score [2,3,4]	Dietary Vitamin B12 Intake (µg per day) [1]			
	1st quartile (0.0-3.8 µg) (n=216)	2nd quartile (3.9-5.0µg) (n=224)	3rd quartile (5.1-6.5µg) (n=220)	4th quartile (>6.5µg) (n=219)
MMSE (total score)	27.4 (27.2, 27.7)	27.6 (27.4, 27.8)	27.6 (27.4, 27.8)	27.6 (27.4, 27.8)
FAB (total score)	15.1 (14.8, 15.3)	15.1 (14.8, 15.3)	15.1 (14.8, 15.4)	15.2 (15.0, 15.5)
RBANS (total score)	91.8 (90.0, 93.6)	93.1 (91.4, 94.9)	93.0 (91.2, 94.8)	94.1 (92.3, 95.9)
RBANS INDEX 1	97.3 (93.8, 100.8)	95.2 (91.8, 98.6)	94.5 (91.1, 98.0)	97.8 (94.3, 101.2)
RBANS INDEX 2	94.4 (92.1, 96.6)	95.7 (93.6, 97.9)	95.7 (93.5, 97.9)	95.4 (93.2, 97.6)
RBANS INDEX 3	91.5 (90.0, 92.9)	91.5 (90.1, 92.9)	92.6 (91.1, 94.0)	91.9 (90.5, 93.3)
RBANS INDEX 4	97.1 (95.1, 99.0)	97.9 (96.0, 99.8)	97.4 (95.4, 99.3)	98.1 (96.2, 100.1)
RBANS INDEX 5	93.0 (90.6, 95.3)	95.0 (92.7, 97.2)	94.3 (92.1, 96.6)	95.0 (92.7, 97.3)

Table 6: Continued

Cognitive Score [2,3,4]	Methylmalonic Acid Status (µmol/L) [1]			
	1st quartile (0.0-0.19µmol/L) (n=246)	2nd quartile (0.2-0.25µmol/L) (n=271)	3rd quartile (0.26-0.33 µmol/L) (n=220)	4th quartile (>0.33µmol/L) (n=240)
MMSE (total score)	27.4 (27.2, 27.7)	27.5 (27.2, 27.7)	27.4 (27.1, 27.6)	27.5 (27.3, 27.8)
FAB (total score)	15.1 (14.8, 15.3)	15.1 (14.8, 15.3)	15.0 (14.7, 15.3)	15.0 (14.8, 15.3)
RBANS (total score)	94.1 (92.4, 95.8)[a]	93.4 (91.8, 95.0)[ab]	90.7 (88.9, 92.4)[b]	91.5 (89.7, 93.2)[b]
RBANS INDEX 1	96.4 (93.1, 99.6)	95.7 (92.7, 98.7)	96.0 (92.6, 99.4)	94.9 (91.6, 98.2)
RBANS INDEX 2	95.7 (93.6, 97.8)	96.5 (94.6, 98.5)	93.1 (90.9, 95.3)	94.1 (92.0, 96.3)
RBANS INDEX 3	93.6 (92.2, 94.9)[a]	92.1 (90.8, 93.3)[ab]	90.3 (88.9, 91.7)[b]	90.2 (88.8, 91.5)[b]
RBANS INDEX 4	97.5 (95.6, 99.4)	97.8 (96.0, 99.5)	96.8 (94.9, 98.8)	95.8 (93.9, 97.7)
RBANS INDEX 5	95.1 (92.9, 97.4)	93.6 (91.5, 95.7)	92.7 (90.4, 95.1)	93.8 (91.5, 96.1)

Table 6: Continued

Cognitive Score [2,3,4]	Serum B12 Status (pmol/L) [1]			
	1st quartile (0.0-183.5pmol/L) (n=214)	2nd quartile (183.6-277.5pmol/L) (n=242)	3rd quartile (277.6-378.3pmol/L) (n=241)	4th quartile (>378.3pmol/L) (n=241)
MMSE (total score)	27.7 (27.4, 27.9)	27.3 (27.1, 27.6)	27.4 (27.2, 27.7)	27.4 (27.2, 27.7)
FAB (total score)	15.1 (14.8, 15.4)	15.2 (15.0, 15.5)	15.0 (14.7, 15.3)	14.9 (14.6, 15.2)
RBANS (total score)	92.6 (90.8, 94.4)	92.2 (90.5, 94.0)	92.2 (90.5, 93.9)	93.0 (91.2, 94.9)
RBANS INDEX 1	94.5 (91.1, 97.9)	94.0 (90.8, 97.3)	96.3 (93.1, 99.5)	98.5 (95.0, 101.9)
RBANS INDEX 2	94.9 (92.7, 97.1)	93.8 (91.7, 95.9)	94.6 (92.5, 96.7)	96.5 (94.3, 98.8)
RBANS INDEX 3	91.6 (90.2, 93.1)	91.6 (90.2, 92.9)	91.3 (90.0, 92.6)	91.9 (90.5, 93.4)
RBANS INDEX 4	97.5 (95.6, 99.5)	97.4 (95.5, 99.3)	97.1 (95.1, 99.2)	97.1 (95.1, 99.2)
RBANS INDEX 5	94.7 (92.3, 97.0)	94.3 (92.0, 96.5)	93.0 (90.7, 95.2)	93.5 (91.1, 95.9)

1 Dietary intake or biomarker status is split into quartiles, each quartile is then compared with each other.

2 The median cognitive score, 25th and 75th centiles are displayed.

3 Log transformed or square root of the raw data was used as appropriate for statistical analysis using MANCOVA controlling for the influence of age, gender, education, waist hip ratio, stroke, TIA, gastrointestinal disorders, alcohol, smoking status, depression score and vitamin D supplement use. Scores that differ significantly are denoted by different letter in superscript (p<0.05), rows without these letters have no values of statistically significant difference.

4 Mini mental state examination (MMSE), frontal assessment battery (FAB), repeatable battery for the assessment of neuropsychological status

Chapter 6

Discussion

6.1. Folate, Folic Acid and B12 intake and status

Analysis of dietary folate and folic acid (FA) intake, and interpretation of biomarker status indicated that folate status declined significantly as reflected by the decreasing RCF concentration and the greater prevalence of elevated homocysteine despite the increase in reported use of folic acid supplements from 11.1% to 14.1%. However, folic acid supplement use was reported in just 2.8% of older Irish adults by the TILDA study (n>5000) (Laird *et al.*, 2018) and thus our population may not be truly represent the free living population. This trend in reduced folate status, despite average intakes being meeting the RNI, was also found by the National Diet and Nutrition Survey (NDNS) (Bates, B, 2019). The median red blood cell folate (RCF) was 766.0nmol/L, which is higher than the guidelines from the World Health Organisation (WHO) for both reducing the risk of macrocytosis (<226.5nmol/L) and elevated homocysteine (<340nmol/L) (WHO, 2015a). However, 34.9% of participants in this study had elevated homocysteine levels despite exceeding the WHO guidelines.

At present, Ireland and the UK have a voluntary, but liberal food fortification policy and it is estimated that between 1.9% and 6.2% of foods in Ireland are fortified with one or more micronutrient (Hannon, Kiely and Flynn, 2007; Hennessy *et al.*, 2011). It is estimated that fortified foods contribute to 28.1% of total folate intake in males and 26.2% of total folate intake in females in Ireland (Hennessy, Walton and Flynn, 2013). This analysis has demonstrated a 54.0% higher folate intake in those consuming the highest amount of fortified foods compared to those who were non-consumers which resulted in significant improvement in folate status. This also resulted in a decrease in

the proportion of participants falling below the RNI from 49.0% to 0.0% and an improved

biomarker status of RCF which increased by 31.7% and homocysteine which decreased

by 18.8% between non—consumers and those in the highest quartile for consuming FA

fortified foods. Similar improvements in B vitamin status were found in other studies

(Hoey *et al.*, 2007; Hennessy, Walton and Flynn, 2013). Furthermore, the median

quantity of dietary folate equivalents (DFE) gained from consuming FA fortified foods

was 84µg and this increased to 210.9 µg for those in the highest quartile. In America, it

has been estimated that mandatory fortification has led to an average increase of 380

µg DFE which resulted in a 50% reduction in fasting homocysteine levels in those who

had elevated homocysteine levels and also a reduction in prevalence of low folate

biomarker status (Jacques *et al.*, 1999; Quinlivan and Gregory, 2003; Stover, 2008). This

improvement between non-consumers and high consumers in our study, and with

consideration to the benefits seen following the mandatory FA fortification in America,

suggests further improvements in oral intake and biomarker status could be achieved in

the UK and Ireland by implementing a mandatory fortification policy.

The average vitamin B12 intakes (mean=5.7µg and median=5.0 µg) were in excess of the

RNI (1.5µg). Low B12 intake were almost non-existent as only 0.5% of the participants

fell below the RNI. Moreover, B12 supplement use increased to 10.5% which is

substantially higher than the 2.6% reported by the TILDA study (Laird *et al.*,

2018).Median serum B12 increased from 270 to 279pmol/L and methylmalonic acid

(MMA) increased from 0.33 to 0.34µmol/L. The average serum B12 suggests an

improvement in status, however the MMA suggests a decrease in status, although only

119 participants had MMA measured at both time points and the prevalence of deficiency according to MMA status (>0.37µmol/L) stayed the same. . For serum B12, there was an increase from 8.5% to 15.8% for those falling below 148pmol/L indicating deficiency. The prevalence of deficiency is similar to what has been found in other studies (Hunt, Harrington and Robinson, 2014; Laird *et al.*, 2018).

The use FA fortified foods was associated with higher B12 intake and status, as other B vitamins (including B12) are often added in addition to FA (Hoey *et al.*, 2007). Research has shown fortified food use contributes to approximately 11% of total B12 intake (Hennessy, Walton and Flynn, 2013). Our study found statistically higher B12 intake in high consumers than non-consumers of fortified foods. This was coupled with a 16.9% increase in serum B12 (p<0.05) between non-consumers and high consumers and a 12.0% decrease in MMA between low consumers and high consumers of fortified foods (p<0.05).

In respect to the increase in deficiency in TUDA participants, it is worth considering the significant increase since the original study in reported metformin and PPI use which are known to reduce the absorption of Vitamin B12 (Porter *et al.*, 2016). Additionally, it is possible that with their increase in age, efficiency of absorption may be reduced increased, particularly as gastrointestinal disease has also been more frequently reported (Wong, 2015).

Fortifying commonly consumed foods with both FA and vitamin B12 could reduce the risk of masking vitamin B12 deficiency which is associated with high intakes of FA that may result in increased risk of cognitive impairment. Particularly, as B12 does not appear to impose safety concerns in high amounts as there is no upper limit for B12 intake (EFSA, 2016b). Instead, it may promote lower incidence of cognitive impairment which is associated with normal B12 status and high folate (Morris *et al.*, 2007; Selhub and Paul, 2011). Fortifying foods with B12 would also help to improve B12 status in the older population, who may have poorer absorption of the protein bound B12 in food. This strategy has been recommended for older Americans in the prevention of malabsorption of vitamin B12 (Stover, 2008).

6.2. B vitamins and cognitive performance

Similar to other studies, we found the effect of B vitamin intake and status on cognition to be variable. Cognitive scores in the mini mental state examination (MMSE) improved with increasing folate intake. Those with a total folate intake between 0 μg and 206.1μg had a significantly lower cognitive score than those whose intake was between 206.2μg and 357.3μg, (which exceeds the RNI). Whilst intakes higher than 357.3 μg did not offer further cognitive benefit. This differs from the another study, which found no significant difference in MMSE score in relation to folate intake or status and both studies had an average total folate intake of approximately 300μg but the sample size was small (n=155) and the follow up period was approximately 4 years whilst on average TUDA participants were followed up after 6 years. The same study found a beneficial effect related to B6 intake and status (Hughes *et al.*, 2017). Furthermore, a meta-analysis

found significant associations between low serum folate and cognitive impairment using the MMSE (Michelakos *et al.*, 2013). However, we found that total folate intake did not statistically impact scores in either the frontal assessment battery (FAB) or the repeatable battery for the assessment of neuropsychological status (RBANS).

Although total folate was associated with MMSE performance, RCF was not, nor was it associated with FAB scores. There was, however, an improvement in RBANS. Those with the lowest RCF, 0.0-568nmol/L had a statistically lower total score than those with higher RCF concentrations (>568nmol/L). Furthermore, in two of the RBANS's domains there was an association between RCF and improved scores. Index 3 which concentrates on language, those with RCF concentrations above 568nmol/L scored significantly higher than those with concentrations below this. In index 5, delayed memory, the highest score was found in those with RCF levels between 568.1 and 1061.0nmol/L, those below this range had a statistically lower score, and although those above this scored better than those in the lowest quartile it was not significant. Our findings differ from another study which found improvement in 2nd index, the visuospatial/constructional cognitive domain, after 2 years of supplementation with 400μg folic acid, 12 μg B12, 10mg B6 and 10mg B2 but no improvement in total scores (Moore *et al.*, 2018).

It has been established that severe B12 deficiency causes cognitive impairment, however, the impact of suboptimal status is still unclear (Stover, 2008). Some reviews have concluded that there was a beneficial cognitive impact in those with low or

suboptimal B vitamin status prior to B vitamin supplement intervention but no significant changes for those with normal status at baseline (Forbes *et al.*, 2015; Mcgrattan *et al.*, 2018). A study of older people with hyperhomocysteinemia, which indicates suboptimal B vitamin status, found improved cognitive scores after 14 weeks of folate, B6 and B12 supplementation (Cheng *et al.*, 2014). A review (Kennedy, 2016) has highlighted several studies that have implicated homocysteine as risk factor in the development of cognitive impairment and dementia. This study, however, did not indicate any significant differences between the quartiles of homocysteine concentration and cognitive performance.

This study found that although vitamin B12 intake was not associated with changes in cognitive test scores, MMA was. Increased MMA indicates poorer B12 status, and there was a decrease in both total RBANS score and index 3, the language domain. This was between the lowest MMA quartile, which equates to the higher B12 status, and the highest MMA quartile >0.26µmol/L. This is concerning as B12 deficiency is only diagnosed at >0.37µmol/L and therefore the impact on cognition is detected even within what is thought to be sufficient B12 status. Other studies have supported this finding as MMA tends to be a more sensitive measure of B12 status (O'Leary, Allman-Farinelli and Samman, 2012; Doets *et al.*, 2013). In this study, we did not find significant change in MMSE scores in relation to MMA, serum B12 or dietary B12, although Zhang et al speculate that the MMSE may not be sensitive enough to detect slight changes in cognition (Zhang, Ye and Mu, 2017).

The Dietary reference values were met by the vast majority of participants (99.5%) in this study for vitamin B12 intake. These values are in place as a guide to prevent deficiency disease in the general population. Therefore, it is worth considering that simply meeting the guideline amount may not be enough to attain the potential benefits such as protection against cognitive decline associated with optimising intake (Kennedy, 2016). Furthermore, malabsorption of vitamin B12 is commonly found in older people and it is associated with food-bound vitamin B12. Therefore, dietary intake may not give a true reflection of biomarker status and subsequently, may not be reflected the individual's cognitive performance (Wong, 2015). In which case, the cognitive performance for those participating in this study who have included vitamin B12 fortified foods and vitamin B12 supplements (which is recommended in America for older adults) may give a better indication of influence of vitamin B12 in cognition through ageing(Stover, 2008).

Since baseline there has been an increase in anaemia and macrocytosis. Anaemia and macrocytosis conditions increase with age, particularly with accelerated biological ageing (frailty), which in turn is impacted by nutritional factors such as B vitamin and iron deficiency, genetic risk factors and chronic diseases. Those who are affected by anaemia and macrocytosis have a higher risk of cognitive impairment (Morris *et al.*, 2007; WHO, 2017).

Chapter 7

Final Conclusions

Study Strengths and Limitations

It would be expected that in most scenarios cognitive decline develops slowly therefore the long period between the two recruitment points is an important strength of the study. Additionally, we utilised a comprehensive battery of cognitive tests which strengthens the quality of our data. The FAB assesses the frontal lobe solely and the MMSE assesses global cognitive function. The RBANS also assesses global cognition and enables assessment of individual cognitive domains which enables more specific investigation of the impact B vitamin intake and status has on cognition.

This study utilised several biomarkers which is often recommended to give a fuller picture of one's micronutrient status. MMA and serum B12 were used which gives an indication of both deficiency by low status and low function. RCF provided an indication of longer-term folate status and total homocysteine indicates functional deficiency that can be as a result of either B vitamin.

During the research appointment we asked participant about their diet using a food frequency questionnaire and we also asked participants to complete a four-day food diary. Participants were asked to give an indication of their portion size using the instructions provided, rather than weighing their food, as the latter would have likely reduced the return of completed food diaries. Furthermore, having a seven-day food diary in this study would have reduced the risk of over-representing the weekend, however, this would be time consuming for the participant and may have increased the incidence of under-reporting and reduced the rate of food diary return. At least 20% of

the food diaries returned were under-reported as the average energy intake fell below the energy requirements as indicated by the basal metabolic rate. This may explain, in part, the poor correlation with the biomarkers and may mean the influence of total folate and vitamin B12 on cognition may be more pronounced if intakes were reported more accurately. For publication, the analysis will be repeated so that the 20% can be excluded. Additionally, numeracy and literacy skills may have been a factor in determining the likelihood the participant agreed to complete the food diary. Perhaps if the participants who did not return a food diary were offered a chance to complete the dietary recall over the phone this may have increased the completeness of the dietary data. Although, self-reported dietary intake is open to misreporting, the combination of the FFQ and the food diary improved the quality of data from the first recruitment as it enables a more in-depth dietary assessment.

The data gathered through this study was comprehensive as it included questions regarding medication use, medical history, depression scale, ability with activities of daily living which enabled us to control for confounding factors.

Some other components of this study used self-reported data, for example, medication and supplement use, medical history, participation in exercise, smoking status and alcohol consumption. Another area of weakness for this study was the underrepresentation of males.

Nutritics was used for dietary analysis and although this programme is updated frequently it did not contain all the brands and specific products used by the participants, as a result, the researcher chose the most suitable alternative. If the participant did not give an indication of the portion size used a medium serving was assumed which may lead to over or under estimation of dietary intake.

The accuracy of self-reported data is dependent on participant providing an accurate recollection. Although, to help mitigate this we excluded participants who had an MMSE score less than 24 at their initial assessment.

Unfortunately, only 119 of the participants had methylmalonic acid measured on both occasions which limited our analysis of change in vitamin B12 status, although, serum B12 biomarkers were available for analysis for the majority of participants.

Conclusion

This study aimed to assess dietary intake of folate and B12, determine biomarker status and investigate the relationship between B vitamins and cognitive performance.

We identified that a substantial proportion of this cohort are not meeting the RNI for folate which is reflected in the decreasing RCF levels. Despite dietary intakes of B12 typically meeting the RNI between 15.8% and 21% of the cohort are deficient in B12 by

their serum B12 and MMA concentrations respectively, which could be related to reduced absorptive capacity associated with ageing, increased reporting of gastrointestinal disease and use of medications such as PPIs, H2 receptor blockers, metformin and statins.

Over 1/3 of this cohort had elevated homocysteine which is associated with low B vitamin status and this study has shown the consumption of FA fortified foods has an inverse relationship with homocysteine and MMA concentration and a positive relationship with RCF and serum B12.

Further, this thesis has shown that total folate intake, RCF and MMA concentration are associated with cognitive performance in the MMSE and RBANS respectively. As higher MMA status was associated with poorer cognitive performance, it would be expected that dietary intakes of B12 would be influential too. However, this analysis did not indicate this and perhaps this could be due to malabsorption, the effect of which, may be reduced or avoided by used fortified foods.

In conclusion, optimising B vitamin status may have role in preventing or delayed cognitive decline in older people. However, this finding needs to be confirmed with the randomised control trials. One method of optimising folate and B12 status can be achieved through the consumption of fortified foods and this may have beneficial

impact on cognition. Therefore, introduction of a mandatory fortification policy should be considered to support the needs of the ageing population.

Future Research

Further biomarker analysis, namely serum folate, transcobalamin (holoTC) erythrocyte glutathione reductase (vitamin B2 biomarker) and plasma pyridoxal 5-phosphate (vitamin B6 biomarker), is being carried out for this study which would enable further exploration of the role of other B vitamins in cognition.

Further research, if possible, with this cohort would be ideal to further investigate cognitive and dietary change over a longer period. This project focused on folic acid fortified foods in relation to biomarker status and cognitive performance rather than B12 fortified foods. Both folic acid and B12 fortified foods have a greater bioavailability, but for the older population in particular, for whom it is known food-bound malabsorption can be problematic, further research into the impact of B12 fortified foods on biomarker status and cognitive performance would be beneficial.

Further longitudinal research with comprehensive dietary assessment using weighed food diaries and a range of nutritional biomarkers and multiple cognitive assessments and an equal gender distribution will increase the sensitivity of analysis. In addition to randomised control trials with the use of fortified foods or supplements over a longer

period with a range of biomarkers and a battery of cognitive tests would determine if B vitamins can improve rates of cognitive decline in older adults.

Furthermore, brain imaging techniques in relation to the impact of nutrition on cognitive function would greatly improve our understanding of any potential associations.

Appendix

Appendix 1

Patient Information and Consent

Title of study:

The Trinity, University of Ulster and Department of Agriculture (TUDA) Cohort Phenotype / Genotype database – Five-year follow-up study

Introduction:

The Department of Medicine for the Elderly at St. James's Hospital sees many patients each year who suffer from memory problems or osteoporosis (i.e. brittle bones). To try to more fully understand the link between diet and health in Irish people, we are involved in research programmes that aim to explore the relationship between our diet, our genetic make-up and our risk of developing chronic diseases of ageing, including cognitive decline and bone disease. The purpose of the TUDA study is to collect dietary and medical information and blood data from Irish adults over 60 years old, to explore some of the factors contributing to the development of chronic diseases of ageing such as bone disease, impaired cognitive function and hypertension. The original TUDA study was sponsored by the Department of Food, Agriculture and the Marine (DAFM). The data that we collected will help us to understand associations between diet and health that are likely to be of benefit in the prevention of these common diseases.

We now wish to conduct a follow-up study on a group of the original TUDA participants. This follow-up study will help us to understand how our diet and genetic make-up can influence the rates at which changes occur in our physical and mental health as we age. You are one of the original TUDA participant, which was conducted at the Hospital between 2008 and 2012. If you agree to come back for re-assessment, you will be one of 400 people taking part in this 5-year follow-up study.

Procedures;

You may remember that the assessment took about 90 minutes and included a detailed questionnaire of your dietary and medical history, some memory and mood tests. We also took a blood sample to look at your blood count, kidney function, glucose, cholesterol and vitamin levels. For this follow-up study, the procedure would be the same as before and the assessment will be carried out by an experienced researcher. We will measure an expanded list of biochemicals in blood and will carry out a more detailed investigation of your diet.

Your participation requires that you are:

(i)   Willing to answer some questionnaires about your diet, physical and mental health and use of vitamin supplements

(ii)   Willing to have some blood tests

(iii)   Willing to undergo some tests of memory and mood

Benefits:

The main benefit of this study will be to help your doctors better understand the association between the link between diet and health in Irish people. By taking part in the study you will also find out how you perform on a battery of physical and psychological tests and get some clinical blood tests. In the unlikely event that we find any abnormal clinical results we will notify your GP and arrange for you to get treatment as appropriate.

Risks:

An experienced phlebotomist will take a blood sample from you. If you tend to faint after a blood test, please tell the person taking the sample and he/she will make sure you are sitting down. The blood test may be a little uncomfortable, and may, in a small number of cases, result in some bruising. The study does not involve taking any medications.

Exclusion from participation:

You cannot be in this study if you suffer from severe cognitive impairment and are unable to fully appreciate the implications of participation.

Confidentiality:

Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the hospital.

Compensation:

The medical practitioners involved in this study have current medical malpractice insurance cover. Nothing in this document restricts or curtails your rights.

Voluntary Participation:

You have volunteered to participate in this study. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study.

Stopping the study:

You understand that your doctor may stop your participation in the study at any time without your consent.

Permission:

 Approval will be sought from the hospital Research Ethics Committee.

Appendix 2 - Health and Lifestyle Questionnaire

Sex: ☐ Male   ☐ Female   **Timed up and go:**
Age (deciage):

Waist (cm):
Hip (cm):

| Waist:Hip: |
| BMI: |

Hand grip strength (kg): (patient to stand upright and use non dominant hand x 3 measures)
    L / R   1)
              2)
              3)
                   Av:

Hours since your last meal (to the nearest whole hour)
Minutes to blood sample

| Unintentional weight-loss in last 6-months: |

Weight (kg):
(without shoes but in normal clothes)

Have you had any unintentional weight loss in the last 6 months?

Height (cm)
(without shoes)

BP (sitting with arm supported) (circle reference arm)
                                       L   **R**

1st Reading   2nd Reading

(3rd Reading)   (4th Reading)   (5th Reading)

(6th Reading)   **Average:**

(Please allow 5 minutes in seated position with back and arm supported before recording first reading and a further 1-2 minutes before subsequent readings. Continue to take readings until two are within 5 mmHg Systolic of each other and take mean of these two as actual reading). In the event that no two readings are within 5mmhg of each other (after 6 readings) then note this and take mean of last four.

Lives   **Marital Status:**
☐ Alone   ☐ Single
☐ Spouse   ☐ Married
☐ Children   ☐ Common-law
☐ Other   ☐ Separated
                                       ☐ Widow(er)

Driving: **Past Occupation**
☐ Currently
☐ Past
☐ Never   **Education**

Medications *(N.B. tablets, inhalers, injections):*   *All > 6/12   Y/N*

Have you started any of these medication within the past (tick all that apply):			
1 _____	1 ☐	2-6 ☐	>6 ☐ months
2 _____	1 ☐	2-6 ☐	>6 ☐ months
3 _____	1 ☐	2-6 ☐	>6 ☐ months
4 _____	1 ☐	2-6 ☐	>6 ☐ months
5 _____	1 ☐	2-6 ☐	>6 ☐ months
6 _____	1 ☐	2-6 ☐	>6 ☐ months
7 _____	1 ☐	2-6 ☐	>6 ☐ months
8 _____	1 ☐	2-6 ☐	>6 ☐ months
9 _____	1 ☐	2-6 ☐	>6 ☐ months
10 _____	1 ☐	2-6 ☐	>6 ☐ months
11 _____	1 ☐	2-6 ☐	>6 ☐ months
12 _____	1 ☐	2-6 ☐	>6 ☐ months
13 _____	1 ☐	2-6 ☐	>6 ☐ months
14 _____	1 ☐	2-6 ☐	>6 ☐ months
15 _____	1 ☐	2-6 ☐	>6 ☐ months
16 _____	1 ☐	2-6 ☐	>6 ☐ months
17 _____	1 ☐	2-6 ☐	>6 ☐ months
18 _____	1 ☐	2-6 ☐	>6 ☐ months
19 _____	1 ☐	2-6 ☐	>6 ☐ months
20 _____	1 ☐	2-6 ☐	>6 ☐ months

PPI H2 blockers BP Metformin Other DM Med Statin Anti-depressant

Specific Cardiovascular Risk Factors:

- **Hypertension**   Yes ☐   No ☐   Don't Know☐
  If YES, when diagnosed?_____ Age started medication____
  Anti-hypertensive medication last taken:
  <4 hours _____   4-12 hours _____   >12 hours_____
- **Diabetes**   Yes ☐   No ☐   Don't Know☐
  If YES, when diagnosed?   _____
  Compliance with diet?   Yes ☐   No ☐
  Compliance with testing?   Yes ☐   No ☐
- **Hyperlipidaemia/Hypercholestrolaemia**
          Yes ☐   No ☐   Don't Know☐
  If YES, when diagnosed?   _____
- Ishaemic Heart Disease   Yes ☐   No ☐   Don't Know☐
- Angina   Yes ☐   No ☐   Don't Know☐
- Heart attack   Yes ☐   No ☐   Don't Know☐
- Heart failure   Yes ☐   No ☐   Don't Know☐
- Atrial Fibrillation   Yes ☐   No ☐   Don't Know☐
- Angioplasty/CABG   Yes ☐   No ☐   Don't Know☐
- Stroke   Yes ☐   No ☐   Don't Know☐
  If YES, how many and when?_____
- TIA   Yes ☐   No ☐   Don't Know☐
  If YES, how many and when?_____
- Peripheral artery disease, e.g. Intermittent claudication?
          Yes ☐   No ☐   Don't Know☐

Family History of cancer:

Family history means occurrence before the age of 60 years in a first degree relative (Mother, father, brother or sister).

Family History of Stroke:

Family history means occurrence before the age of 60 years in a first degree relative (Mother, father, brother or sister).

Family History of Heart disease:

Family history means occurrence before the age of 60 years in a first degree relative (Mother, father, brother or sister).

Family History of Pre Senile Dementia (onset under 65years):

Family history means occurrence before the age of 60 years in a first degree relative (Mother, father, brother or sister).

Family History of Senile Dementia (onset at or after 65years):

Family history means occurrence before the age of 60 years in a first degree relative (Mother, father, brother or sister).

FALLS

1) Have you fallen in the last year? Yes☐ No ☐
(Note: "A fall is an event in which an individual comes to rest on the ground or another lower level with or without loss of consciousness," (Oxford textbook of Geriatric Medicine 2nd Edition 2000).

If yes: a) How many times in the past year? _____
b) Did you sustain any injuries? Yes☐ No ☐

If yes, what type of injury?
☐Soft tissue injury (bruise / laceration)
State location: _____
☐Fracture
State location: _____

c) Did you need to see your General Practitioner as a result?
Yes☐ No ☐

d) Did you need to go to an Emergency department (A/E) as a result?
Yes☐ No ☐

e) Did you need to be admitted to hospital as a result?
Yes☐ No ☐

2) Do you have to be careful not to stand up too quickly when rising from a sitting or lying position? Yes☐ No ☐

3) Do you feel dizzy if you stand up too quickly? Yes☐ No ☐
If yes, how often?

Several times a day	☐
Several times a week	☐
Several times a month	☐
Several times a year	☐
Less than once a year	☐

4) Do you feel dizzy if you stand for a prolonged period (other than just after standing up)? Yes☐ No ☐
If yes, how often?

Several times a day	☐
Several times a week	☐
Several times a month	☐
Several times a year	☐
Less than once a year	☐

5) Have you ever fainted (i.e. lost consciousness)? Yes☐ No ☐

If yes: a) How many times? _____
b) How many times in the past year? _____

6) Have you ever felt that you were going to faint but did not?
Yes ☐ No ☐

If yes: a) How many times? _____
      b) How many times in the past year? _____

7) Are you afraid of falling?   Yes☐ No☐

8) Do you limit any household activities because you are afraid you might fall?   Yes☐ No☐

9) Do you limit any outside activities because you are frightened you may fall?   Yes☐ No☐

EXERCISE

Have you done any exercise in the past 2 weeks?

    If YES: How many times did you exercise? _____
                On average, how long did you exercise for on each occasion? _____

Please place a tick activities partaken in, in the past 2 weeks:

☐ Walking for exercise
☐ Housework
☐ Jogging/Running
☐ Gardening
☐ Dancing
☐ Calisthenics or General Exercise
☐ Golf
☐ Cycling
☐ Swimming
☐ Other, please specify _____

BONE HISTORY

A)

- Have you ever had a fractured bone?   Yes ☐   No ☐

- Have you ever had a hip fracture?   Yes ☐   No ☐

- Have parent(s) ever had hip fracture?   Yes ☐   No ☐

- Do you suffer with Rheumatoid Arthritis?   Yes ☐   No ☐

- Do you suffer with Osteoporosis?   Yes ☐   No ☐

- Have you ever taken Glucocorticoids for more
  than 3 months?   Yes ☐   No ☐
  If yes, Duration_____
  Mean daily dose_____

- Have you ever suffered from Epilepsy?   Yes ☐   No ☐

- Have you ever taken anti-epileptic medication?   Yes ☐   No ☐
  If yes, for how long (in months) _____
  Name of medication(s)_____

B) Osteoporotic Medication: **Duration (months):**

- <u>Protelos</u>   Yes ☐ No ☐   _____

- <u>Bisphosphonates</u>   Yes ☐ No ☐   _____
  (Alendronate ☐ / Risedronate ☐ / Ibandronic Acid ☐ / Etidronate ☐
  Zoledronic acid ☐)

C) <u>**Have you ever taken**</u>:   **Duration (months):**

1. <u>Aromatase Inhibitors</u>:   Yes ☐ No ☐   _____
   (Arimidex ☐ / Femara ☐)

2. <u>GnRH / LHRH analogues</u>:   Yes ☐ No ☐   _____
   (Zoladex ☐ / Gonapeptyl ☐ / Prostap ☐)

3. <u>Anti-androgen</u>:   Yes ☐ No ☐   _____
   (Casodex ☐)

SMOKING

Smoking status

Current   ☐ (i.e. Smoked in last month)
Past   ☐
Never   ☐

Have you ever smoked cigarettes regularly (at least 1/day) for a period longer than 6 months?   Yes ☐   No ☐

If YES:
At what age did you start smoking?_____

Are you still smoking?   Yes ☐   No ☐

If YES:
> How many cigarettes do you smoke per day? _____
> Or if <u>ROLL</u>, how quickly do you go through a 25g pack of tobacco (in days)?_____

If NO:
> At what age did you stop smoking?_____
> How many cigarettes did you smoked on average (per day)?_____
> Or if <u>ROLL,</u> how quickly do you go through a 25g pack of tobacco (in days)?_____

DIET & SUPPLEMENTS

1. Do you eat any fortified foods? *(Researcher, please refer to Aide Memoire)*   Yes ☐ No ☐

If YES, please specify: ☐ Fortified Breakfast Cereals
                              ☐ Fortified Cereal Bars
                              ☐ Fortified Bread
                              ☐ Fortified Fat Spreads
                              ☐ Fortified Drinks
                              ☐ Marmite or other yeast extracts
                              ☐ Other _____

For each fortified product ticked, name the product and brand below and state how often.

Product 1:   Product 2:   Product 3:

_____   _____   _____

☐ Twice/day or more   ☐ Twice/day or more   ☐ Twice/day or more
☐ Once/day   ☐ Once/day   ☐ Once/day
☐ 5-6 times/week   ☐ 5-6 times/week   ☐ 5-6 times/week
☐ 3-4 times/week   ☐ 3-4 times/week   ☐ 3-4 times/week
☐ 1-2 times/week   ☐ 1-2 times/week   ☐ 1-2 times/week
☐ Other _____   ☐ Other _____   ☐ Other _____

If YES, when did you last eat these products?

Product 1:   Product 2:   Product 3:

_____   _____   _____

2. Do you take milk? Yes ☐ No ☐

If YES:
As a drink ☐   In tea/coffee ☐   With cereal ☐

How often?
☐ Twice/day or more   ☐ Twice/day or more   ☐ Twice/day or more
☐ Once/day   ☐ Once/day   ☐ Once/day
☐ 5-6 times/week   ☐ 5-6 times/week   ☐ 5-6 times/week
☐ 3-4 times/week   ☐ 3-4 times/week   ☐ 3-4 times/week
☐ 1-2 times/week   ☐ 1-2 times/week   ☐ 1-2 times/week
☐ Other _____   ☐ Other _____   ☐ Other _____

If YES: Name the brand of milk you typically take _____

            Do you typically take -

Whole ☐   Low-fat ☐   Skimmed/ Slimline ☐   Soya ☐   Other☐

If YES, when did you last take milk? _____

3. Do you eat –

Meat? Yes ☐ No ☐   **Poultry?** Yes ☐ No ☐

If YES, how often?

☐ Twice/day or more   ☐ Twice/day or more
☐ Once/day   ☐ Once/day
☐ 5-6 times/week   ☐ 5-6 times/week
☐ 3-4 times/week   ☐ 3-4 times/week
☐ 1-2 times/week   ☐ 1-2 times/week
☐ Other _____   ☐ Other _____

4. Do you eat –

White fish? Yes ☐ No ☐   **Oily fish?** Yes ☐ No ☐
(e.g. cod/haddock/plaice/fishfingers)   (e.g. salmon/trout/mackerel)

If YES, how often?

☐ Twice/day or more   ☐ Twice/day or more
☐ Once/day   ☐ Once/day
☐ 5-6 times/week   ☐ 5-6 times/week
☐ 3-4 times/week   ☐ 3-4 times/week
☐ 1-2 times/week   ☐ 1-2 times/week
☐ Other _____   ☐ Other _____

5. Do you eat –

Eggs? Yes ☐ No ☐   **Cheese?** Yes ☐ No ☐   **Yoghurt?** Yes ☐ No☐

If YES, how often?

☐ Twice/day or more	☐ Twice/day or more	☐ Twice/day or more
☐ Once/day	☐ Once/day	☐ Once/day
☐ 5-6 times/week	☐ 5-6 times/week	☐ 5-6 times/week
☐ 3-4 times/week	☐ 3-4 times/week	☐ 3-4 times/week
☐ 1-2 times/week	☐ 1-2 times/week	☐ 1-2 times/week
☐ Other _____	☐ Other _____	☐ Other _____

6. Do you take any vitamin supplements (e.g. vitamins in tablet form, cod liver oil, etc)? Yes ☐ No ☐

If YES, how often?

☐ Twice/day or more
☐ Once/day
☐ 5-6 times/week
☐ 3-4 times/week
☐ 1-2 times/week
☐ Other _____

If on **FOLIC ACID** when last taken?_____

If YES, name the supplement(s) *(Researcher please put product name in capitals)* -

(i)_____   (ii)_____   (iii)_____

How long have you been taking each supplement?

(i)_____   (ii)_____   (iii)_____

Was the name of the supplement(s) verified by researcher *(Researcher please tick)* -

    At interview (examination of product)   ☐
    In a follow-up phone call   ☐
    Not verified   ☐

7. When you are outdoors during the sunny months, do you stay in the sun or do you seek the shade?

I try to avoid staying in direct sunshine	☐
I stay sometimes in the sunshine	☐
I enjoy staying often in the sunshine	☐

8. During the sunny months, how often would you apply sun protection factor?

Never ☐   Rarely ☐   Sometimes ☐   Usually ☐   Always ☐   Other ☐

What sun protection factor do you usually apply? _____

9. Have you been on a sunny holiday in the last 6 months?

Yes ☐ No ☐

If YES, please specify:

(i) where you went, (ii) during which month(s) and (iii) how long (no of days) (*Researcher, ensure response to (iii) is the total number of days of ALL breaks in sunnier climates in the last 6 months*)

_____
_____

10. Apart from the last 6 months, do you generally tend to go on sunny holidays?

Yes ☐ No ☐

If YES, specify how often (e.g. once a year, twice a year, etc)

_____

11. Do you use a sun-lamp or sun-bed regularly?

Yes ☐ No ☐

If YES, specify how often (e.g. weekly, monthly, several times a year, etc)

_____

ALCOHOL

Do you drink alcohol
- ☐ Yes, currently (within the past year)
- ☐ No, but I have in the past (more than 1 year ago)
- ☐ No, never

How often do you drink alcohol? _____ **(days per month)**

How many units (of each type) of alcohol do you consume per week?
(1 unit = ½ pint of beer, 1 glass of wine, 1 measure of spirits; 1 bottle of wine=10 units)

Beer _____

Wine _____

Spirits _____

Total units _____

MEMORY CONCERNS

1) Do you have any concerns with regard to your memory?
Yes ☐   No ☐

2) Does your family have any concerns with regard to your memory?
Yes ☐   No ☐

DEPRESSION (CES-D)

Below is a list of the ways you might have felt or behaved. Please respond on how often you have felt this way during the <u>PAST WEEK,</u> by ticking the most appropriate box.

I was bothered by things that usually don't bother me	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I did not feel like eating; my appetite was poor	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt that I could not shake off the blues even with help from my family or friends	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt that I was just as good as other people	3 Never or Rarely (less than 1 day) 2 Some of the time (1-2 days) 1 Occasionally (3-4 days) 0 Most of the time (5-7 days)
I had trouble keeping my mind on what I was doing	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt depressed	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt that everything I did was an effort	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt hopeful about the future	3 Never or Rarely (less than 1 day) 2 Some of the time (1-2 days) 1 Occasionally (3-4 days) 0 Most of the time (5-7 days)
I thought my life had been a failure	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt fearful	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)

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My sleep was restless	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I was happy	3 Never or Rarely (less than 1 day) 2 Some of the time (1-2 days) 1 Occasionally (3-4 days) 0 Most of the time (5-7 days)
I talked less than usual	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt lonely	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
People were unfriendly	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I enjoyed life	3 Never or Rarely (less than 1 day) 2 Some of the time (1-2 days) 1 Occasionally (3-4 days) 0 Most of the time (5-7 days)
I had crying spells	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt sad	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I felt that people disliked me	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 day) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)
I could not get "going"	0 Never or Rarely (less than 1 day) 1 Some of the time (1-2 days) 2 Occasionally (3-4 days) 3 Most of the time (5-7 days)

TOTAL SCORE:

ANXIETY (HADS)

In general do you ever feel

I feel tense or wound up...	Most of the time	A lot of the time	Occasionally	Not at all
	3	2	1	0
I get a sort of frightened feeling as if something awful is about to happen...	Quite badly	Not too badly	A little	Not at all
	3	2	1	0
Worrying thoughts go through my mind...	A great deal of the time	A lot of the time	From time to time	Only occasionally
	3	2	1	0
I can sit at ease and feel relaxed...	Definitely	Usually	Not often	Not at all
	0	1	2	3
I get a sort of frightened feeling like butterflies in the stomach...	Not at all	Occasionally	Quite often	Very often
	0	1	2	3
I feel restless as if I have to be on the move...	Very much	Quite a lot	Not very much	Not at all
	3	2	1	0
I get sudden feelings of panic...	Very often	Quite often	Not often	Not at all
	3	2	1	0

TOTAL SCORE:

PHYSICAL SELF MAINTENANCE SCALE (PSMS)

1) Do you eat:
    3 Without any help
    2 With some help (e.g. cutting food, etc.)
    1 Someone must feed me

2) Do you dress and undress yourself:
    3 Without help (able to pick out clothes & dress)
    2 With some help
    1 No, someone must dress and undress me

3) Do you take care of your own appearance? (e.g. combing your hair, or for *men*, shaving)
    3 Yes, without help
    2 Yes, with some help
    1 No, someone must help me with this type of thing

4) Are you able to get around your house/apartment without any help?
    3 Yes, without help of any kind (except a cane)
    2 Yes, with some help (from a person, walker, crutches, or chair)
    1 No, I cannot get around my home unless someone moves me

5) Are you able to get in and out of bed yourself?
    3 Yes, without help or aid
    2 Yes, with some help (from a person or device)
    1 No, I cannot get out of bed unless someone lifts me

6) Are you able to bathe, --that is, take a bath, shower, or sponge bath by yourself?
    3 Yes, without help
    2 Yes, with some help (from a person or device)
    1 No, someone must bathe me and lift me in and out of the bath

7) a. Do you ever have trouble getting to the bathroom on time?
    1 Yes
    2 No

b. About how often would you wet or soil yourself during the day or night?
    4 Never
    3 Less than once a week
    2 Once or twice a week
    1 Three times a week or more

TOTAL SCORE:

INSTRUMENTAL ACTIVITIES OF DAILY LIVING (IADL)
1) Can you the use the telephone?
     3 Yes, without help
     2 Yes, with some help
     1 No, I am completely unable to use the telephone

2) Can you get to places out of walking distance?
     3 Yes, without help
     2 Yes, with some help
     1 No, I am completely unable to travel unless special arrangements are made

3) Can you go shopping for groceries?
     3 Yes, without help
     2 Yes, with some help
     1 No, I am completely unable to do any shopping

4) Can you prepare your own meals?
     3 Yes, without help
     2 Yes, with some help
     1 No, I am completely unable to prepare meals

5) Can you do your own housework?
     3 Yes, without help
     2 Yes, with some help
     1 No, I am completely unable to do my own housework

6) Can you do your own handyman work?
     3 Yes, without help
     2 Yes, with some help
     1 No, I am completely unable to do my own handyman work

7) Can you do your own laundry?
     3 Yes, without help
     2 Yes, with some help
     1 No, I am completely unable to do any laundry at all

8) a. Do you take or use any medications?
     1 Yes
     2 No
    b. Do you take your own medicine?
    c. If you had to take medicine, can you do it:
     3 Without help, taking the right dose at the right time
     2 With some help (e.g. someone prepares it for you, or reminds you)
     1 I am completely unable to take my own medicines

9) Can you manage your own money?
     3 Yes, without help
     2 Yes, with some help
     1 No, I am completely unable to handle money   TOTAL SCORE:

Appendix 3 - Mini Mental State Examination (MMSE)

Maximum Score	Score	
		ORIENTATION
5	( )	What is the (year) (season) (date) (day) (month)?
5	( )	Where are we: country, county, town, street, room?

REGISTRATION

3	( )	Name 3 objects: (APPLE, TABLE, PENNY) 1 second to say each. Then ask the volunteer to repeat all three after you have said them. Give 1 point for each correct answer. Then repeat the trials until he/she either learns all 3 or has had 6 trials. Count all trials and record them.

No of Trials.....................

ATTENTION AND CALCULATION

5	( )	Begin with 100 and count backwards by subtracting 7. Stop after 5 answers. Score 1 point for each correct answer. Alternatively spell "world" backwards.

RECALL

3	( )	Ask for the three objects learned above. Give 1 point for each correct answer.

LANGUAGE

2	( )	Name a pencil and a watch.
1	( )	Repeat the following "No ifs ands or buts".
3	( )	Follow a 3-stage command: "Take a sheet of paper in your right hand, fold it in half, and put it on the floor".
1	( )	Read and obey the following: CLOSE YOUR EYES
1	( )	Write a sentence.
1	( )	Copy a design.

CLOSE

YOUR

EYES
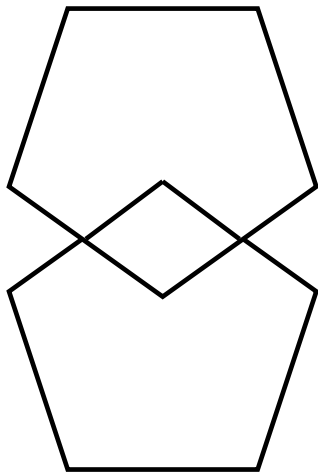
Appendix 4 - Frontal Assessment Battery (FAB)

1. Similarities (conceptualization)

"In what way are they alike?"
A banana and a orange (In the event of total failure: "they are not alike" or partial failure "both have a peel", help the patient by saying "both a banana and an orange are…."; but credit 0 for the item; do not help the patient for the tow following items)

> A table and a chair
> A tulip, a rose, and a daisy

Score: only category responses (fruits, furniture, flowers) are considered correct.
> Three correct: 3
> Two correct: 2
> One correct: 1
> None correct: 0

2. Lexical fluency (mental flexibility)

"Say as many words as you can beginning with the letter 'S', any words except surnames or proper nouns."

If the patient gives no response during the first 5 seconds, say: "For instance, snake." If the patient pauses 10 seconds, stimulate him by saying: "any word beginning with the letter 'S'. The time allowed is 60 seconds.

Score: word repetitions or variations (shoe, shoemaker), surnames, or proper nouns are not counted as correct responses.

> More than nine words: 3
> Six to nine words: 2
> Three to five words: 1
> Less than three words: 0

3. Motor series (programming)

"Look carefully at what I'm doing."

 The examiner, seated in front of the patient, performs alone three times with his left hand the series of Luria "fist-edge-palm." "Now, with your right hand do the same series, first with me, then alone." The examiner performs the series three times with the patient, and then says to him/her: "Now, do it on your own."

Score:

Patient performs six correct, consecutive series alone: 3
Patient performs at least three correct consecutive series alone: 2
Patient fails alone, but performs three correct consecutive series with the examiner: 1
Patient cannot perform three correct consecutive series even with the examiner: 0

4. Conflicting instructions (sensitivity to interference)

"Tap twice when I tap once."

To be sure that the patient has understood the instructions, a series of three trials is run: 1 – 1 – 1. "Tap once when I tap twice."   To be sure the patient has understood the instructions, a series of three trials is run: 2 – 2 – 2. The examiner performs the following series: 1 – 1 – 2 – 1 – 2 – 2 – 2 – 1 – 1 – 2.

Score:

No error: 3
One or two errors: 2
More than 2 errors: 1
Patient taps like the examiner at least four consecutive times: 0

5. Go-No-Go (inhibitory control)

"Tap once when I tap once."

To be sure that the patient has understood the instructions, a series of three trials is run: 1 – 1 – 1. "Do not tap when I tap twice." To be sure the patient has understood the instructions, a series of three trials is run: 2 – 2 – 2. The examiner performs the series: 1 – 1 – 2 – 1 – 2 – 2 – 2 – 1 – 1 – 2.

Score:
No errors: 3
One or two errors: 2
More than two errors: 1
Patient taps like the examiner at least four consecutive times: 0

1. Prehension behavior (environmental autonomy)

"Do not take my hands."

The examiner is seated in front of the patient. Place the patient's hands palm up on his/her knees. Without saying anything or looking at the patient, the examiner brings his/her hands close to the patient's hands and touches the palms of both the patient's hand, to see if he/she will spontaneously take them. If the patient takes the hands, the examiner will try again after asking him/her: "Now, do not take my hands."

Score:
Patient does not take the examiner's hands: 3
Patient hesitates and asks what he/she has to do: 2
Patient takes the hands without hesitation: 1
Patient takes the examiner's hands even after he/she has been told not to do so: 0

SCORE:_____

Appendix 5 - Repeatable Battery for the Assessment of Neuropsychological Status

Manual not shown due to copyright.

FOOD DIARY INSTRUCTIONS

Please record **<u>ALL</u>** the foods you eat on the days requested.

For each food please describe:
1. The **method of cooking** e.g. fried, boiled, grilled, stewed, microwaved etc.
2. If possible please give the **brand and name** of the product used and the weight given on the packet were appropriate.
3. For **milk** please state type i.e. whole milk, low fat, skimmed/slimline or soya and alternatives.
4. For **butter, margarine, cooking oil** etc please give exact brand name.
5. Describe the **portion size** according to the instructions below.

1.PORTION SIZES

To estimate a portion size use the following household measurements:

RICE/PASTA
Estimate the portion size as small/ medium/ large, or give the number of tablespoons of cooked food eaten.

BREAD
State the number of slices/ rolls/ crisp breads eaten.

BREAKFAST CEREALS
Estimate the portion size as small/ medium/ large, or give the number of tablespoons eaten.

MEAT & FISH
Estimate the portion size as small/ medium/ large. For fish fingers, state the number eaten.

FRUIT
For whole fruit give the number eaten and estimate the size of the fruit as small/ medium/ large.
For tinned fruit estimate the number of tablespoons eaten or state the size of the can if the whole tin is consumed.
For dried fruit estimate the amount consumed in tablespoons.
For grapes estimate the number eaten.

6. VEGETABLES
Estimate the portion size as small/medium/large or give the number of tablespoons eaten.

7. NUTS
Estimate the number eaten or give the size of the bag.

8.   MILK
Estimate the amount consumed in pints.

9.   CHEESE
Estimate the number of slices eaten and state whether the slices were small medium or large.

10. EGGS
State the number eaten.

11. BISCUITS
State the number eaten.

12. CAKES
Estimate the size of the slice as small, medium or large.

13. CRISPS, SWEETS, CHOCOLATE, FIZZY DRINKS ETC
Report the brand name and weight as stated on the packet.

14. DRINKS
State whether cup or mug. For glasses state whether small, medium ($^1/_2$ pint) or large (pint).

15. SAUCES, CHUTNEYS ETC
Estimate the number of tablespoons consumed.

16. SUGAR, JAMS ETC
Estimate the amount consumed in teaspoons.

OTHER FOOD
Estimate the number of teaspoons consumed.

An example of how to complete a food diary is provided for you on the next page.

THANK YOU FOR YOUR HELP !

EXAMPLE

Day: Monday   Date: 12/10/14   **FOR OFFICE USE ONLY**

	CODE	WT (g)
BREAKFAST 1 cup coffee (1 teaspoon sugar & Dale farm skimmed milk) 6 tablespoons Kelloggs cornflakes with ¼ pint skimmed milk 1 slice of Brennans white bread toasted with flora margarine thinly spread and 1 teaspoon strawberry jam		
LUNCH Cheese and tomato sandwich 2 slices Hovis wholemeal bread, spread thinly with flora, 3 medium thick slices of Coleraine cheddar cheese, 1 small tomato, 2 teaspoons HP brown sauce) 1 packet cheese and onion crisps (Tayto) 1 small orange and 1 medium sized banana 1 can of coca-cola (330ml)		
EVENING MEAL 1 medium sized chicken breast (without skin) fried in vegetable oil 3 medium sized potatoes, 5 tablespoons boiled carrots 3 tablespoons bisto gravy (made with bisto gravy granules) 1 small glass white wine medium slice of appletart (homemade) & coffee made as above		
BETWEEN MEAL SNACKS 2 cups of tea (each cup 1 teaspoon sugar and skimmed milk) 1 mars bar 1 pot yoplait full fat strawberry yoghurt 4 McVities chocolate digestive biscuits		

Day: Date:

	CODE	WT (g)
BREAKFAST		
LUNCH		
EVENING MEAL		
BETWEEN MEAL SNACKS		

Appendix 7

TUDA Study: Height, Weight & Circumference Measurements WI
TUDA/WI004/001

Table of Contents

1.   THEORY

Body composition is used to describe the percentages of fat, bone and muscle in human bodies. Because muscular tissue takes up less space in our body than fat tissue, our body composition, as well as our weight, determines leanness. Two people at the same height and same body weight may look completely different from each other because they have a different body composition.

Directly measuring body composition overcomes the limitations of anthropometric measurements such as body mass index (BMI) and waist circumference (WC). In the research setting, body composition can be measured to determine normal or healthy growth, to evaluate nutritional status, to determine the effect of a nutrition intervention or to assess the effect of a disease state.

2.   RESPONSIBILITIES

Chief Investigator (CI)

To ensure that all researchers are aware of and comply with the TUDA Study Height, Weight and Circumferences WI (TUDA/WI004/001) and all related standard

operating procedures, work instructions and risk assessments for their designated study tasks.

Researcher/ User   To ensure that all procedures relating to use of measurement of body composition are performed in accordance with T TUDA Study Height, Weight and Circumferences WI (TUDA/WI004/001). To adhere to all standard operating procedures, work instructions and risk assessments for their designated study tasks.

3.   CONSUMABLES (materials available stores)

Alcohol wipes 3.65

4.   PROCEDURE

Seca model 888 weighing scales, Seca 213 stadiometers and Seca 200 circumference measuring tapes are to be used for the TUDA Study. Researchers are responsible for all equipment when it is signed out and must return the equipment in good condition.

4.1   Weight

6.1.1   Ask volunteer to remove any heavy loose clothing and shoes.

4.1.2   Ask volunteer to step onto scales, drop their hands by their side, look straight ahead and remain as still as possible.

4.1.3   Record the weight on the TUDA Study Questionnaire.

4.1.4   Notify Shauna Harte or Kim Martin if any participant's weight exceeds the 136kg weight limit on the DXA Prodigy.

4.2   Height

4.2.1   Ask volunteer to remove shoes and step onto stadiometer, with their back to the measuring stick.

4.2.2   Lower the height indicator onto their head and ask them to look straight ahead with their arms by their side.

4.2.3   Once in position, ask volunteer to bend their knees and step out from under the height indicator.

4.2.4   Record the volunteers' height on the TUDA Study Questionnaire.

4.3   **Waist and Hip Circumferences**

4.3.1   With participant standing straight and using specific seca measuring tape, ask volunteer to lift their top until belly button is exposed.

4.3.2   Wrap tape around volunteers' waist at point of belly button with tape horizontal and record measurement on the TUDA Study Questionnaire.

4.3.3   With participant standing straight wrap tape around the widest part of the hips. The widest girth of the hips is usually around the greater trochanters of the femur, but may vary according to the shape of the subject.

4.6.4   With the tape horizontal record hip measurement on the TUDA Study Questionnaire.

5.   **Quality Control**

•   **Calibration documents for all relevant equipment**

6.   **Troubleshooting**

Problem: **Scales fail to work**
- Replace battery pack and/or
- Obtain new set
Problem: **Stadiometer arm does not stay in place**
-   **Replace stadiometer**

NB: All faults must be reported to the HISU office (W3010) as soon as possible

Appendix 8 – Blood processing protocol

1.REAGENTS

Reagent

L-Ascorbic acid   Sigma

Cryovials + lids

PBS

2.CONSUMABLES

Refrigerated centrifuge

Ice box

Ice/ice packs

Pipettes

Tips (yellow and blue)

Plastic transfer pipettes

Racks

Virakon (1tablet in 500mls water; blood lab sink cupboard)

Cryovials and coloured lids

Starstedt boxes

Blood collection tubes -   2 x 9ml EDTA (purple)

- 1 x 8ml gel separator (red)

- 1 x 4ml lithium heparin (green)

- 1 x 4ml Serum (gold)

- 1 x 4ml Sodium Fluoride (grey)

- 2 x 4ml EDTA (purple)

Needles (21G butterfly needles)

Cotton wool

Plasters

Micropore tape

Pillow (+extra cover)

Tourniquet

Gloves

Blood spill kit

Alcohol swabs

TUDA participant pack

3.PROCEDURE

• Only fully trained and qualified phlebotomists can perform blood collection procedures for the TUDA Study.

• The researcher must always achieve positive participant identification before the blood sample is taken. The volunteer must be asked to state their name, address and date of birth. The information provided by the participant must match that contained in the participant pack and the details entered on the hospital blood bag/referral form.

• A total of 8 blood tubes are collected per participant.

Research:

• 1 x 9ml EDTA (purple; refrigerated sample with coloured sticker)

• 1 x 9ml EDTA (purple; room temperature)

• 1 x 8ml gel separator (red; serum)

• 1 x 4ml lithium heparin (green)

Routine (for hospital lab):

• 1 x 5ml Serum (gold; renal, liver, lipid & bone function)

• 1 x 2ml Fluoride oxalate (grey; glucose)

• 2 x 4ml EDTA (purple; full blood count and HBA1c (Glycosylated Haemoglobin)

• All research blood collection tubes must be labelled with the unique subject ID number.

• All routine blood collection tubes must be labelled with the participant name, date of birth and UU/TUDA

• One 9ml EDTA must be kept refrigerated (labelled with coloured sticker). All other samples can remain at room temperature until processed.

4. Pre-processing

• Place required number of aliquots of 1% solution of ascorbic acid (450µl in each; 2 aliquots per participant) on roller mixer to defrost. Cover with tinfoil.

• Label 22 cryovials with pre printed sample tube labels. Labels contain study identifier, unique subject ID number and the sample type. Set up in order on rack.

• Make up Virakon solution. Dissolve1 tablet in a beaker containing 500mls of water.

•Set centrifuge to 4°C.

5. 8ml gel seperator (serum)

• Allow the 8ml serum tube to sit for a minimum of 30 minutes at room temperature before centrifuging at 3000rpm for 15 minutes.

• Aliquot 0.5ml (vitamin D), 1.0ml (B12 and folate) and 0.25ml (HoloTC) of serum into three 1.5ml cryovial tubes, cap with red cap and store at -80°C.

• Divide the remainder of the serum into two cryovial tubes

• Store at -80°C.

5.1 9ml EDTA Sample (room temperature plasma, red cell folate, washed red cells)

• Place sample on a roller for 10 mins to ensure adequate mixing with the EDTA.

• Remove 50µl of whole blood from EDTA tube and place in cryovial labelled RCF1 (defrosted 1% Ascorbic Acid aliquot; red cell folate). Repeat above for RCF2. Cap with a yellow cap and mix by inversion. Leave to stand on the bench for 60 minutes at room temperature. Store at -80°C.

• Centrifuge the remaining sample at 3000rpm for 15 mins.

• Remove the plasma with a disposable pasteur pipette and divide into three 1.5ml cryovial tubes. Cap with purple cap and store at -80°C.

• Using a disposable pipette transfer the buffy coat into a labelled 1.5ml cryovial tube, cap with white cap and store at -80°C.

• Washing the red cells

- Add PBS to the remaining red cells in the blood collection tube (up to the black mark on the outside of the tube).

- Cap the tube and mix by inversion.

- Centrifuge at 3000rpm for 15mins at room temp.

- Remove the supernatant with a disposable pasteur pipette.

- Repeat steps adding PBS and centrifuging sample twice at 2000rpm for 10mins.

- Divide the washed red cells into two 1.5ml cryovial tubes and cap with

- Clear cap for storage at –80°C for (tissue B12)

5.2 9ml EDTA Sample (refrigerated plasma, washed red cells)

• Centrifuge the sample at 3000rpm for 15 mins 4°C temp.

• Aliqout 0.5ml of plasma (homocysteine) into a 1.5ml cryovial tube, cap with green cap and store at -80°C.

• Aliquot 0.75ml of plasma (MMA) into a 1.5ml cryovial tube, cap with green cap and store at -80°C.

• Remove the remainder of the plasma with a pasteur pipette and divide into two 1.5ml cryovial tubes, cap with green cap and store at -80°C.

• Transfer the buffy coat into a labelled 1.5ml cryovial tube, cap with white cap and store at -80°C.

• Washing the red cells

- Add PBS to the remaining red cells in the blood collection tube (up to the black mark on the outside of the tube).

- Cap the tube and mix by inversion.

- Centrifuge at 3000rpm for 15mins at 4°C temp.

- Remove the supernatant with a disposable pasteur pipette.

- Repeat steps adding PBS and centrifuging sample twice at 2000rpm for 10mins.

- Divide the washed red cells into two 1.5ml cryovial tubes and cap with

- brown cap for storage at –80°C for (EGRac, B6)

5.3 4ml lithium heparin (plasma)

• Centrifuge the sample at 3000rpm for 15mins at 4°C temp.

• Divide plasma between two 1.5ml cryovial tube, cap with blue cap and store at -80°C.

5.4 Labelling

Apply unique ID labels to tube caps (small round labels available from designated folder in lab). Double check and verify that tube label and cap labels are correct and that the IDs match.

5.5 Storage

Store all samples in TUDA freezer. Update sample storage map.

5.6   Bloods to hospital

The following blood tubes are sent to Hospital Laboratories:

• 1 x 5ml Serum (gold; renal, liver, lipid & bone function)

• 1 x 2ml Fluoride oxalate (grey; glucose)

• 2 x 4ml EDTA (purple; full blood count and HBA1c (Glycosylated Haemoglobin)

Hospital Laboratories

• All blood tubes must be labelled with participant name, date of birth and UU/TUDA.

• Hospital blood bags must be labelled in accordance with NHSCT laboratory request form completion and specimen labelling (NHSCT/10/333).

• The researcher must verify the at the details provided on the blood tube are correct and match those on the blood bag prior to sending the samples to the hospital laboratories.

Transportation of blood samples

• All unscreened blood samples must be packaged and transported in accordance with UN P650 packaging instruction. P650 approved containers for the transport of blood samples must be used.

• If a private vehicle is to be used it is the responsibility of the registered owner to ensure that their insurance is valid for these purposes.

5.7   Disposal of blood tubes

Once processing is complete all blood tubes must be discarded into the incineration bin in the lab.

6.0 QUALITY CONTROL

• Records of problems/issues encountered during blood processing must be noted in the TUDA study laboratory book and brought to the attention of the CI where appropriate.

• Laboratory and blood collection consumables should be monitored regularly to ensure they are within the expiry date and that adequate stock levels are maintained. Out of date consumable/reagents should not be used and must be disposed of as appropriate.

7.0 TROUBLESHOOTING

Problem: centrifuge fault
- Halt cycle, remove blood tubes and place in another centrifuge. Report fault to a member of technical staff.

Problem: blood spillage
- Solution: Obtain blood spillage kit from laboratory. Follow protocol provided with kit.

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